FLOW-MICROCALORIMETRIC DETERMINATION OF ENZYMATIC ACTIVITIES OF THE TRICHODERMA VIRIDAE-CELLULASE COMPLEX

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A method for measuring enzymatic activities of the Trichoderma viridae cellulase complex is described. The LKB 2277 Thermal activity monitor and a flow-mix mode were used.

Enzymatic activities of samples of a crude cellulose complex have been determined using three substrates: cellobiose, carboxymethyl cellulose (CMC) and xylan. Some kinetic constants for cellobiase activity have been evaluated from the obtained results. A process of the end-product inhibition of cellobiase resp. carboxymethyl cellulase activity by glucose has been observed too.

The described method allows a direct determination of various enzymatic activities of the cellulase complex. Because of the high sensitivity and the simplicity, the method is a very suitable tool for studying the cellulase complex, determining the optimal conditions of enzymatic break-down of the cellulosic materials and observing various mechanisms of the feedback control by products of enzymatic action. The procedure is completely general in nature and is applicable to other enzymatic systems.

Keywords: cellulase complex, enzymatic activities, flow-microcalorimetry, kinetics

Introduction

The characterization of cellulase enzymes poses special problems to the enzymologist. Kinetic studies are difficult since the natural substrate is both insoluble and structurally variable, and thus relatively undefined with respect to concentration and chemical form. Often a magnitude of endo- and exo-glucanases act in synergy and in a complex manner still poorly understood, and a variety of end-products and trans-glycosylation species are frequently formed, involving various mechanisms of feedback control. The presence of β-glucosidase or other enzymes which are required for cellulose metabolism and enhance cellulose hydrolysis, but which are not, strictly speak-

ing, cellulases, further complicates the picture. Moreover, there has been comparatively little elucidation of the differences in the modes of action of the cellulase system of various organisms.

Commonly used standard assays, such as carboxymethyl cellulase assay, cellobiase assay and filter paper assay are all based on the same principle of estimating a fixed amount of glucose from the relevant substrate [1]. A large disadvantage of these methods is that they are rather elaborate and time-consuming. That is why we have developed the method of flow-microcalorimetric determination of some of the enzymatic activities.

Many enthalpimetric methods concerning the analysis of enzymatic reactions have been described during the last twenty years [2–10]. The use of calorimetry in the determination of enzymatic activities has many attractive features, such as the possibility of continuous-flow operation, insensivity to the optical properties of the sample, simple procedures, a general detection principle, a dynamic monitoring range of several orders of magnitude in substrate concentrations, and high sensitivity.

It is a well-known fact that biological macromolecules, particularly proteins, are dynamic, fluctuating entities. One of the possible consequences of the fluctuating nature of macromolecules is that certain small molecules (for example allosteric effectors) may, upon binding, be able to alter the population of accessible states of the macromolecule, and thus induce changes in its chemical and physical properties, including its affinity and reactivity towards other ligands. Ligand-induced conformational changes may serve very important functional roles, such as promoting proper orientation of catalytic groups at an enzyme's active site, mediating the linked binding groups at on enzyme's active site, mediating the linked binding of ligands, or storing free energy in an enzyme-substrate complex for later release during a catalytic process. While structural alterations may be directly visualized by crystallographic studies, it is still difficult to assess the energetics associated with the conformational changes. The relationship between structure and thermodynamics is a fundamental physical chemical key to understanding a mechanism of enzymatic action. However, the binding of ligand to a macromolecule will often be coupled to other side-reactions and experimentally observed thermodynamic quantities will contain contributions from these coupled reactions. Deducing the correct interpretations can be a large problem. Nevertheless, such thermodynamics information can aid greatly in the development of a molecular interpretation of complex biological systems including enzymes [11].

Calorimetry in conjunction with other analytical techniques is a very valuable tool for observing the thermodynamic aspects of enzymatic action. Our contribution is one of the examples of the utilization of microcalorimetry for the study of enzymatic kinetics.

Experimental

Materials

The industrial enzymatic preparation P10 Kolin was produced using mesophilic fungal species Trichoderma viridae 9123 in the Food Industry Research Institute in Prague. The culture medium was separated on a through-flow separator and the super-natant was filtered. The pellucid filtrate was concentrated on an ultra-filter (the compounds with m.w. lower than 10.000 passes through a membrane) and spray dried. The obtained preparation contained 64% of proteins and these enzymatic activities:

CMC act.	Xylanase act.	Cellobiase act.	Filter paper act.
IU. g^{-1}			
2303	2098	16.63	585

The carboxymethyl cellulase TS20 (LOVOSA) and xylan FLUKA 95590 were used for determining the respective enzymatic activities. All other chemicals were commercial products of analytical grade.

Standard assay procedures for cellulase enzyme system

CMC and cellobiase activities of the enzymatic preparation were determined according to standard assay procedures for cellulase enzyme system proposed by the Commission on Biotechnology in 1984 [1]. Both methods are based on the principle of estimating a fixed amount of glucose from the respective substrates. Xylanase activity was determined in a similar way [12].

Apparatus and operative conditions

Microcalorimetric experiments were performed by using the LKB 2277 Thermal Activity Monitor (LKB Produkter AB, Bromma, Sweden), a free-standing multichanel microcalorimeter. Continuous heat leakage measurements are conducted in an isothermal system. The instrument is equipped with the LKB 2132 MicroPerpex Pump. A single circuit for flow-through and a double circuit for flow-mix experiments are available. We used the continuous flow-mix system for our experiments. The total volume of the double circuit is appr. 1.5 ml (including measuring cell). The effective volume of the measuring cell is appr. 0.6 ml. All measurements were performed at $50\pm.10^{-4}$ °C and flow rate of 27.5 ml·h^{-1}.

The buffer used for all solutions for both enzyme and substrate was 0.05 M citrate buffer with pH 5.0. The flow-through system was filled with distilled water and sealed.

Determination of cellobiose activity

The microcalorimeter was calibrated by using the static calibration procedure at 50°C according to the instruction manual. A stable base line on the chart recorder and zero were then established using the clean 0.05 M citrate buffer and solutions of the enzymatic preparation in the citrate buffer under flow conditions. Both the solutions were located outside the microcalorimeter at room temperature under continuous stirring and pumped with exactly the same rate into the microcalorimeter. Both the solutions were then mixed in the flow-mix cell of the instrument. After mixing, the mixed flow passed through the measuring cell. When the stable base line was obtained, the clean buffer was changed step by step for solutions with different concentrations of cellobiose in the buffer. After mixing in the flow-mix cell, the enzymatic reaction of splitting cellobiose to glucose took place. The heat effect generated by the enzymatic reaction (or also by other side-reaction) was measured in the measuring cell. When the stable base line was obtained on the chart recorder, the stable signal output was read on a built-in digital voltmeter. The obtained signal outputs were corrected by substraction of the heat effects of dilution of cellobiose. The dilution heat effects were obtained by measuring the heat effects generated after mixing of the cellobiose solution with clean buffer. Before that, the stable base line and zero were established by using only the clean buffer.

Determination of glucose inhibition of cellobiose activity

Determination of glucose inhibition was carried out in a way similar to the determination of the sole cellobiose activity, but different amounts of glucose were added to each cellobiose solution.

The obtained signal outputs had to be corrected by substraction on the heat effects of dilution of cellobiose and glucose. The dilution heat effects of glucose were measured in a way similar to the cellobiase dilution effects.

Determination of CMC-activity

1% (w/v) of CMC was prepared by quickly stirring CMC in the buffer at 60°C. The stable base line and zero were established by mixing the clean buffer and solutions with different concentrations of CMC. When the stable base line was obtained, the clean buffer was changed for the solution of the enzymatic preparation (0.15 g of the preparation was dissolved and topped up to 100 ml with the buffer). The obtained stable signal outputs were corrected by substraction of the heat effect of dilution of the enzyme solution. The dilution heat effect was obtained by measuring the heat effect generated after mixing the enzyme solution with the clean buffer.

Determination of glucose inhibition of CMC-activity

Different amounts of glucose were added to each CMC solution. The further course of the determination was the same as during the determination of the sole CMC-activity.

The obtained stable signal outputs had to be corrected by substraction of the dilution heat effects of enzyme and glucose.

Determination of xylanase activity

0.2% (w/v) solution of xylan was prepared by boiling xylan in the buffer for 10 minutes. The procedure of the determination was the same as the procedure of the CMC-activity determination but the xylan solutions were used instead of the CMC solutions.

Results and discussion

Cellobiose activity

The saturation curve for the cellobiase activity 1.25 $IU \cdot ml^{-1}$ is reported in Fig. 1. The detection limit is about $1 \cdot 10^{-5}$ $mole \cdot l^{-1}$ of cellobiose in this case. The obtained curve has the typical hyperbolic shape for Michaelis-Menten kinetic behaviour. That is why we extrapolated from the Michaelis-Menten theory to interpret the results. We replaced the well-known Eq. (13)

$$V = \frac{V_{max} \cdot [S]}{K_M + [S]}$$

with an analogical equation

$$Q = \frac{Q_{max} \cdot [S]}{K_M + [S]}$$

where Q is heat flow ($J \cdot s^{-1}$ and Q_{max} is the heat flow under conditions of total saturation of an enzyme with a substrate. The values of Q_{max} and K_M were determined by using the computer program ENZFITER for enzymatic kinetics. The method according to Eady [14] was used for the computation. Figure 1 shows an application of the method too. We determined the values
$25.10 \cdot 10^{-6} \pm 1.47 \cdot 10^{-6}$ $J \cdot s^{-1}$ for Q_{max} and $1.103 \cdot 10^{-3} \pm 0.23 \cdot 10^{-3}$ $mole \cdot l^{-1}$ for K_M for this enzyme concentration.

The heat flows Q were determined at the same cellobiose concentration for different concentrations of enzyme and the Q_{max} values were then calculated according to the equation mentioned above. Because we found the linear dependence Q_{max} on enzyme concentration (Fig. 2), we replaced the equation $V_{max} = k_{+2} \cdot [E]$ [14], with an analogical equation $Q_{max} = k \cdot [E_{IU}]$, where enzyme concentration $[E_{IU}]$ is expressed in International Units.

The resulting equation will be

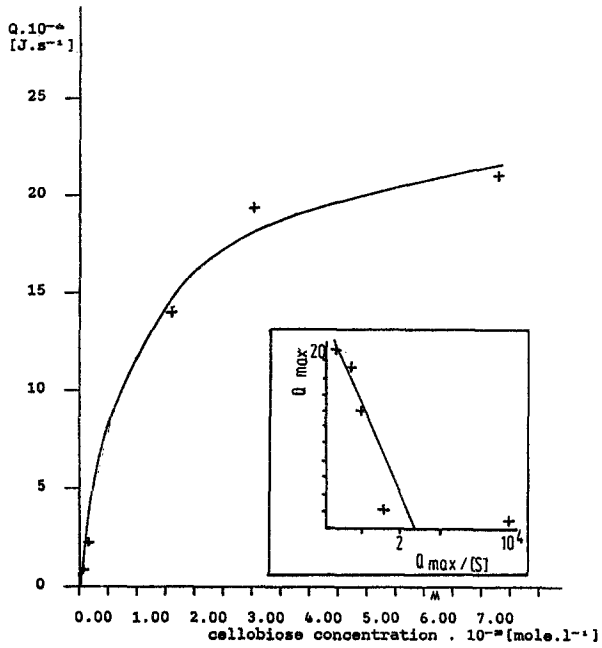**Fig. 1** Saturation curve of cellobiose activity (1.25 $IU \cdot ml^{-1}$)
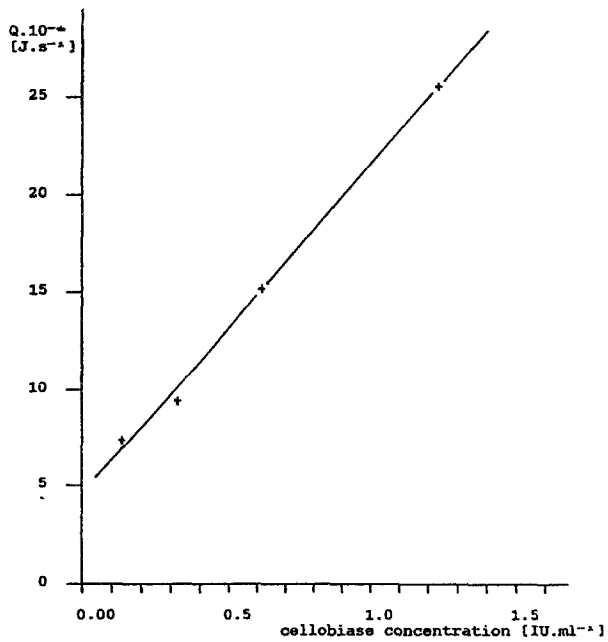

Fig. 2 Dependence Q_{max} on concentration of cellobiose activity

$$Q = \frac{k \cdot [E_{IU}] \cdot [S]}{K_m + [S]}$$

The value $k = 16.89 \pm 1.17$ J·s^{-1}·IU^{-1} was determined as the slope from Fig. 2. The constant k characterizes a substrate under method conditions.

Determination of glucose inhibition of cellobiase activity

Figure 3 shows saturation curves of cellobiose activity with different concentrations of glucose. The first three curves for glucose concentration lower than 0.5% (w/v) appear to have the typical courses for the end-product inhibition. However, when the glucose concentration was increased above 2.5% (w/v), the curves have atypical courses. Unexpected large heat responses appears at cellobiose concentrations higher than appr. $1.5 \cdot 10^{-3}$ mole·l^{-1}. It is very interesting, that a similar effect was not observed when only glucose and enzyme were used, without cellobiose. Therefore, all three components (cellobiose, glucose and enzyme) must be presented in a solution to generate the discussed heat response. That is why we suppose that the heat effect is a possible consequence of conformational changes of the enzyme. Similar behaviour is typical of
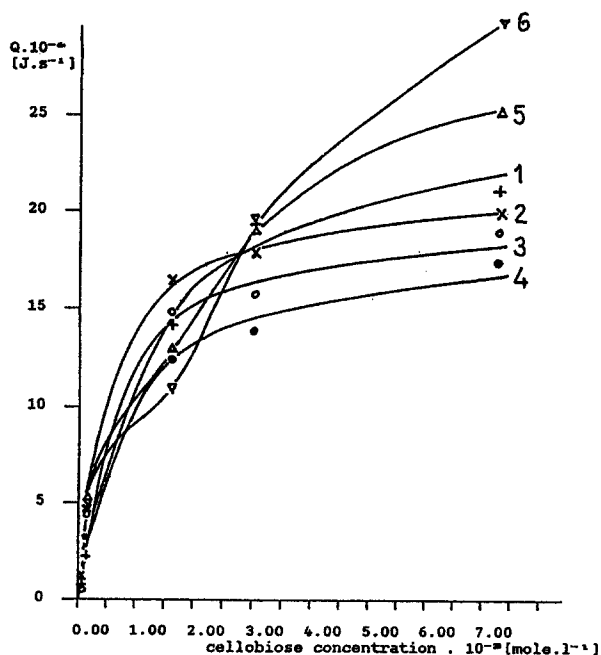

Fig. 3 Inhibition influence of glucose on cellobiase activity (1.25 IU·ml^{-1}). Glucose was added to cellobiose solutions. Glucose concentrations in the reaction mixture: 1: 0.000% (w/v), 2: 0.005% (w/v), 3: 0.050% (w/v), 4: 0.500% (w/v), 5: 2.500% (w/v), 6: 5.000% (w/v)

acompetitive enzyme inhibitions, when an inhibitor may be bound only when a binding substrate changes the enzyme conformation suitably [14].

Table 1 contains so called apparent values Q_{max} and K_M for the three lowest glucose concentrations (Fig. 3). The lower values of the apparent K_M for the inhibitory curves, in comparison with the uninhibited curve, support the hypothesis of acompetitive inhibition.

Table 1 Glucose inhibition of cellobiose activity (1.25 $IU \cdot ml^{-1}$)

	Glucose concentration (% w/v)			
	0.000	0.005	0.050	0.500
$Q_{max} / J \cdot s^{-1} \cdot 10^{-6}$	25.10±1.47	21.15±0.47	19.11±0.68	18.52±0.96
$K_M /$ mole$\cdot l^{-1} \cdot 10^{-4}$	11.02±2.30	4.76±0.54	4.44±0.82	7.03±1.62

But this assumption must be confirmed by other experiments, probably in conjunction with other analytical techniques. For an exact determination of the real kinetic constants of glucose inhibition it would be probably necessary to distinguish the heat contributions of the real enzyme reaction and the conformational change.

Determination of CMC-activity and glucose inhibition influence

Figure 4 shows a saturation curve of CMC-activity (3.45 $IU \cdot ml^{-1}$) and saturation curves with 2.5 and 5.0% (w/v) of glucose. Because of the polymeric nature of the substrate, the simultaneous action of endo- and exo-glucanases and the variety of intermediate products, it is very difficult to interpret these curves and to determine kinetic constants. It will probably be necessary to separate individual activities and to use other analytical techniques for further study of cellulase activities and mechanisms of feedback control. The courses of the inhibitory curves have the typical goosenecked shape corresponding to the cooperative action of the allosteric inhibitors.

Determination of xylanase activity

Figure 5 shows a saturation curve of xylanase activity (3.15 $IU \ ml^{-1}$). Similar problems, as in the previous case, such as the polymeric structure of substrate and the variety of intermediate products, complicate the interpretation of the results. Another problem is the low solubility of xylan.

Conclusions

The described method allows one to determine directly the cellobiose activity, or the cellobiose concentration, by simply measuring the heat involved in the cellobiose
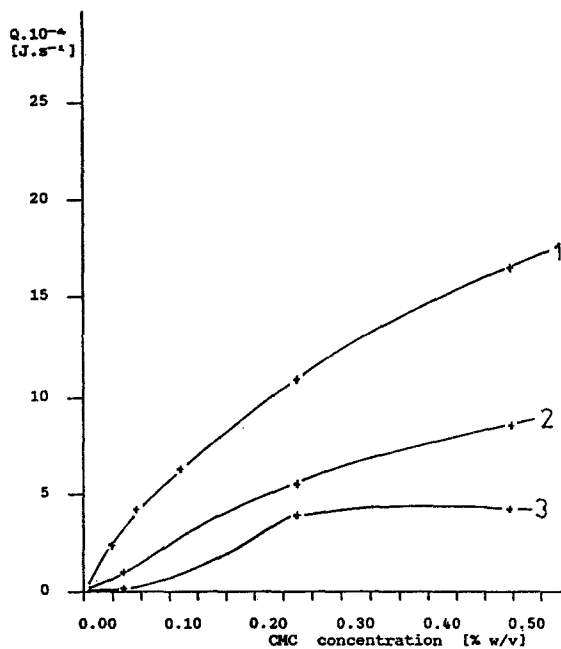
Fig. 4 Saturation curve of CMC-activity and inhibition of glucose ($3.45\ IU \cdot ml^{-1}$). Glucose was added to CMC solutions. Glucose concentrations in the reaction mixture: 1: 0.000% (w/v), 2: 2.500% (w/v), 3: 5.000% (w/v)
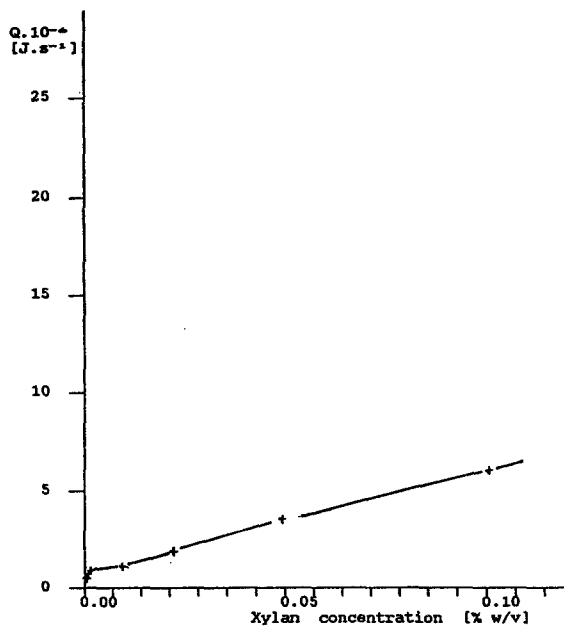

Fig. 5 Saturation curve of xylanase activity ($3.15\ IU \cdot ml^{-1}$)

hydrolysis. The high sensitivity of the thermal activity monitor, the simplicity of the analytical procedure and the general detection principle make the widespread application of these calorimetric method possible.

The method is a very suitable tool for observing various mechanisms of feedback control by products of enzymatic action. The observation of the glucose inhibition of the cellobiase activity described here is a demonstration of the use of the method in this way. The obtained results suggest the acompetetive nature of the inhibition.

Because of the polymeric nature of the substrates and the other problems described under Results and discussion, it is very difficult to interpret the results of the determination of the CMC- and xylanase activity. Nevertheless, we consider microcalorimetry to be a very suitable tool for the study of enzyme complexes, especially in combination with other analytical techniques. Our contribution is only an example of the possibilities of utilizing microcalorimetry in this field.

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Zusammenfassung — Es wird eine Methode zur Bestimmung der enzymatischen Aktivität des Trichoderma viridae Zellulase Komplexes beschrieben. Dazu wurde ein LKB 2277 Thermoaktivitätsmonitor und ein kontinuierlicher Flüsigkeitsmischer verwendet.
Bei der Bestimmung der enzymatischen Aktivität von Proben des rohen Zellulasekomplexes wurden drei Substanzen verwendet: Zellobiose, Karboxymethylzellulose (CMC) und Hemizellulose. Anhand der Ergebnisse wurden einige kinetische Parameter für die Zellobiaseaktivität ermittelt. Außerdem wurde eine Endprodukt-Inhibition von Zellobiase sowie Karboxymethylzellulaseaktivität durch Glukose beobachtet.
Das beschriebene Verfahren erlaubt eine direkte Bestimmung der verschiedenen Enzymaktivitäten des Zellulasekomplexes. Wegen ihrer hohen Empfindlichkeit und Einfachheit handelt es sich hier um ein sehr nützliches Werkzeug zur Untersuchung des Zellulasekomplexes, indem die optimalen Bedingungen für die enzymatische Spaltung zelluloser Substanzen bestimmt und verschiedene Mechanismen von Feedback-Kontrolle durch die Produkte der Enzymtätigkeit beobachtet werden. Das Verfahren trägt vollkommen allgemeinen Charakter und kann auch bei anderen Ezymsystemen Anwendung finden.