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Kinetics and Mechanism of the Hydrolysis of Sodium Carboxymethylcellulose (Na-*CMC*) by a Cellulase Complex*

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The *Somogyi-Nelson* colorimetric method is applied in a new manner more suitable for evaluating the kinetics of the enzyme hydrolysis of sodium carboxymethylcellulose (Na-*CMC*) catalyzed by the cellulase complex. By means of selective inhibition of a chosen enzyme from the cellulase complex it became possible to trace the effect of the other enzymes included in its composition.

(Keywords: Cellulase complex; Endo-β-1,4-glucanase; Exo-cellobiohydrolase; Exo-β-1,4-glucanase; β-Glucosidase; Cellobiase; Inhibition of cellulase enzymes; Lactose; Condurrite-B-epoxide)

Kinetik und Mechanismus der Hydrolyse von Natriumcarboxymethylcellulose (Na-CMC) durch einen Cellulase-Komplex

Die kolorimetrische Methode nach *Somogyi* und *Nelson* wird nach einem neuen Verfahren zur Verfolgung der Kinetik der hydrolytischen Spaltung von Natriumcarboxymethylcellulose (Na-*CMC*), katalysiert durch den Cellulase-Komplex, angewandt. Durch selektive Inhibierung eines bestimmten Enzyms des Cellulase-Komplexes kann man die Wirkung der anderen zu seiner gesamten Zusammensetzung gehörenden Enzyme verfolgen.

Introduction

The intensive research of the cellulase complex of certain microorganisms is connected with the role and significance of these enzymes for cellulose hydrolysis with a view to the production of fuel (ethanol), food

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(single cell protein) and other chemicals[1—7]. It was shown by a number of authors that the cellulase complex consists of at least three enzymes: cellulase or *endo*-β-1,4-glucanase (EC 3.2.1.4), *exo*-cellobiohydrolase or *exo*-β-1,4-glucanase (EC 3.2.1.91) and β-glucosidase or cellobiase (EC 3.2.1.21), which act in a strictly defined synergism[8—13]. The enzyme mechanism and the kinetics of cellulose degradation have been the object of a great number of studies[14—18]. Investigations on the inhibition of the enzymes of the cellulase complex have shown that each one of them can be inhibited separately by a suitable selective inhibitor, namely: cellulase by lactose[19,20], *exo*-cellobiohydrolase by calcium chloride[21] and β-glucosidase by condurrite-B-epoxide[22].

In our preceding paper[23] the *Somogyi-Nelson* arsenomolybdate method was applied in a new manner to analyse the kinetics of the cellobiose hydrolysis catalyzed by β-glucosidase.

In this paper we describe the kinetics and mechanism of sodium carboxymethylcellulose (Na-*CMC*) hydrolysis catalyzed by a cellulase complex. This work indicates that, by means of selective inhibition of a chosen enzyme from the cellulase complex, it is possible to trace the effect of the other enzymes included in its composition.

Materials and Method

Enzyme Source. As a source of the cellulase complex the commercial cellulase preparation "Luizym"® from *Aspergillus oryzae* ("Luitpoldwerk", FRG) was used, which contains: cellulase (E'), *exo*-cellobiohydrolase (E'') and β-glucosidase (E''').

Substrate. As substrate was used Na-*CMC* (preparation "Tylose C 10"®, "Hoechst AG", FRG) with DS = 0.7 and M_s = 30 000 g/mol.

Inhibitors. As specific inhibitors of the enzymes of the cellulase complex were used: lactose (I') for E', calcium chloride (I'') for E'' and condurrite-B-epoxide or 3,5/4,6-tetrahydroxy-cyclohexenoxid (I''') for E'''.

Chemicals. The chemicals used in this work were purchased from Koch-Light Laboratories Ltd., England.

Measurement of Reducing Power. The reducing power of the sugars (cellobiose and higher cellodextrins) was determined by the unmodified *Somogyi-Nelson* method[23].

Kinetic Measurements. The enzyme-substrate reaction (both with and without an inhibitor) was carried out in a 1 cm cell (total volume 2.5 ml) in the recording two-beam spectrophotometer "Specord UV-VIS" ("Carl Zeiss", GDR) at pH = 5.0 (0.1 M acetate buffer) and a temperature of 37 °C. Right at the beginning of the reaction a colour reagent, consisting of solutions of *Somogyi*'s (A : B = 25 : 1) and *Nelson's* reagents[23] in a volume ratio of 1 : 1, was added to a mixture of a buffer solution (0.1 M acetate buffer, pH = 5.0), Na-*CMC* and a cellulase preparation (with or without a corresponding inhibitor of the same concentration as that of the cellulase preparation). In a control experiment this composition was retained with the enzyme omitted. The continuous change in the absorption of the sample was

automatically recorded at 760 nm. The time required to record a single curve was 1 min.

Paper Chromatography. The sugars were separated by one-dimensional paper chromatography on Filtrak No. 1 ("VEB Spezialpapierfabrik Niederschlag", GDR) using *n*-butanol : acetic acid : water (4 : 1 : 1) as solvent[24]; Locating reagent: spraying with a solution of anilinhydrophthalate and heating at 100 °C for 10 min[25].

Results and Discussion

According to our method previously described[23] the molar absorption coefficients (ε) for cellobiose, cellotetraose and cellohexaose were determined (Table 1).

Table 1. *The molar absorption coefficients for some sugars with known molecular mass*

Sugars	Molecular mass (g/mol)	Molar absorption coefficient $\varepsilon (M^{-1}\,cm^{-1})$
D-glucose[23]	180	$4.50 \cdot 10^4$
Cellobiose	342	$4.87 \cdot 10^4$
Cellotetraose	666	$4.75 \cdot 10^4$
Cellohexaose	990	$4.80 \cdot 10^4$
	Average	$4.68 \cdot 10^4$

The relation between the change in absorbance (ΔA) at 760 nm and the reaction time (0–7 h) during the hydrolysis of Na-*CMC* catalyzed by the cellulase enzyme complex, with different weight concentrations $[E]_w$ of the latter, is shown in Fig. 1 *A*. In the same figure (1 *B*) the relation obtained at different substrate concentrations $[S]_0$ is represented. At increased concentrations of enzyme or substrate the kinetic curves show a clear three step behaviour, corresponding to the effect of the different components (E', E'' and E''') of the cellulase enzyme complex.

The proposed mechanism of cellulase action which is in accordance with the literature data[1–13] is given in Fig. 2 and consists of the following: a) under the catalytic effect of the enzyme cellulase, the glucoside bonds in the molecule of *Na-CMC* (*S*) are hydrolysed randomly to give oligosaccharides (P'_∞) with a final degree of polymerization $3 \leqslant DP \leqslant 6$; b) under the catalytic effect of the enzyme *exo*-cellobiohydrolase molecules of cellobiose (P''_∞; $DP = 2$) are separated from the reducing end of the chains (Na-*CMC* and oligosaccharides); c) the enzyme β-glucosidase catalyzes the hydrolysis of the disaccharide cellobiose to *D*-glucose (P'''_∞; $DP = 1$).

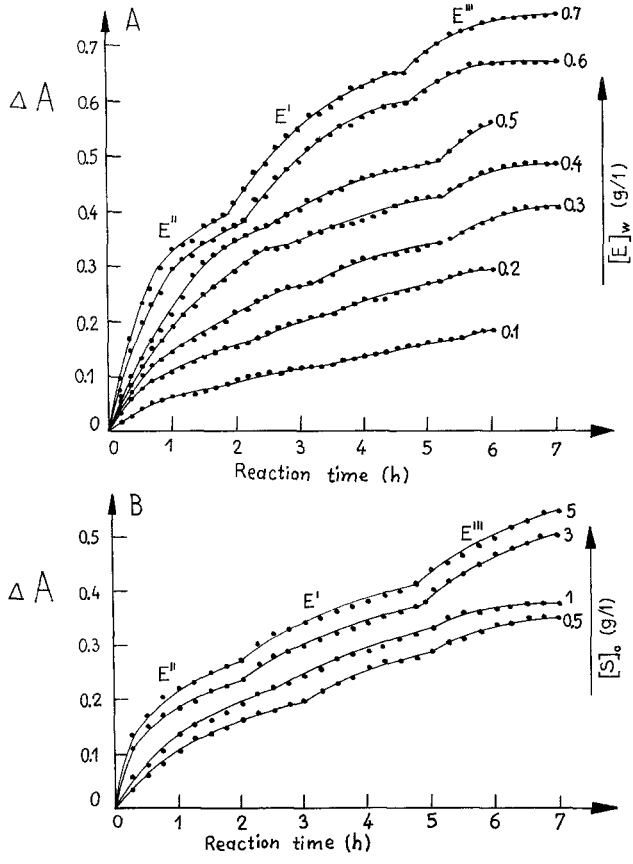

Fig. 1. Kinetic curves of the reaction "Na-*CMC*—cellulase enzyme complex": *A* at different concentrations of the cellulase complex and a constant substrate concentration 1.5 g/l; *B* at a constant concentration of the same enzyme complex (0.4 g/l) and at different substrate concentrations

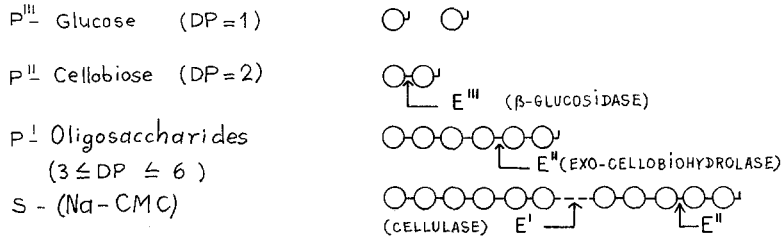

Fig. 2. Mode of action of the enzymes of the cellulase complex catalyzing the hydrolysis of Na-*CMC*

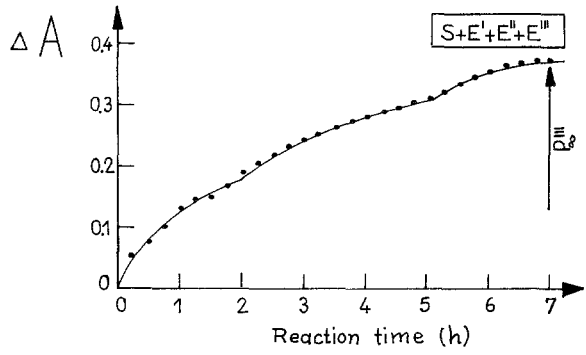

Fig. 3. Three-step kinetic curve of the enzyme hydrolysis of Na-*CMC* catalyzed by the enzymes of the cellulase complex. Reaction conditions: $[S]_0 = 1.0$ g/l, $[E]_w = 0.4$ g/l, $pH = 5.0$, 37 °C

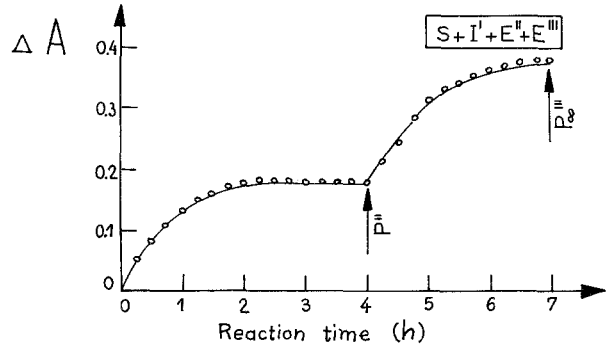

Fig. 4. Two-step kinetic curve of enzyme reactions catalyzed by *exo*-cellobiohydrolase (E'') and β-glucosidase (E''') in the presence of the inhibitor lactose (I') under reaction conditions: $[S]_0 = 1.0$ g/l, $[E]_w = [I'] = 0.4$ g/l, $pH = 5.0$, 37 °C

Fig. 3 shows a three-step kinetic curve obtained in our experiments, resulting from the simultaneous participation of the three enzymes in the catalytic reaction. The probable kinetic mechanism of the separate reactions with the participation of the listed enzymes of the cellulase complex can be represented as follows:

$$
\begin{array}{l}
P'' + E'' \overset{k''_{+2}}{\leftarrow} (E''\,S) \overset{K''_s}{\rightleftharpoons} E'' + S + E' \quad \overset{K'_s}{\rightleftharpoons} (E'\,S) \overset{k'_{+2}}{\rightarrow} E' + {}^{\circ}P' \\
\qquad + E''' \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad + E'' \\
\qquad \Uparrow\Downarrow K'''_s \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \Uparrow\Downarrow K''_s \\
\qquad (E'''\,P') \overset{k'''_{+2}}{\rightarrow} E''' + P'''_{\infty} \qquad\qquad\qquad\qquad\qquad\qquad P'' + P'''_{\infty} + E'' \overset{k''_{+2}}{\leftarrow} (E''\,P') \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad + E''' \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \Uparrow\Downarrow K'''_s \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad (E'''\,P'') \overset{k'''_{+2}}{\rightarrow} E''' + P'''_{\infty}
\end{array}
$$

In order to determine the individual catalytic participation of each enzyme of the cellulase complex and its corresponding step on the kinetic curve, a partial inhibition of a definite enzyme component was carried out with the respective selective inhibitor[19—22].

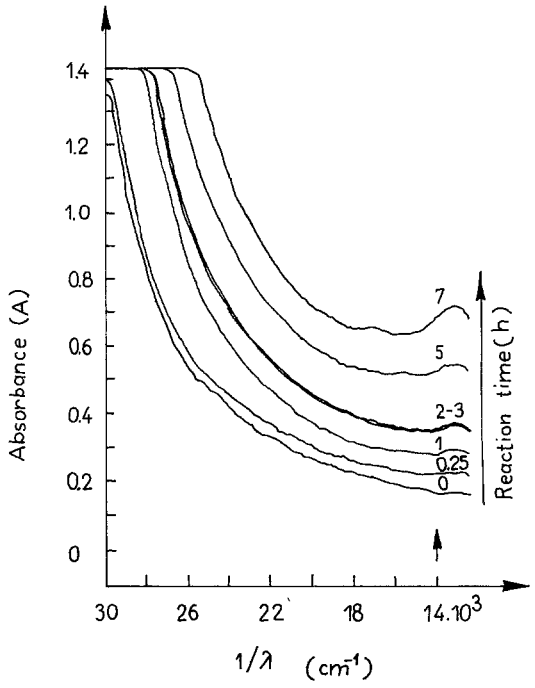

Fig. 5. Absorption spectra of the colour developed by means of our method[23] at various times (0 to 7 h); Reaction mixture: $[S]_0 = 5.0$ g/l, $[E]_w = [I] = 0.4$ g/l; Reaction conditions: $pH = 5.0$, temperature 37 °C and active enzymes-*exo*-cellobiohydrolase (E') and β-glucosidase (E''')

With the inhibition of enzyme E' a two-step kinetic curve is obtained, as represented in Fig. 4, corresponding to the effect of enzymes E'' and E'''. The probable kinetic mechanism of the reactions, with the latter's participation, can be represented as:

$$\begin{array}{lll} & (E''' P''') \xrightarrow{k'''_{+2}} E''' + P'''_{\infty} \\ & \downarrow\uparrow K'''_s & \\ & + E''' & \\ (E' S) \xrightarrow{k'_{+2}} E' + P' & & \\ \downarrow\uparrow K'_s & & \\ S + E' & E' + I' \xrightarrow{K_I} (E' I') & \end{array}$$

In the curves of Fig. 4 it is noteworthy that the enzyme β-glucosidase (E''') begins to be active after the incubation period of about 4 hours. It is shown in Fig. 5 that in the second and third hours of the reaction time no enzyme-substrate reaction takes place, i.e. the absorbance is the same, but at about the fourth hour hydrolysis continues. This fact, quite interesting from the enzymological point of view, shows that a certain accumulation of the reaction product (cellobiose) is necessary in order to begin its degradation under the effect of the enzyme β-glucosidase. Through a paper chromatographic method[24,25] it was ascertained that the product

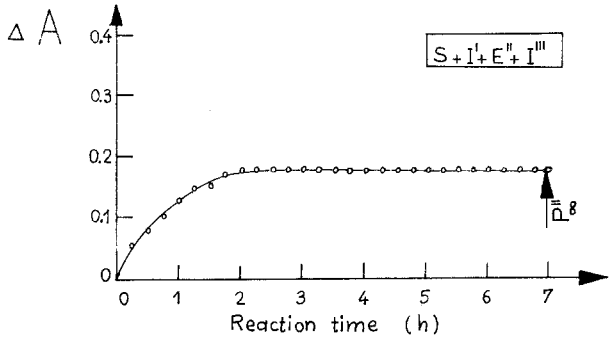

Fig. 6. One-step kinetic curve of enzyme reaction catalyzed by *exo*-cellobiohydrolase (E') in the presence of the inhibitors; lactose (I') and condurrite-B-epoxide (I''); Reaction conditions: $[S]_0 = 1.0$ g/l, $[E]_w = [I'] = [I''] = 0.4$ g/l, $pH = 5.0$, 37 °C

obtained after three hours reaction time was predominantly cellobiose and after more than four (in this case seven) hours D-glucose.

When enzymes E' and E''' are inhibited, a one-step kinetic curve is obtained (see Fig. 6), corresponding to the effect of enzyme E'' (*exo*-cellobiohydrolase). The probable kinetic mechanism of the reaction catalyzed by the latter is:

$$\begin{array}{lll} (E''\,S) \overset{k''_{+2}}{\rightarrow} E'' + P''_{\alpha} & & \\ \uparrow\downarrow K''_s & E' + I' \overset{K'_I}{\rightarrow} (E'\,I') & \\ S + E'' & & \\ & E''' + I''' \overset{K'''_I}{\rightarrow} (E'''\,I''') & \end{array}$$

After 7 hours reaction time the product obtained was cellobiose (paper chromatography).

In Fig. 7, the two-step kinetic curve obtained by the inhibition of enzyme E''' is represented, which corresponds to the effect of enzymes E'

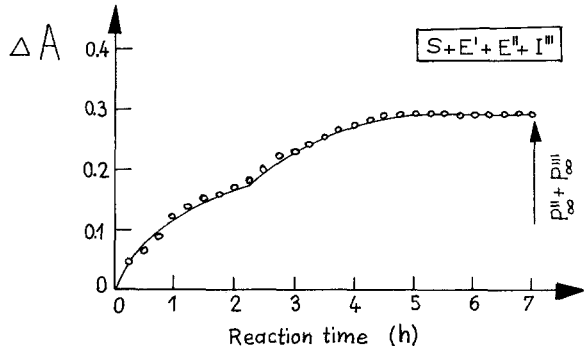

Fig. 7. Two-step kinetic curve of reactions catalyzed by cellulase (E') and *exo*-cellobiohydrolase (E'') in the presence of the inihibitor condurrite-B-epoxide (I''); Reaction conditions: $[S]_0 = 1.0$ g/l, $[E]_w = [I''] = 0.4$ g/l, $pH = 5.0$, 37 °C

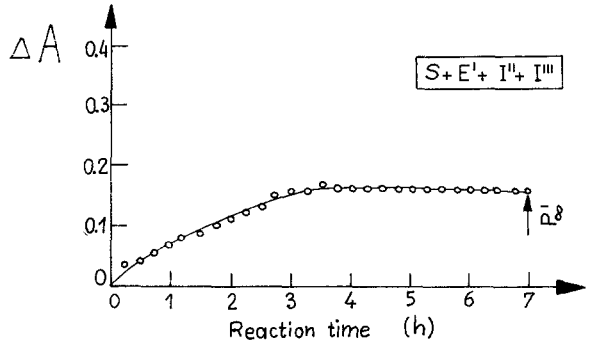

Fig. 8. One-step kinetic curve of enzyme reaction catalyzed by cellulase (E') in the presence of the inhibitors: calcium chloride (I') and condurrite-B-epoxide (I''); Reaction conditions: $[S]_0 = 1.0$ g/l, $[E]_w = [I'] = [I''] = 0.4$ g/l, $pH = 5.0$, 37 °C

and E''. Remarkably, there is the synergistic effect with these two enzymes, which has been repeatedly ascertained by other authors[9,26,27]. The probable kinetic mechanism of the reactions catalyzed by the enzymes examined can be represented in the following way:

$$
\begin{array}{lcl}
(E''S) \overset{K_s''}{\rightleftharpoons} E'' + S + E' \overset{K_s'}{\rightleftharpoons} (E'S) \overset{k'_{+2}}{\rightarrow} E' + P' & & \\
\downarrow k''_{+2} & & + E'' \\
E'' + P''_{\infty} & & \Downarrow K_s'' \\
 & P'''_{\infty} + P''_{\infty} + E'' \overset{k''_{+2}}{\leftarrow} (E''P') & \\
E''' + I'' \overset{K_I''}{\rightarrow} (E'''I'') & &
\end{array}
$$

After 7 hours reaction time, the products obtained were cellobiose and *D*-glucose (paper chromatography).

In Fig. 8 the one-step kinetic curve obtained by the inhibition of enzymes E'' and E''' is represented, which corresponds to enzyme E'

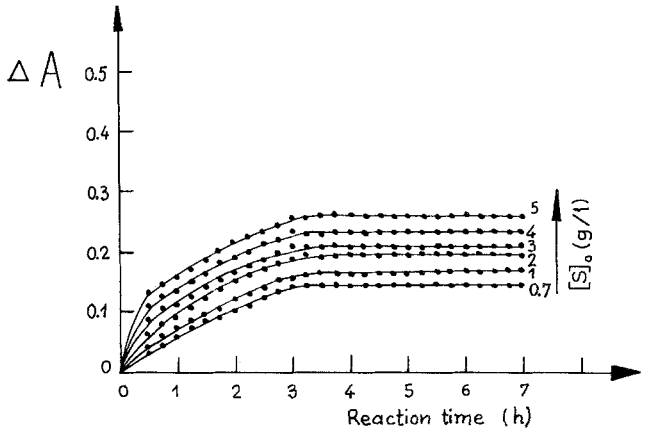

Fig. 9. Kinetics of hydrolysis of Na-*CMC* with different weight concentrations, catalyzed by cellulase (E')

(cellulase). The probable kinetic mechanism of the reaction catalyzed by the latter is:

$$S + E' \overset{K'_s}{\rightleftharpoons} (E'\,S) \overset{k'_{+2}}{\rightarrow} E' + P'_{\infty}$$

$$E'' + I' \overset{K''_I}{\rightarrow} (E''\,I')$$

$$E''' + I'' \overset{K'''_I}{\rightarrow} (E'''\,I'')$$

The paper chromatographic investigation of the products obtained showed the presence of oligosaccharides with $DP = 3$–6.

From the kinetic curves depicted on Figs. 1, 4, and 7, the synergistic effect of the enzymes of the cellulase complex is obvious. The concentrations of the end products of the reaction "Na-*CMC*—cellulase enzyme complex" proved to be equal, both according to the simple *Somogyi-Nelson* colorimetric method and the one we proposed[23]. These results

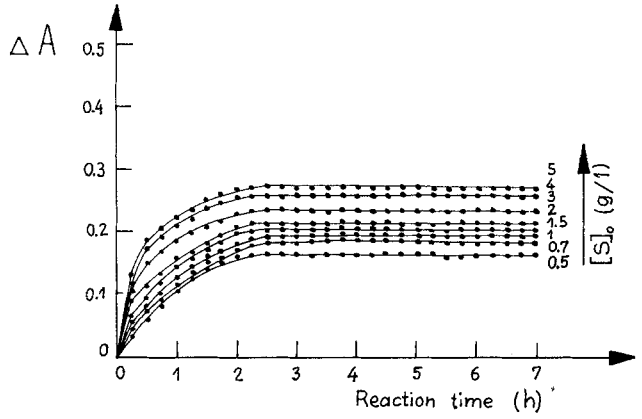

Fig. 10. Kinetics of hydrolysis of Na-*CMC* with different weight concentrations, catalyzed by *exo*-cellobiohydrolase (*E''*)

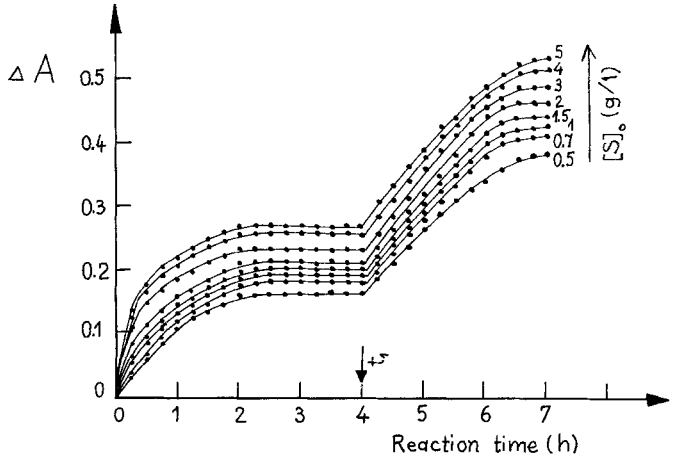

Fig. 11. Kinetics of hydrolysis of Na-*CMC* with different weight concentrations, catalyzed by *exo*-cellobiohydrolase (*E''*) and β-glucosidase (*E'''*)

enabled us further to examine the effect of the separate enzymes of the cellulase multicomponent system.

In Fig. 9 the kinetic curves of the Na-*CMC* hydrolysis with various initial substrate concentrations are represented, as catalyzed by the enzyme cellulase (*E'*), (see Fig. 8). The end products of the reaction were oligosaccharides with a polymerization degree of 3–6.

In Fig. 10 the kinetic curves of the Na-*CMC* hydrolysis with various initial substrate concentrations are represented, as catalyzed by the enzyme *exo*-cellobiohydrolyase (*E''*), (see Fig. 6). The end reaction product was cellobiose.

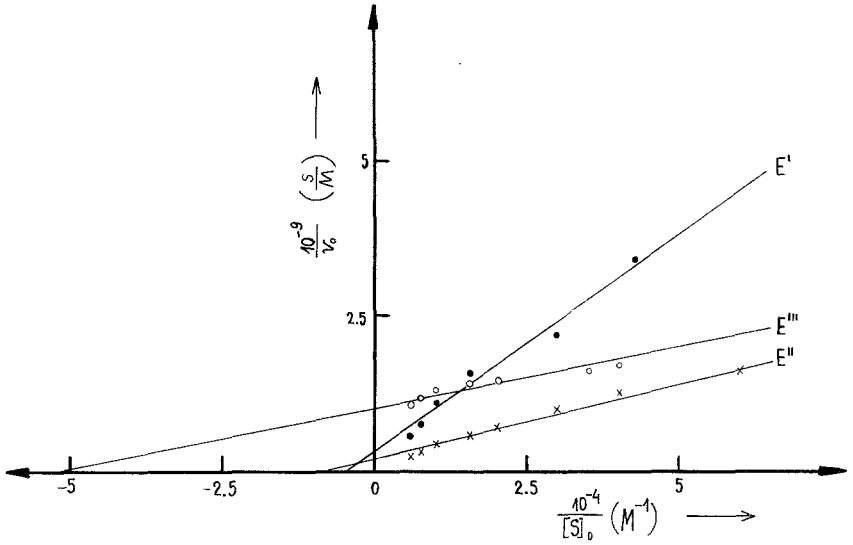

Fig. 12. *Lineweaver-Burk* plots of the reaction "Na-*CMC*—cellulase enzyme complex" obtained from Fig. 9 (for cellulase, *E'*), 10 (for *exo*-cellobiohydrolase, *E''*) and 11 (for *β*-glucosidase, *E'''*)

Table 2. *Michaelis parameters for cellulase, exo-cellobiohydrolase and β-glucosidase*

Enzymes of the cellulase complex	In the presence of the specific inhibitors	$Km(M)$	$V(M/s)$
Cellulase (*E'*)	$I' + I''$	$2.50 \cdot 10^{4}$	$3.30 \cdot 10^{-9}$
exo-cellobiohydrolase (*E''*)	$I + I'''$	$1.25 \cdot 10^{4}$	$5.00 \cdot 10^{-9}$
β-glucosidase (*E'''*)	I	$0.20 \cdot 10^{4}$	$0.95 \cdot 10^{-9}$

In Fig. 11 the kinetic curves of the Na-*CMC* hydrolysis with certain initial substrate concentrations are represented, with consecutive catalysis by *exo*-cellobiohydrolase (*E''*) and *β*-glucosidase (*E'''*), as in Fig. 4. The end product of the first stage of this reaction was cellobiose, which was hydrolyzed to *D*-glucose in the second stage.

Fig. 12 shows *Lineweaver-Burk*[28] plots of data from the reaction "Na-*CMC*—cellulase enzyme complex" obtained from Fig. 9 (for cellulase), Fig. 10 (for *exo*-cellobiohydrolase) and Fig. 11 (for β-glucosidase). The determined values for *Km* and *V* from these data are presented in Table 2.

Acknowledgements

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Symbols Used

E	enzyme (E'—cellulase; E''—*exo*-cellobiohydrolase; E'''—β-glucosidase)
$[E]_w$	weight concentration of enzyme E
S	substrate (Na-*CMC*—sodium carboxymethylcellulose)
$[S]_0$	weight concentration of substrate S
I	inhibitor (I'—lactose; I''—calcium chloride; I''—condurrite-B-epoxide)
P	product (P'—oligosaccharides; P''—cellobiose; P'''—*D*-glucose)
P_∞	end product (P'_∞, P''_∞, P'''_∞)
DP	degree of polymerization
DS	degree of substitution
ES	enzyme-substrate complex ($E'S$, $E''S$, $E'''S$)
EP	enzyme-product complex ($E'P'$, $E''P''$)
EI	enzyme-inhibitor complex ($E'I'$, $E''I'$, $E'''I''$)
M_s	molecular mass of substrate S
K_s	substrate constant (K'_s, K''_s, K'''_s)
K_I	inhibitor constant (K'_I, K''_I, K'''_I)
K_m	*Michaelis-Menten* constant
k_{+1}, k_{+2}	(k'_{+2}, k''_{+2}, k'''_{+2}) forward rate constants
k_{-1}	reverse rate constant
v_0	initial rate of reaction
V	maximal reaction rate
ΔA	change in absorbance
ε	molar absorption coefficient
λ	wavelength

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