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Kinetics of Enzymatic Hydrolysis of Sodium Carboxymethylcellulose with Different Degrees of Polymerization by Cellulase

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The reaction cellulase (EC 3.2.1.4)—sodium carboxymethylcellulose (Na-*CMC*) with different degrees of polymerization ($n = 140$, 640 and 900) was investigated by the use of a modified *Michaelis-Menten* equation, valid for enzymatic hydrolysis of linear homopolymers. The *Michaelis-Menten* constant [$Km'(M) = 6.31 \cdot 10^{-2}$ mol/dm^3] and the reaction rate constant ($k'_{+2} = 4.07 \cdot 10^{-6}$ s^{-1}), which correspond to the enzymatic hydrolysis of a single bond in the homopolymer substrates are determined. The free energy ($\Delta G^{\neq} = 101$ kJ/mol), which corresponds to the degradation and formation of a single bond in the enzyme—polymer substrate is also estimated. This energy expressed in electron-volt units is $\Delta E^{\neq} = 1.39$ eV. The ratio between the effective cross section of the reactive substrate bond and the active enzyme center is $\alpha = 1.22$.

(Keywords: Cellulase; Cellulase kinetics; Sodium carboxymethylcellulose; Linear homopolymer substrates; Michaelis-Menten constant; Reaction rate constant; Free energy)

Kinetik der enzymkatalysierten Hydrolyse von Natriumcarboxymethylcellulose mit verschiedenem Polymerisationsgrad durch Cellulase

Die modifizierte Gleichung nach *Michaelis-Menten* wird bei der durch Cellulase (EC 3.2.1.4) katalysierten hydrolytischen Spaltung von Natriumcarboxy-methylcellulose (Na-*CMC*) verschiedenen Polymerisationsgrades ($n = 140$, 640 und 900) angewandt. Es wurde die *Michaelis-Menten*-Konstante [$Km'(M) = 6.31 \cdot 10^{-2}$ mol/dm^3] und die Reaktionsgeschwindigkeitskonstante ($k'_{+2} = 4.07 \cdot 10^{-6}$ s^{-1}), die der enzymatischen Hydrolyse einer Einfachbindung im homopolymeren Substrat entspricht, berechnet. Die freie Energie ($\Delta G^{\neq} = 101$ kJ/mol), die dem Abbau und der Bildung einer Einfachbindung im Enzym—Polymer-Substrat entspricht, wurde bestimmt. Diese Energie — ausgedrückt in Elektronvolt-Einheiten — beträgt $\Delta E^{\neq} = 1.39$ eV. Das Verhältnis

zwischen den effektiven Querschnitten der reaktiven Substratbindung (σ_S) und des aktiven Enzym-Zentrums (σ_E) beträgt $\alpha = 1.22$.

Introduction

When the kinetics of enzyme reactions involving the hydrolysis of linear homopolymers is examined, the *Michaelis-Menten* equation[1] is inadequate since it does not take into account the degree of polymerization or the molecular mass of the substrate. The application of the *Michaelis-Menten* equation for reactions of enzymatic hydrolysis of polymers results in an estimation of the maximum reaction rate (V) and the *Michaelis-Menten* constant (Km) which varies with the degree of polymerization of the substrate.

Recently a new theory was proposed[2] which permits the derivation of a modified *Michaelis-Menten* equation. This equation can be applied to kinetic studies of the enzymatic hydrolysis of linear homopolymers with different degrees of polymerization. The modified *Michaelis-Menten* equation makes it possible to define V which increases with the molecular mass of the substrate and Km (expressed in molar concentration) which decreases with the increase in the number of bonds in the substrate molecule. The modified equation allows the definition of the rate constant k'_{+2} and $Km'(M)$ which corresponds to a single hydrolyzed substrate bond. These kinetic parameters can be used as a basis for the estimation of the real kinetic properties of the enzyme.

In the present report the modified equation of *Michaelis-Menten* is used to study some kinetic parameters of the hydrolysis of sodium carboxymethylcellulose (Na-*CMC*) with different degrees of polymerization catalyzed by cellulase.

Experimental

Materials

A commercial cellulase preparation obtained from *Aspergillus sp.* (Celluzym®, Nagase & Co., Japan) was used as a source of cellulase (EC 3.2.1.4). The commercial preparation was partially purified by low speed centrifugation followed by extensive dialysis. The protein was lyophilized and dissolved in a sodium citrate-phosphate buffer ($pH = 5.0$) at a concentration of $[E]_0 = 25\,\mathrm{mg/dm^3}$.

Commercial preparations of Na-*CMC* (Tylose C®, Hoechst AG, FRG) with a degree of substitution of 0.7 and with different degrees of polymerization (n) were used:

Substrate	M_S	n
Tylose C-10	30 000	140
Tylose C-1000p	140 000	640
Tylose C-6000	200 000	900

The Na-*CMC* preparations were additionally purified from water-insoluble substances as described by *Datta* et al.[3]. The purified substrates were dried and dissolved before the assay of hydrolysis at different initial concentrations $[S]_0 = 2\text{–}25\,g/dm^3$.

Kinetic Measurements

The hydrolysis of Na-*CMC* with different degrees of polymerization catalyzed by cellulase was examined at 37 °C and $pH = 5.0$. The reducing sugars formed after hydrolysis were determined by the arsenomolybdate method according to published procedures[4—6]. The initial velocity (v_0) was determined 5 min after the start of the enzyme reaction. *Km* and *V* for each substrate were estimated according to the double reciprocal linear plot of the *Michaelis-Menten* equation[7]:

$$\frac{[S]}{v_0} = \frac{1}{V}[S] + \frac{Km}{V} \tag{1}$$

$1/V$ and *Km* were evaluated by linear regression analysis[7,8]. The standard deviations were determined from an average of 28 kinetic experiments at different substrate concentrations.

The *Michaelis-Menten* constant $Km'(M)$, expressed in molar concentr., and the reaction rate constant k'_{+2} which correspond to a single hydrolysed substrate bond were graphically estimated by the plotting of $Km(M)$ and (k_{+2}) vs. the number of β-1,4-glucoside bonds in the substrate[2]. The free energy of the formation and degradation of the enzyme-substrate complex (ΔG^{+}), the free energy of degradation of a single substrate bond (ΔE^{+}) in electron-volts (eV), as well as the ratio (α) between the effective cross sections of the reactive substrate bond (σ_S) and the active enzyme center (σ_E) were determined as described previously[2].

Results and Discussion

Km (in weight or in molar concentration), *V* and k_{+2} estimated for the enzyme catalyzed reactions of hydrolysis of Na-*CMC* with different degrees of polymerization are presented in Table 1.

The *Michaelis-Menten* constant $Km'(M)$ and the reaction rate constant k'_{+2}, each corresponding to a single degraded substrate bond, were determined by using a recently derived modification of the *Michaelis-Menten* equation, valid for the type of enzyme reactions examined[2].

$$v_0 = \frac{V[S]}{Km + [S]} = \frac{k'_{+2}[E](1 + M_S/M_E)(b—b_\infty)[S]}{\frac{k'_{-1} + k'_{+2}}{k'_{+1}} \frac{a}{(C_b^a)^\alpha} \frac{M_S}{N_A} + [S]}, \tag{2}$$

where $[E]$ and $[S]$ are the weight concentrations of the enzyme and the substrate, k'_{+1} and k'_{+2} are the forward rate constants, while k'_{-1} is the reverse rate constant (these rate constants correspond to a single degraded substrate bond), M_E is the molecular mass of the enzyme, b is the number

Table 1. *Kinetic data for the enzymatic degradation of* Na-*CMC with different degrees of polymerization*

Na-*CMC* sub-strates (*n*)	Number of reactive substrate bonds (*b*)	*Km* (g/dm^3)	M_S (g/mol)	*Km*(*M*) (mol/dm^3)	*V* (g/ml · min)	k_{+2} (s^{-1})
140	139	4.07 ± 0.34	3.10^4	$1.36 \cdot 10^{-4}$	14.47	$0.96 \cdot 10^{-2}$
640	639	2.94 ± 0.15	14.10^4	$0.21 \cdot 10^{-4}$	17.12	$1.15 \cdot 10^{-2}$
900	899	2.80 ± 0.16	20.10^4	$0.14 \cdot 10^{-4}$	19.03	$1.27 \cdot 10^{-2}$

of reactive bonds in a single substrate molecule, b_∞ is the number of unbroken bonds of the substrate molecule, a is the number of active centers in a single enzyme molecule, (C_b^a) is the combination free of repetition and N_A is the *Avogadro* number.

The *Michaelis-Menten* constant in molar concentration can be defined from equation (2) as:

$$Km(M) = \frac{Km}{M_S} = \frac{k'_{-1} + k'_{+2}}{N_A k'_{+1}} \frac{1}{b^\alpha} = Km'(M) \frac{1}{b^\alpha} \tag{3}$$

with $a = 1$.

The maximum reaction rate is defined by the expression:

$$V = k'_{+2}[E]\left(1 + \frac{M_S}{M_E}\right)(b - b_\infty) \tag{4}$$

$Km'(M)$ and k'_{+2} were determined graphically by plotting $Km(M)$ and (k_{+2}) against the number of reactive bonds (b) according to equations (3) and (4). The plots presented in Fig. 1 lead to the following values:

$$Km'(M) = 6.31 \cdot 10^{-2}\,\text{mol/dm}^3$$
$$k'_{+2} = 4.07 \cdot 10^{-6}\,\text{s}^{-1}.$$

The free energy $(\Delta G^{\neq})$, which corresponds to the formation and degradation of a single bond in the enzyme—polymer substrate reaction, was determined from the values for $Km'(M)$ and k'_{+2} according to the equation:

$$\Delta G^{\neq} = RT \ln \frac{kTKm'(M)}{hk'_{+2}} = 101\,\text{kJ/mol}, \tag{5}$$

where R, k, h and T are the gas constant, the *Boltzmann* constant, the *Planck* constant and the absolute temperature of the enzyme reaction. This energy can be expressed in electron-volts (eV) according to the equation:

$$\Delta E^{\neq} = \frac{\Delta G^{\neq}}{1.2 \cdot 10^{-19} N_A} = 1.39\,\text{eV}. \tag{6}$$

The ratio (α) between the effective cross sections of the reactive substrate bond and of the active enzyme center was determined:

$$\alpha = \frac{\sigma_S}{\sigma_E} = -\frac{\Delta \ln Km'(M)}{\Delta \lg b} = 1.22. \tag{7}$$

The kinetic parameters obtained, which correspond to the hydrolysis of a single glucoside bond $[Km'(M), k'_{+2}, \Delta G^{\neq}, \Delta E^{\neq}$ and $\alpha]$ for the examined cellulase preparation of *Aspergillus sp.*, are comparable to the parameters obtained for a cellulase fraction from *Trichoderma viride*[9,10].

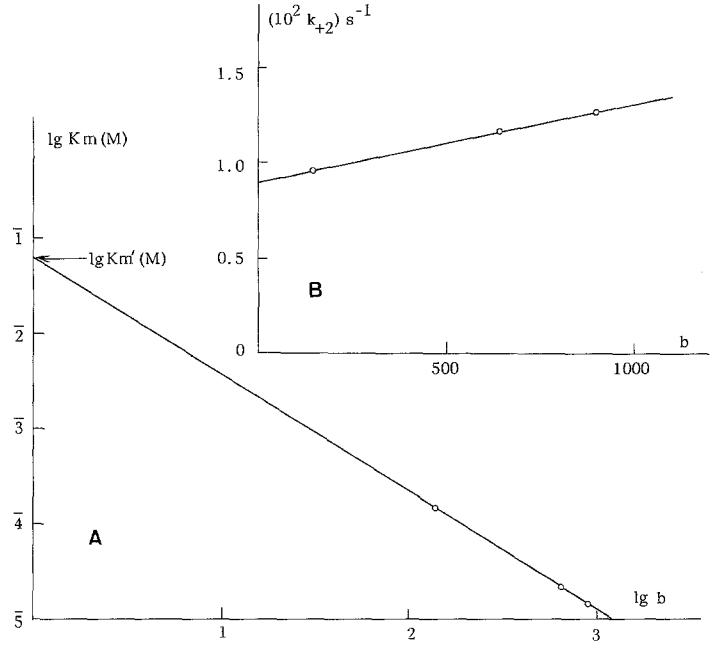

Fig. 1. A) Plot of $Km(M)$ and B) plot of k_{+2} against the number of bonds (b) in the reaction cellulase—Na-*CMC* with different degrees of polymerization

Although the substrates used in both cases are widely different in respect to the degree of polymerization, the modified *Michaelis-Menten* equation permits the comparison of the enzyme kinetic properties on the basis of the degradation of a single substrate bond.

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