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# ACTIVE GROUPS OF EXTRACELLULAR ENDO-D-GALACTURONANASE OF *ASPERGILLUS NIGER* DERIVED FROM pH EFFECT ON KINETIC DATA

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## Summary

In an attempt to characterize the groups essential for the catalytic action of extracellular endo-D-galacturonanase of *Aspergillus niger* (poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) the behaviour of the kinetic parameters as a function of pH was examined. The dependence of $k_{cat}$ and $k_{cat}/K_m$ on pH suggests that two dissociable groups are involved, for which the p$K$ values of about 3.0 and 5.0 in the free enzyme and 3.06 and 5.72 in the catalytic complex were found at 30°C. These values and the value of the heat of ionization of the acidic group, $\Delta H_i$ 6.48 kcal/mol, resulting from the p$K_a$ values obtained at 20°C (5.91) and at 30°C (5.72) suggest the participation of a carboxylate group and a protonated imidazole group of histidine in the reaction catalyzed by endo-D-galacturonanase.

## Introduction

Endo-D-galacturonanase * (poly-(1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) catalyzes the random hydrolytic splitting of internal glycosidic α-1,4-linkages in D-galacturonan chains of pectic substances. At present, very little is known about the mechanism of the enzymic reaction, the mode of binding of a substrate by the enzyme, and the character of active groups participating in the catalytic reaction.

On the basis of the inhibitory effect of several compounds on different microbial endo-D-galacturonanases, amino groups [1,2], sulphydryl and tyrosyl groups [3], and histidine [4] are regarded as essential for the activity of the enzymes. In *Aspergillus niger* extracellular endo-D-galacturonanase photooxidative inactivation paralleled by the decomposition of imidazole groups indicated that one of the seven histidine residues present in the enzyme molecule [6] is essential for the enzyme activity. On the basis of pH vs. activity profiles, Koller

* So far called endopolygalacturonase.

[7] considered that, in *A. niger* endo-D-galacturonanase, the carboxyl groups of glutamic or aspartic acid together with the imidazole group are part of the active centre. In the substrate molecule, carboxyl groups [8] and some secondary alcoholic groups [9] are presumed to be involved in the interaction with the enzyme.

Differences in the action pattern of various endo-D-galacturonanases towards oligomeric substrates indicate that the active site, in particular the number of binding subsites of endo-D-galacturonanases is not the same [10]. It appears that extracellular endo-D-galacturonanase of *A. niger* interacts with a substrate by means of four binding subsites [11] and of some secondary loci of contact, of lower affinity [12].

In an attempt to further characterize the active site of *A. niger* extracellular endo-D-galacturonanase the kinetics of the enzyme action were studied as a function of pH. Because of limited solubility of high-molecular-weight substrates at acidic pH heptagalacturonic acid was used in kinetic measurements as a substrate.

## Experimental

### *Materials and Methods*

Endo-D-galacturonanase was isolated from the filtrate of a surface culture of *A. niger* growing on Czapek-Dox nutrient medium containing 1.5% citrus pectin as the carbon source. The isolation procedure included salting-out of protein fraction by $(NH_4)_2SO_4$ (0.9 saturation), precipitation with 76% ethanol, affinity chromatography on cross-linked pectic acid [13], and gel chromatography on Sephadex G-100 eluted with 0.5 M NaCl/0.1 M acetate buffer (pH 4.2). Products obtained after two last purification steps were freeze-dried, desalted on Sephadex G-25 (grade Medium), and again freeze-dried.

Heptagalacturonic acid was isolated from a partial acid hydrolysate of pectic acid by gel chromatography on Sephadex G-25 Fine as described elsewhere [14]. The purity of the preparation was tested by thin-layer chromatography on silica gel [15], as well as on the basis of content of reducing groups (spectrophotometrically [16]) and carboxyl groups (titrimetry).

Pectic acid (D-galacturonan content 89.8%, average molecular weight 27 000) was prepared from citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) by repeated alkaline de-esterification with 0.1 M NaOH, followed by precipitation at pH 2.5.

*Enzyme assay.* Endo-D-galacturonanase activity was assayed at various pH values by measuring the increase of reducing groups at different time intervals, by spectrophotometric method using Nelson—Somogyi reagent [16]. Initial velocities were determined at five substrate concentrations ranging from 0.1 to 1.0 mM and at enzyme concentration 0.025 mg/ml. Enzyme kinetics were measured at 30°C in pH range 3.2—5.8 and at 20°C at pH 4.0—5.8 (0.05 M acetate buffers). D-galactopyranuronic acid standards used at each pH allowed correction for variations in colour yield. The temperature of the reaction mixture was kept constant with the accuracy of ±0.1°C. The pH was measured at room temperature with a Radiometer PHM 28 instrument. The initial velocities defined in moles of reducing groups liberated by 1 kg of protein per s were cal-

culated on a computer using a program for the polynomial procedure of Booman and Niemann [17]. Protein was determined by the method of Lowry et al. [18] using human serum albumin as standard. $K_m$ and $V$ were calculated on a computer using the least-square procedure of Wilkinson [19]. For the calculation of catalytic constant, $k_{cat}$, a molecular weight of the enzyme of 35 000 was used [6].

## Results and Discussion

*A. niger* extracellular endo-D-galacturonanase resembles other glycan hydrolases on being active in weekly acidic region. The pH vs. activity profile for the reaction with heptagalacturonic acid is characterized in the measured range (pH 3.2—5.8) at all substrate concentrations by a bell-shaped curve with a maximum at pH 4.1—4.2 (Fig. 1).

In some endo-D-galacturonanases, the pH optimum was found to depend on the degree of polymerization of the substrate. With decreasing the degree of polymerization the pH optimum of these enzymes was shifted towards acidic side [20—22]. However, in the case of extracellular endo-D-galacturonanase of *A. niger*, such a shift in the pH optimum was not observed. Similar profile, with the same pH optimum as found for heptasaccharide was obtained with sodium pectate (degree of polymerization 153) when used as substrate (Fig. 2).

The pH vs. activity profiles with both substrates indicate that the activity of the enzyme is controlled, similarly as in other glycan hydrolases [23—26] by a

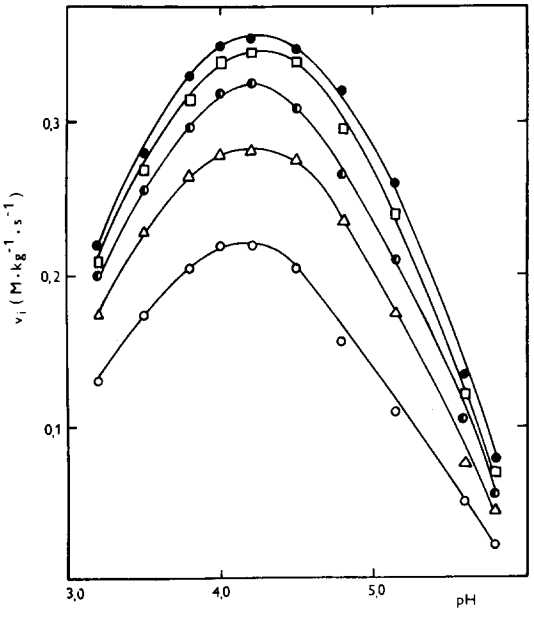


Fig. 1. The pH vs. activity profiles for the reaction of *A. niger* endo-D-galacturonanase with heptagalacturonic acid. Reaction conditions: 30°C, 0.05 M acetate buffers of respective pH, enzyme concentrations 0.025 mg/ml, substrate concentrations: 0.2 mM ○, 0.4 mM △, 0.6 mM ◐, 0.8 mM □, 1.0 mM ●.

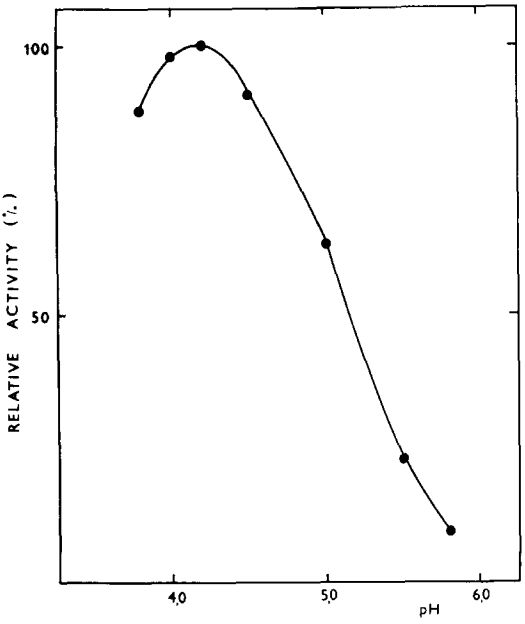


Fig. 2. The pH vs. activity profile for reaction of *A. niger* endo-D-galacturonanase with sodium pectate (d.p. 153). Reaction conditions: 30°C, 0.5% sodium pectate of respective pH (0.05 M acetate buffers), enzyme concentration 0.025 mg/ml.

protonated group which ionizes at the alkaline side and an unprotonated group ionizing on the acidic side of the pH vs. activity range.

In an attempt to characterize the essential groups the behaviour of the kinetic parameters of the degradation of heptagalacturonic acid by endo-D-galacturonanase as a function of pH was examined. Fig. 3 summarizes the obtained kinetic data by correlating $pK_m^{\sim}$, log $k_{cat}^{\sim}$, and log $k_{cat}^{\sim}/K_m^{\sim}$ with pH [27] $K_m^{\sim}$ and $k_{cat}^{\sim}$ correspond to pH-dependent kinetic parameters. At all pH values heptagalacturonic acid exhibited Michaelinian behaviour. The value of $K_{m,app}^{\sim}$ for the reaction was constant ($0.9 \cdot 10^{-4}$ M) at pH 3.2—4.0 and further increased with the increasing pH. The apparent catalytic constant, $k_{cat}^{\sim}$, as well as the values of $k_{cat}^{\sim}/K_m^{\sim}$ for the reaction decreased on both sides of the pH optimum. The pH range at the acidic side could not be extended far enough because at pH lower than 3.2 less reproducible values of activity were obtained with Somogyi reagent.

In simple cases one can assign the dissociation constants of essential ionizing groups of the free enzyme or substrate ($pK_a$, $pK_b$) from the log $k_{cat}^{\sim}/K_m^{\sim}$ vs. pH plot and the dissociation constants of essential groups of the enzyme · substrate complex ($pK'_a$, $pK'_b$) from the plot of log $k_{cat}^{\sim}$ vs. pH [27] ($pK_a$, $pK'_a$ correspond to acidic ionizing groups, $pK_b$, $pK'_b$ to basic ionizing groups). The bell-shaped profiles of both plots shown in Fig. 3 again indicate that at least two dissociable groups participate in the catalysis. The break on the alkaline side of the log $k_{cat}^{\sim}/K_m^{\sim}$ curve characteristic for the ionizing constants of the free enzyme, or substrate occurs approximately at pH 5.0. In addition, assuming that the curve is symmetrical, extrapolation at the acidic side indicates the

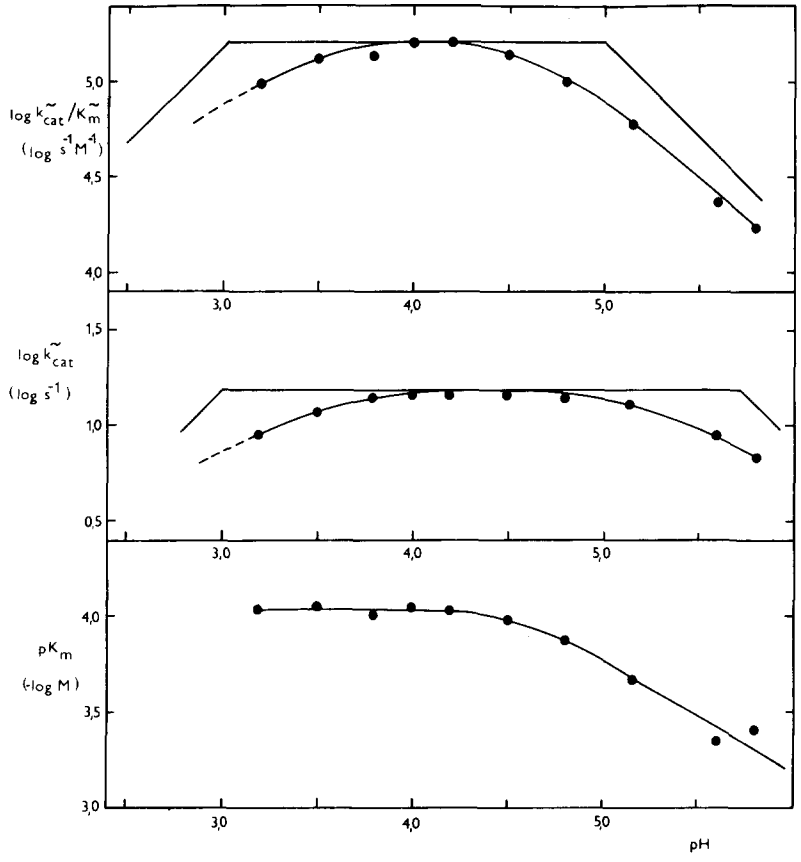


Fig. 3. Variation of kinetic parameters $pK_m$, log $k_{cat}$, and log $k_{cat}/K_m$ of the reaction of endo-D-galacturonanase with heptagalacturonic acid as a function of pH, at 30°C.

involvement of a group having $pK_b$ of about 3.0.

In the log $k_{cat}$ plot a shift of the alkaline arm of the curve and consequently of respective $pK'$ value from mentioned $pK$ 5.0 to about 5.7 occurs. The $pK'$ value of the basic group seems to be unaffected by the enzyme-substrate complex formation; $pK'_b \approx 3.0$. The slope on the alkaline side, as well as the presumed slope on the acidic side of the curve equal approximately to one suggesting that a single protonated group and a single unprotonated group control the catalytic action of the enzyme.

Similar values of dissociating constants were obtained by plotting $1/k_{cat}$ versus $1/[H^+]$ and $[H^+]$, resp. according to the equations:

$$1/k_{cat} = 1/k_{cat}(1 + [H^+]/K'_{b,app})$$

and

$$1/k_{cat} = 1/k_{cat}(1 + K'_{a,app}/[H^+]), \text{ ref. 28 ,}$$

in which $k_{cat}$ represents pH independent catalytic constant. By this method determined $pK'_b$ equals to 3.06 and $pK'_a$ to 5.72. By the same methods were

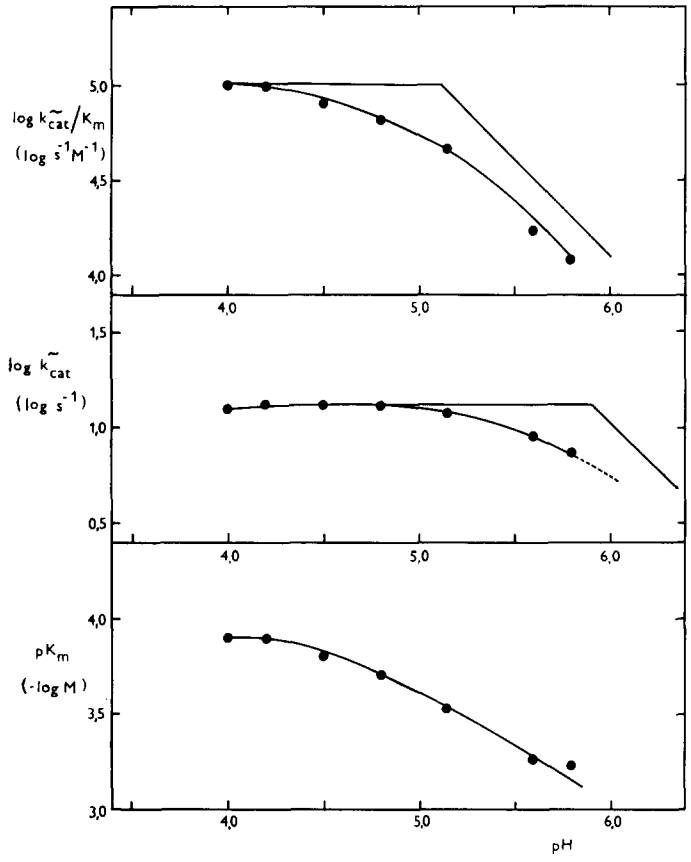


Fig. 4. Plots of $pK_m^{\sim}$, log $k_{cat}^{\sim}$, and log $k_{cat}^{\sim}/K_m^{\sim}$ against pH for the reaction of endo-D-galacturonanase with heptagalacturonic acid, at 20°C.

determined $pK_a$ and $pK_a'$ at 20°C (Fig. 4). Their values were found to be 5.14 and 5.91, resp.

The results obtained indicate an unprotonated group which has a p$K$ 3.0 both in the free enzyme and catalytic complex and a protonated group having at 30°C a p$K$ 5.0 in the free enzyme, and 5.72 in the catalytic complex, as the catalytic residues of the enzyme. From a comparison of p$K$ values obtained with those given by Dixon and Webb [28] for various groups of proteins a most probable candidate for the unprotonated group having p$K$ 3.0 could be a carboxylate (p$K$ of carboxyl ≈ 3.0—4.7). Since the group dissociates both in the free enzyme and enzyme · substrate complex it is very likely to be a component of the catalytic site, not of the binding site of the enzyme. On the same account it cannot be a component of the substrate molecule.

If one takes into account that the p$K$ value within the proteins can be shifted over more than 2 units from the value in the free state, the acidic group ionizing at pH 5.0 and 5.72, resp. might be either undissociated carboxyl with high p$K$, similarly as it is the case of Glu-35 of lysozyme [29] or protonated imidazole group with low p$K$ value (p$K$ of imidazole ≈ 5.6—7.0).

An additional information about the character of the acidic group was pro-

vided by the determination of heat of ionization $\Delta H_i$ which is characteristic for ionizing groups of proteins [28]. The difference between $\Delta H_i$ reported for the carboxyl group (±1.5 kcal/mol) and for the imidazole group (7 kcal/mol) allows an easy discrimination.

The heat of ionization was calculated according to Van 't Hoff's equation

$$\Delta H_i = -2.303\, RT^2 \frac{\mathrm{d}pK}{\mathrm{d}T}$$

using $pK'_a$ value of 5.91 found at 20°C and 5.72 found at 30°C. The resulting $\Delta H_i$ 6.48 kcal/mol is very close to the value of heat of ionization of imidazole group of histidine.

The results of kinetic measurements at two temperatures indicate the importance of carboxylate and protonated imidazole group of histidine in the catalytic action of endo-D-galacturonanase, similarly as it is supposed in some other glycan hydrolases [23—26]. In those enzymes a reaction mechanism is assumed involving general acid catalysis by protonated imidazole and the stabilization of the developing oxycarbonium ion intermediate by a carboxylate group. It is probable that endo-D-galacturonanase operates by the same mechanism.

As already mentioned the importance of the imidazole group for the catalytic action of endo-D-galacturonanase of *A. niger* has been evidenced by chemical modification of the enzyme [4,5]. The lower $pK$ value resulting from the kinetic measurements, in comparison with the value in free histidine might be due to the presence of some cationic groups occurring in the proximity of the catalytic site. The presence of such groups is possible; they could take part in the interaction of the enzyme with carboxylate groups of D-galactopyranuronic acid units of substrate molecule [1]. The independence of pH optimum of endo-D-galacturonanase on the substrate concentration (Fig. 1) supports the assumption of ionic interaction of the enzyme with substrate.

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