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Identification of Catalytically Active Groups of *Penicillium canescens* F-436 β-Galactosidase

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Abstract—The functional groups of *Penicillium canescens* F-436 β-galactosidase have been identified. The p*K* values and heats of ionization of these groups and photoinactivation of the enzyme with methylene blue indicate that the active site contains carboxyl and imidazole groups. A mechanism for the participation of these groups in the cleavage of the glycoside bond in lactose is proposed.

Key words: β-galactosidase, hydrolysis of lactose, photoinactivation, mechanism of action, *Penicillium canescens*

β-Galactosidase (EC 3.2.1.23) is a typical enzyme of catabolism. It is related to carbohydrases (*o*-glycoside hydrolases) and glycosidases.

The enzyme hydrolyses β-D-galactoside bonds with the cleavage of galactoside groups of such galactosides as oligosaccharides, polysaccharides, glycolipids, glycopeptides, and mucopolysaccharides [1]. As this takes place, the bond between the C_1-atom of a monosaccharide residue and the oxygen atom of the glycoside is cleaved [2].

Bacteria, yeasts, and fungi actively produce β-galactosidase. Unlike bacteria and yeasts, fungi synthesize both intra- and extracellular enzymes [3, 4].

Extracellular β-galactosidases produced by microscopic fungi differ sharply in their properties from the intracellular enzymes produced by bacteria and yeasts [5, 6].

Although there are many publications concerning β-galactosidases in microorganisms, data on the structure of their active sites are few and contradictory; this especially concerns the identification of functional groups in the active sites of β-galactosidases isolated from micromycetes [3, 7, 8]. The mechanism of action of β-galactosidase on various galactosides is not well established for any of the purified enzymes.

We have carried out studies on the identification of functional groups involved in lactose hydrolysis by β-galactosidase from *Penicillium canescens* F-436. The experimental data (p*K* values, heats of ionization, photooxidation) quite reliably indicate the chemical nature of these groups.

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MATERIALS AND METHODS

An active β-galactosidase producer was chosen from 40 strains of micromycetes from different taxonomic groups obtained from the Russian Collection of Microorganisms. The micromycetes were grown in submerged culture conditions in the following medium (wt. %): soybean flour, 2; $Na_2HPO_4 \cdot 12H_2O$, 1.74; citric acid, 1.67; $(NH_4)_2SO_4$, 0.2; $MgSO_4$, 0.015; KCl, 0.05; pH 5.0. The medium was poured into 700-ml Erlenmeyer flasks (100 ml per flask), and the cultures were grown on a laboratory shaker for 96 h at 240 rpm and 28-30°C.

Six to seven day old cultures on wort agar were used as stock material. Cultures were inoculated with conidial suspension containing no less than 2 million conidii per ml medium. Enzymatic activity was determined as the initial hydrolysis rate of 5% lactose solution at 30°C and pH 4.5. Products of hydrolysis were measured by the method of Bertran and converted to lactose by multiplying by the coefficient 0.95. The quantity of β-galactosidase hydrolyzing 1 μmole of lactose per minute was taken as 1 unit of specific activity. Citrate-phosphate buffer with ionic strength 0.06, which has sufficient buffering capacity in the pH range 2.0-8.0, was used in the experiments.

The fungus *P. canescens* F-436, which was stored on Rowlen–Tom medium with maize extract, displayed the most effective synthesis of β-galactosidase. This micromycete was then used as the active producer of β-galactosidase, and all subsequent procedures for purification of the enzyme and studies of catalytic groups in its active site were performed with this microorganism.

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The culture was subjected to ultrafiltration on Amicon (USA) equipment at 0.2-0.3 MPa using a membrane with pore size of 0.05 μm.

The enzyme was precipitated from the culture medium with ethanol at 2-4°C. The mixture was kept for 15-20 min to form a pellet. The pellet was collected using a refrigerated centrifuge and then dried with air.

Then the pellet was dissolved in a minimum volume of citrate-phosphate buffer, pH 4.5, and fractionated with ammonium sulfate (75-95% saturation).

The enzyme was then purified by gel filtration on Sephadex G-25 (Pharmacia, Sweden) [9]. Enzyme solution was loaded onto the column (1.4 × 60 cm) and eluted with 0.15 M citrate-phosphate buffer, pH 4.5, at flow rate 25-30 ml/h. Eluted fractions (5-6 ml per fraction) were collected using a fraction collector, and then protein concentration by Lowry and β-galactosidase activity were determined.

For the final purification step, the β-galactosidase was subjected to gel filtration on a Sephadex G-100 column (2.4 × 30 cm). The enzyme was eluted with the same buffer solution at 6-8 ml/h.

The homogeneity of the most active enzymatic fractions was evaluated by electrophoresis in polyacrylamide gel [10]. The electrophoresis was performed in 7.5% gel at pH 7.5 (Tris-glycine buffer) for 2-2.5 h at 260 V and 5 mA. The gels were stained with 10% Amido black 10B. Chemicals from Reanal (Hungary) were used for electrophoresis; all other chemicals were of analytical or high purity grade (Russia).

The kinetics of lactose hydrolysis by β-galactosidase was studied at the optimum pH and temperatures of 40, 50, and 55°C. In all experiments, the same amount of the enzyme (19 units per g lactose) was added.

The imidazole group of histidine was detected by photoinactivation of the enzyme in the presence of methylene blue [11].

RESULTS AND DISCUSSION

Table 1 displays the results of the purification of the β-galactosidase. An enzyme preparation with 93-fold purity and specific activity of 427.1 units per mg protein was obtained. The enzyme was homogeneous as judged from the electrophoresis according to Davies [12].

When evaluating the molecular weight of the β-galactosidase by gel filtration on the column with Sephadex G-200, the following proteins with known molecular weights were used: lysozyme (17 kD), serum albumin (70 kD), γ-globulin (130 kD), glucose oxidase (150 kD), and catalase (250 kD). Column free volume (V_o) was determined using dextran blue. The molecular weight of the β-galactosidase from *Penicillium canescens* F-436 was 145 kD.

Catalytically active groups of the β-galactosidase. Specific criteria exist for determining the presence of chemically active groups in the active sites of carbohydrases. For example, the use of specific labeled irreversible inhibitors that form covalent enzyme–inhibitor complexes with the active group of a particular amino acid involved in the active site of the enzyme was suggested [13].

The chemical nature of catalytic groups is often deduced from their ionization constants, p*K* values, or

Table 1. Purification of *P. canescens* F-436 β-galactosidase

Purification step	Volume, ml	Protein, mg	Activity		Purification degree	Yield, %
			total, units	specific, units per mg protein		
Culture medium	160.0	3328.0	15231.2	4.58	1.00	100.0
Ultrafiltration through 0.05-μm membrane	50.0	2465.2	14830.0	6.01	1.31	97.3
Precipitation with ethanol	20.0	527.1	11312.9	21.46	4.69	74.1
Fractionation with ammonium sulfate	4.0	187.4	8769.7	46.80	10.22	57.6
Sephadex G-25	15.5	113.7	8130.1	71.42	15.59	53.3
Sephadex G-100 (the most active fraction)	4.0	2.5	1067.8	427.1	93.25	7.0

heats of ionization or from the influence of pH and ionic strength on the enzymatic activity [3, 4, 7, 14].

Experimental studies have indicated an important role of a SH-group in some β-galactosidases [8, 15] and carboxyl group [3] or imidazole group [3, 16] in some other β-galactosidases.

Using site-specific mutagenesis, Richard, Huber, and others [17-19] investigated the details of the mechanism of cleavage of a β-galactoside by *E. coli* β-galactosidase. The carbonyl group of Glu461 and the ionized carboxyl group of Glu537 were found to be involved in galactoside cleavage, wherein the carboxyl group of Glu461 acts as a proton donor and ionized carboxyl of Glu537 serves as a base. In our opinion, these data are controversial. For successful electrophilic–nucleophilic attack on a glycoside bond with its cleavage by the Glu461-COOH...$^{-}$OOC-Glu537 system, the ΔpK of the system is supposed to be at least 2-3 units. In the glycoside bond cleavage process, the two groups have to come together within the length of the bond. Carboxyl groups with low pK values are unlikely to differ significantly in ΔpK. Other neighboring chemical groups are expected to have virtually the same influence on their ionization.

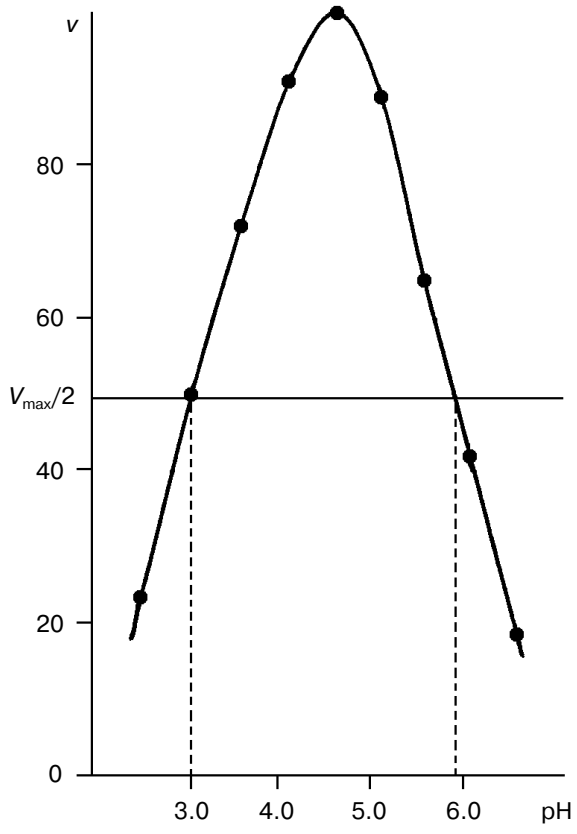

Fig. 1. The function $v = f$(pH) for *P. canescens* F-436 β-galactosidase at 30°C.

It should be stressed that a single experimental criterion was used for identification of the catalytically active groups of β-galactosidases in most studies. It is difficult to judge the significant participation of some group in the enzyme active site from a single criterion; several independent criteria should be used to establish a certain conclusion about the chemical nature of the group. We have used this approach for identification of catalytically active groups in *Penicillium canescens* F-436 β-galactosidase.

Determination of pK values for catalytically active groups of β-galactosidase. pK values were determined from the dependence of enzymatic activity on pH [20]. Figure 1 shows a dependence curve, $v = f$(pH), where v is the initial rate of lactose hydrolysis with β-galactosidase. The pH optimum for the enzyme is 4.5. The shapes of the ascending and descending branches of the curve are characteristic for the dissociation of ionizable groups and evidence for the participation of two catalytically active groups in the cleavage of the β-1,4-glycoside bond of lactose. The straight line drawn parallel to the abscissa at the distance $V_{max}/2$ intersects the branches of the curve $v = f$(pH) at points corresponding to the pK values of the catalytically active groups [21]. As seen from Fig. 1, these are: $pK_1 = 3.0$ and $pK_2 = 5.8$. These pK values are consistent with the carboxyl group of either the C-terminal or glutamate or aspartate and with the imidazole group of a histidine residue [20, 21].

Determination of heats of ionization for the catalytically active groups of β-galactosidase. The participation of carboxyl and imidazole groups in the cleavage of the β-1,4-glycoside bond of lactose was also supported by the heats of ionization of these groups. The values were calculated from the van't Hoff equation:

$$\Delta H = 2.303\ R\ (pK_1 - pK_2)\ \frac{T_1 \cdot T_2}{T_2 - T_1}, \qquad (1)$$

where ΔH is heat of ionization for a group, R is the universal gas constant (8.315 J/mole·K), and pK_1 and pK_2 are ionization constants at temperatures T_1 and T_2, respectively.

The values of pK_1 and pK_2 were determined from the shift of the branches of the curve $v = f$(pH) measured at temperatures 10 and 30°C, a temperature range within which β-galactosidase is quite stable. The results are presented in Table 2.

As seen from Table 2, there was very little if any shift in the pK_1 value for the ascending branch; the measured values lay within the range of experimental errors (ΔpK = 0.1 and ΔH = 8.2 kJ/mole). However, the small ΔpK and ΔH magnitudes suggest that a carboxyl group is involved in the cleavage of the glycoside bond of lactose. As Dixon and Webb showed [20], the ΔH value for a carboxyl group is 6.3 kJ/mole and is the lowest in comparison with that for other ionizable groups in proteins, which is in agree-

Table 2. Values of pK and ΔH for the catalytically active groups of *P. canescens* F-436 β-galactosidase

Branch of curve	pK		ΔpK	ΔH, kJ/mole
	10°C	30°C		
Ascending	3.1	3.0	0.1	8.2
Descending	6.2	5.8	0.4	32.8

ment with our data. As for the descending branches of the curves, the pK_2 shift is significant (ΔpK = 0.4) and respective heat of ionization (ΔH = 32.8 kJ/mole) is consistent with that for an imidazole group.

Photoinactivation of β-galactosidase. The presence of an imidazole group of histidine in the active site of β-galactosidase is supported by its photoinactivation in the presence of methylene blue. As seen from Fig. 2a, β-galactosidase was photoinactivated in the presence of methylene blue (curve *1*), whereas in controls with methylene blue in the darkness (curve *2*) and without methylene blue in the light (curve *3*) the β-galactosidase activity remained unchanged. The dependence $\log A/A_\tau = f(\tau)$ is linear (Fig. 2b) and is described by the first-order equation:

$$K = \frac{2.303}{\tau} \log \frac{A}{A_\tau}, \qquad (2)$$

where K (h^{-1}) is the photoinactivation rate constant and A and A_τ are the β-galactosidase activities at the initial time and at time τ, respectively.

The loss of catalytic function of β-galactosidase results from the photooxidation of the imidazole group of histidine. In the light, methylene blue accepts electrons from the imidazole group, which becomes oxidized with the breaking of the imidazole heterocycle. However, both the phenyl group of tyrosine and indole group of tryptophan are susceptible to the same reaction. But, unlike the latter, the imidazole heterocycle has a resonance structure and can act as an H^+ carrier in biochemical reactions. At high H^+ concentrations, it is protonated and does not give an electron to methylene blue. In other words, the oxidation rate of the heterocycle depends on pH. Neither the phenyl group of tyrosine nor the indole group of tryptophan has this property [22, 23].

We investigated the dependence of β-galactosidase photoinactivation rate on H^+ concentration within the pH range 3.0-8.0 at temperatures of 10 and 30°C.

As seen from Fig. 3, the $k = f$(pH) curves have the characteristic sigmoidal shape for the titration of ionizable groups.

Within the pH range 7.0-8.0, the curves reach a plateau corresponding to the maximum photoinactivation constant. The shape of the curves satisfies the equation:

$$K = K_0 (1 + 10^{\mathrm{pH}-\mathrm{p}K_2}), \qquad (3)$$

where K (h^{-1}) is the inactivation rate constant at the corresponding pH value and K_0 (h^{-1}) is the limiting photoinactivation rate constant.

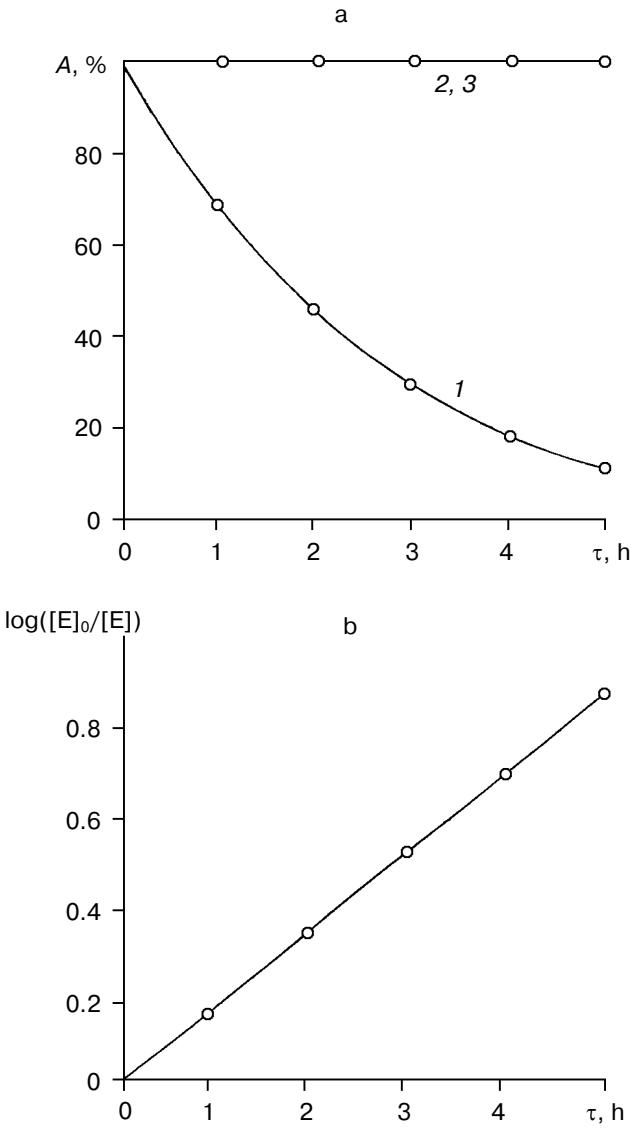

Fig. 2. a) Photooxidative inactivation of *P. canescens* F-436 β-galactosidase: *1*) in the presence of methylene blue under illumination at pH 5.0; *2*) without methylene blue under illumination; *3*) in the presence of methylene blue in darkness. b) The function $\log([E]_0/[E]) = f(\tau)$ at pH 5.0 ($[E]_0$ and $[E]$ are the initial enzyme activity and the enzyme activity at time τ, respectively, determined as the initial reaction rate (%); τ is incubation duration (h)).

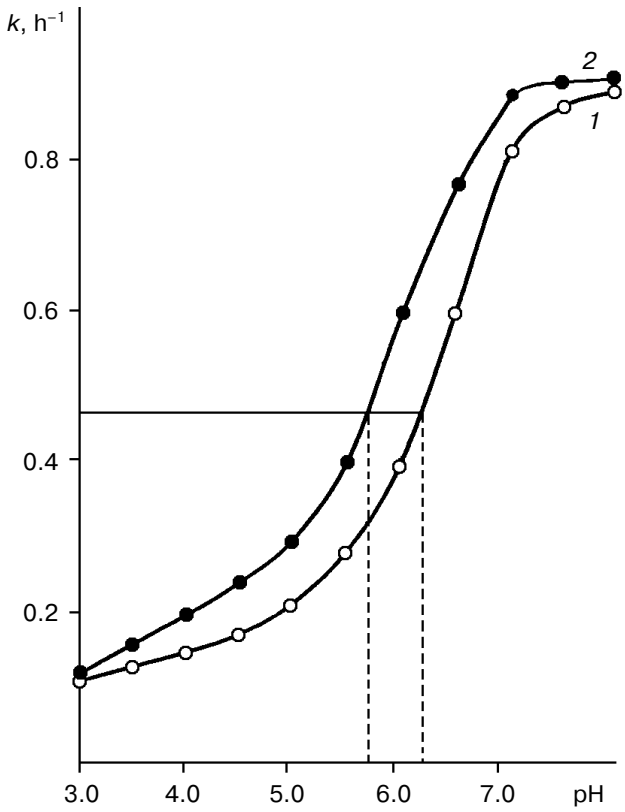

Fig. 3. The function $k = f(\mathrm{pH})$ during the photoinactivation of *P. canescens* F-436 β-galactosidase at 10 (*1*) and 30°C (*2*).

According to Eq. (3), $K = K_0/2$ when $\mathrm{pH} = \mathrm{p}K_2$. As seen from Fig. 3, a straight line drawn parallel to the abscissa at the distance $K_0/2$ intersects curves *1* and *2* at points corresponding to $\mathrm{p}K_2' = 6.2$ and $\mathrm{p}K_2'' = 5.7$ with relative curve shift $\Delta\mathrm{p}K = 0.5$. The value of ΔH calculated from Eq. (1) is 41.1 kJ/mole. These data also support the participation of an imidazole group of histidine in lactose hydrolysis by β-galactosidase.

On the mechanism of glycoside bond cleavage in lactose. The pH optimum for the activity of β-galactosidase is 4.5, and p*K* values for the carboxyl and imidazole groups are within the ranges 3.0-3.1 and 5.7-6.2, respectively, So, the carboxyl group in the active site of β-galactosidase is in its negatively charged carboxylate ion form and the imidazole group is in the form of protonated imidazolium. These two groups are involved in the cleavage of the glycoside bond of lactose as the $COO^-...H^+Im$ (Im is imidazole residue) system. The carboxylate acts as a nucleophilic group and the imidazolium as an electrophilic (proton donating) group.

A scheme proposed for the mechanism of catalysis of cleavage of lactose by β-galactosidase is presented in Fig. 4. It is based on complementation between the carboxylate–imidazolium system and the C_1–O bond in the lactose molecule. A characteristic feature of this bond is inequality of electron density distribution in the C_1 and O atoms. The oxygen atom is very electrophilic (electronegativity 3.5), much higher than that of the carbon atom (electronegativity 2.5) [24]. Therefore, the electron densi-

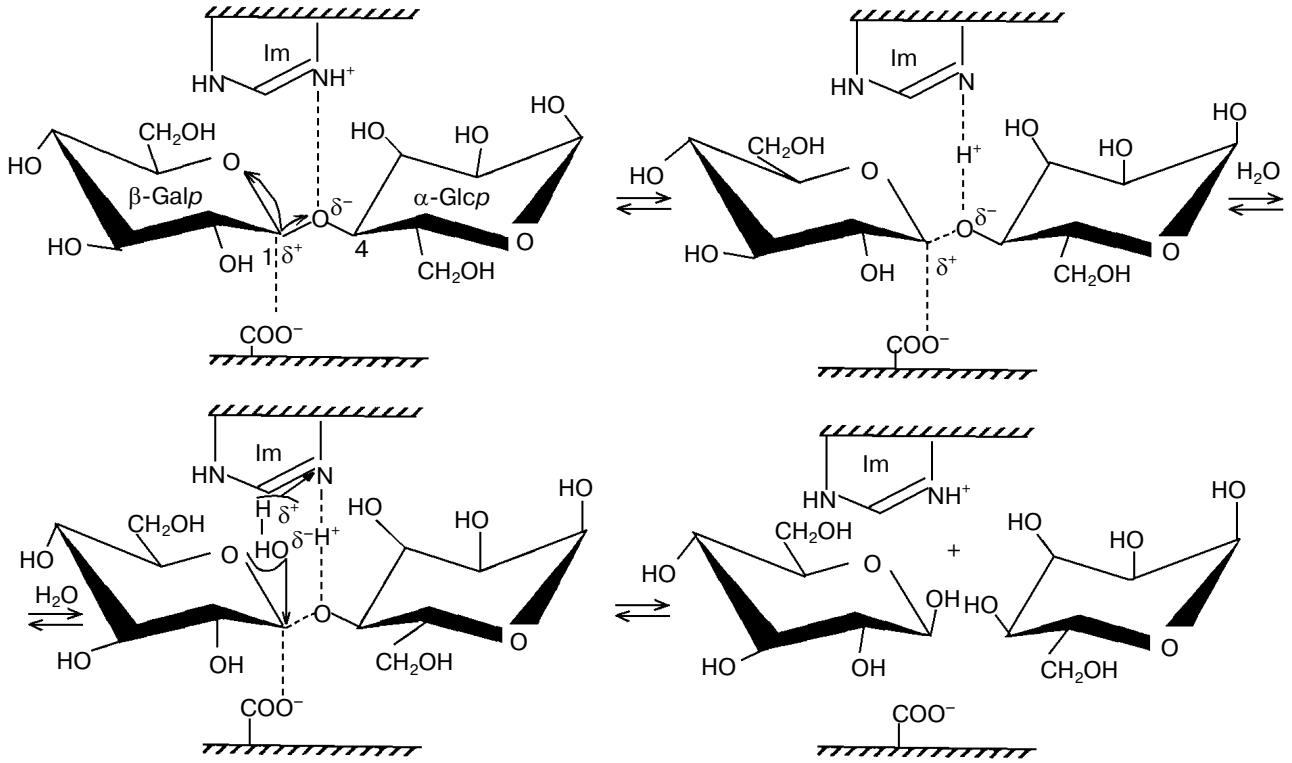

Fig. 4. Mechanism of glycoside bond cleavage in the lactose molecule by the β-galactosidase from *P. canescens* F-436 (β-Gal*p* and α-Glc*p* are residues of β-D-galactopyranose and α-D-glucopyranose molecules, respectively; Im, imidazole group).

ty of the O atom in the glycoside bond of the lactose molecule will be higher than in the C_1 atom of a β-D-galactose residue. Decrease of electron density in the C_1 atom is also due to the fact that it is under inductive influence of the O atom of the galactose pyranose ring. The dashed and hatched lines on the scheme presented in Fig. 4 depict complex formation between the C_1–O bond and the $COO^-...H^+Im$ system and redistribution of electron density in the complex. Nucleophilic attack of the C_1 atom with a H_2O molecule accomplishes the bond cleavage.

In conclusion, we would like to point out that the system $COO^-...H^+Im$ seemingly plays an important role in the hydrolysis of oligo- and polysaccharides by carbohydrases. It participates in starch hydrolysis by amylases [25], inulin hydrolysis by inulinase [21], and sucrose hydrolysis by β-fructofuranosidase [26].

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