# MODE OF ACTION AND PARTIAL PURIFICATION OF THE ACTIVE CENTRE OF exo-POLY-α-D-GALACTURONOSIDASE FROM *Selenomonas ruminantium*

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In the presented paper are summarized results of the study of the mode of action, dimensions and arrangement of the active centre of the exo-poly-α-D-galacturonosidase, (poly(1,4-α-D-galactosiduronate) digalacturonohydrolase, E.C. 3.2.1.82) produced by the bacteria *Selenomonas ruminantium*. With this aim we determined experimentally values of Michaelis constants and limiting rates for the catalytic hydrolysis of linear oligo(D-galactosiduronates) of the degree of polymeration in the range of 3 to 8, at pH 7.0 and the temperature 30 °C. We calculated molecular activities $k_0$ and parameters $k_0/K_m$ from these values. From the dependence of $\log k_0$ and $\log k_0/K_m$ on the degree of polymerization five subsites of the active centre were determined, with the catalytic site being situated between the second and third one. Kinetic data were used for the calculation of the affinities of the fourth and fifth subsites $A_4$ and $A_5$ in accordance with the theory of Hiromi. Product analysis of non-labelled oligo(D-galactosiduronates) and compounds labelled with [1-$^3$H] at the reducing end enabled to ascertain approximately the value for the first subsite $A_1$ of the active centre and to study the mode of action of the exo-poly-α-D-galacturonosidase from *Selenomonas ruminantium*.

A characteristic property of glycanohydrolases is the specificity of their action[1-8]. Its detail study carried out during the last few years resulted in the introduction of the term "subsite of the active centre" into the scientific literature. In the case of glycanohydrolases it means that part of the active centre which binds the substrate glycosyle unit. The quantitative measure of the specificity of the subsite is expressed by the amount of the free energy change of the enzyme–substrate complex formation corresponding to one subsite during binding of the monosaccharide segment of a certain type ($\Delta G_i$). The subsites are arranged at both sides of the catalytic site and are evaluated on the basis of their position towards the catalytic site[9-16]. The independence of $k_{int}$ (real rate constant of the hydrolysis of substrate bonds in the productive complex) of the degree of substrate polymerization ($n$) and of the type of the binding

enzyme–substrate is the basic presumption for the validity of the theory of subsites, confirmed also for several amylases[17 - 23].

Two theories of the mode of action of glycanohydrolases based on the idea of subsites have been presented in literature lately. Hiromi et al.[1,12] suggested a method for the calculation of affinities of subsites from the dependence of the degradation rate on the degree of substrate polymerization. Thoma et al.[9,10] proposed a method based on the analysis of terminal-labelled reaction products. For enzymes with terminal mode of action which form usually a single simple productive complex, the method of Hiromi[24] seems to be more suitable. The exo-poly-α-D-galacturonosidase, (poly(1,4-α-D-galactosiduronate) digalacturonohydrolase, E.C. 3.2.1.82) produced by bacteria catalyzes specifically the degradation of glycosidic α-(1 → 4) bonds of sodium pectane, resulting in the production of a single disaccharide. The enzyme attacks substrates from the non-reducing end of the molecule. The rate of the cleavage of the bonds depends on the polymerization degree of linear oligo(D-galactosiduronates)[25,26]. Based on the similarity of basic characteristics of this enzyme and β-amylase, we applied partially kinetic process described by Kato et al.[27] in our studies of the mode of action and active site of the exo-poly-α-D-galacturonosidase. We also employed the method based on the analysis of labelled reaction products published by Suganuma et al.[28] for β-amylase from corn bran.

## EXPERIMENTAL

### Materials

exo-Poly-α-D-galacturonosidase produced by the bacteria *Selenomonas ruminantium*, strain 777 (isolated from bovine rumen) was purified by the method already described[26]. The method consists of precipitation with ammonium sulfate, centrifugation, dialysis, desalting of the crude product on a Sephadex G-25 column Medium, separation of the protein complex on a QAE Sephadex A-50, permeation chromatography on Sephadex G-75 Fine, desalting of the purified enzyme on Sephadex G-25 Fine and lyophilization. The specific activity of the purified enzyme at the pH optimum 7.0 and 30 °C towards sodium pectane was 37 nkat/mg and its relative molecular weight was 26,000. The enzyme specificity was confirmed on the basis of the analysis of degradation products formed from polymeric and oligomeric substrates and on the basis of the frequency of the cleavage at the reducing end of [1-$^3$H] labelled oligo(D-galactosiduronate) molecules.

Oligo(D-galactosiduronates), α-D-(1 → 4) bound units of sodium salt of the D-galactopyranuronic acid, with a polymerization degree (DP) of 3 to 8 were prepared by enzyme hydrolysis of sodium pectane (poly(1 → 4)-α-D-galactosidpyranuronate) by resolution of the complex of oligo(D-galactosiduronates) on Sephadex G-25 Fine and subsequent desalting of the isolated products on Sephadex G-10 (ref.[29]). Homogeneity of the oligo(D-galactosiduronates) was confirmed by chromatography on thin layer of silica gel (Silufol plates, Czechoslovakia) using the system 1-butanol–formic acid–water (2 : 3 : 1), on the basis of values of log ($R_F$/(1 – $R_F$)) linearly dependent on the polymerization degree of oligo(D-galactosiduronates)[30]. The purity of substrates was determined from the ratios of the numbers of carboxylic and reducing groups[31]. [1-$^3$H]oligo(D-galactosiduronates) labelled at the reducing end were prepared by means of catalytic tritiation of non-labelled oligo(D-galactosiduronates) according to Evans et al.[32]. Individual oligo(D-galactosiduronates) were [1-$^3$H] labelled at the substrate reducing unit at the Institute for the

Investigation, Synthesis and Utilization of Radioisotopes in Prague. [1-$^3$H] labelled oligo(D-galactosiduronates) were purified by means of gel chromatography on Sephadex G-25 Fine using the method described for the isolation of non-labelled compounds. The purity of the radioactive labelled oligo(D-galactosiduronates) was 96 – 98% after the procedure. Samples were stored at –20 °C and no free radioactivity was detected in the course of three months. The specific activity of the purified samples was as follows: 7.44 . $10^7$ MBq for di(D-galactosiduronate), 2.85 . $10^7$ MBq for tri(D-galactosiduronate), 3.19 . $10^7$ MBq for tetra(D-galactosiduronate) and 2.55 . $10^7$ MBq for penta(D-galactosiduronate), related to 1 mol of substrate.

### Methods

Activity of exo-poly-α-D-galacturonosidase was determined on the basis of measurement of the increase in the concentration of reducing groups according to Somogyi[33], using a calibration curve for D-galactopyranuronic acid. The solution of sodium pectate (0.5%) in phosphate buffer (0.1 mol/l, pH 7.0) served as the substrate (relative molecular weight 27,000). A volume of 0.5 ml of the enzyme solution was incubated with a same volume of substrate solutions at 30 °C.

The reaction with [1-$^3$H] labelled substrates was carried out in small test tubes. The non-labelled substrate was dissolved in 50 μl of 0.05 mol/l phosphate buffer pH 7.0, then a constant amount of the labelled oligo(D-galactosiduronate) (0.05 MBq) and an appropriate amount of the enzyme were added. Aliquots were withdrawn from the reaction mixtures during the incubation at 30 °C and the enzyme reaction was stopped by 10 min boiling. For the kinetic studies, 1.5 μl samples of the reaction mixture were subjected to TLC chromatography on silica gel as described above. After the detection of reaction products with aniline–phthalate reagens, the activity in the individual spots (cut out and eluted with water) was determined in scintillation vessels with a scintillation solution SLD-31 (Chemapol, Spolana Neratovice). The measurement was carried out on a liquid scintillation counter LKB WALLAC 1214 RACKBETA. All the measured values were corrected towards the blank and backround. Frequency of the cleavage of the [1-$^3$H] oligo(D-galactosiduronate) bonds was determined at concentrations 0.2 mmol/l of the tri(D-galactosiduronate) and 0.025 mg/ml enzyme, 0.1 mmol/l of the tetra(D-galactosiduronate) and 0.005 mg/ml enzyme, 0.05 mmol/l of the penta(D-galactosiduronate) and 0.0025 mg/ml enzyme, at pH 7.0 and incubation temperature 30 °C. The initial formation of the products, i.e. the degree of degradation at the time $t = 0$ is equal for this reaction to the frequency of the bond cleavage. It was determined according to Allen and Thoma[34]. The activity of the individual products formed from the [1-$^3$H] oligo(D-galactosiduronates) used as substrates was related to the total activity of the substrate and products. The activity of the products related to the total activity is given by the formula:

$$R = \frac{[1-{}^3H](GalpA)_i}{\sum_i^n [1-{}^3H](GalpA)_i} ,$$

where $[1-{}^3H](GalpA)_i$ is the activity of the studied product.

## RESULTS

### *Kinetic Data*

In order to ascertain the kinetic parametres $K_m$ and $V$ for the individual oligo(D-galactosiduronates) we determined the initial reaction rates $v$ at six to eight substrate

concentrations within the range of 0.025 to 0.5 μmol/ml, at pH 7.0 and reaction temperature 30 °C. Individual sets of experimental data $v$ and $s$ were submitted to the direct non-linear regression analysis with a regression function $v = V s /(s + K_m)$ on a personal computer[35]. Table I summarizes results of these calculations: $K_m$ and $V$ together with standard deviations, correlation coefficients and standard deviations of the regression. In the Table I are also the values of enzyme molar concentrations $e_0$, molecular activities $k_0 = V/e_0$ and of the ratios $k_0/K_m$.

From the dependencies of $\log k_0$ and $\log k_0/K_m$ on the degree of polymerization, graphically presented in Fig. 1, it follows that the rate constants increase with the increasing degree of polymerization if $n < 5$ and remain constant if $n \geq 5$. It was ascertained that there are five subsites in the active centre of the exo-poly-(α-D-galacturonosidase from *Selenomonas ruminatum*. From the mode of degradation of the polymeric and oligomeric substrates and on the basis of the determined frequency of cleavage of [1-$^3$H] oligo(D-galactosiduronate) bonds at low substrate concentrations which exclude the participation of bimolecular processes) presented in Fig. 2 it is evident that the catalytic site of the studied enzyme is located between the second and third subsites.

TABLE I

Experimental values of kinetic parameters with standard deviations and regression analysis statistical characteristics for exo-poly-α-D-galacturonosidase catalyzed hydrolysis of oligo(D-galactosiduronates) at the temperature of 30 °C and pH 7.0. $n$ degree of polymerization related to a galactopyranuronate unit, $K_m$ Michaelis constant, $V$ maximum reaction rate and $k_0$ molecular activity. For details see Experimental

| Substrate | $n$ | $K_m$, μmol/l<br>$V$, nmol/s | Correlation coefficient | Standard deviation of regression | $e_0$, mmol/l<br>$k_0$, 1/s | $k_0/K_m$, l/mmol s |
|---|---|---|---|---|---|---|
| Tri(D-galactosiduronate) | 3 | 502 ± 38<br>25.4 ± 1.2 | 0.998 | $1.68 . 10^{-10}$ | 865<br>0.029 ± 0.0021 | 0.0580 ± 0.0071 |
| Tetra(D-galactosiduronate) | 4 | 262 ± 16<br>132 ± 4 | 0.997 | $1.34 . 10^{-9}$ | 346<br>0.382 ± 0.0123 | 1.46 ± 0.13 |
| Penta(D-galactosiduronate) | 5 | 101 ± 8<br>148 ± 3 | 0.993 | $2.12 . 10^{-9}$ | 216<br>0.684 ± 0.014 | 7.84 ± 0.68 |
| Hexa(D-galactosiduronate) | 6 | 78.7 ± 4.5<br>149 ± 2 | 0.995 | $1.55 . 10^{-9}$ | 216<br>0.688 ± 0.009 | 8.73 ± 0.62 |
| Octa(D-galactosiduronate) | 8 | 76.5 ± 3.3<br>149 ± 3 | 0.999 | $9.33 . 10^{-10}$ | 216<br>0.690 ± 0.014 | 9.02 ± 0.57 |

*Calculation of Subsite Affinities*

Calculation of the affinities of subsites located at the reducing side from the catalytic centre was carried out on the basis of the theory and the algorithm derived from it, which were described in the study of the catalytic action of β-amylase by Kato et al.[27].

The following equation expresses the reaction mechanism of the hydrolysis of oligo(D-galactosiduronates) by exo-poly-α-D-galacturonosidase:

$$E + S_n \underset{K_{n,q}}{\overset{K_{n,p}}{\rightleftharpoons}} ES_{n,p} \xrightarrow{k_{int}} E + P \qquad (1)$$
$$ES_{n,q}$$

where E is the enzyme, P are products, $ES_{n,p}$ is a symbol for the productive complex, $ES_{n,q}$ for the nonproductive complex, $k_{int}$ is the rate constant of the hydrolysis in each productive complex. Symbols p and q represent the type of binding and are equal to the subsite numbers towards which the non-reducing end of the monomer is situated during

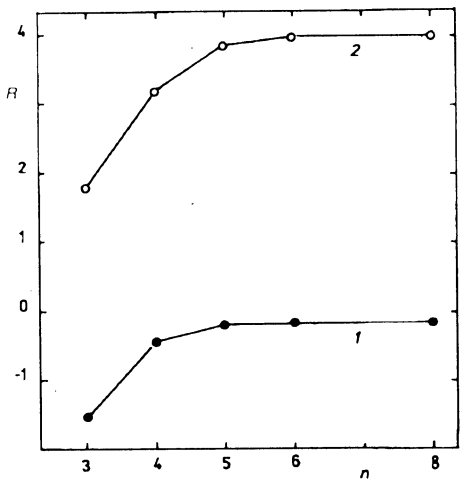


FIG. 1

Dependence of $R$ on the degree of polymerization of oligo(D-galactosiduronates) $n$. $R$ means *1* $\log k_0$ (molecular activity) of exo-poly-α-D-galacturonosidase and *2* $\log k_0/K_m$

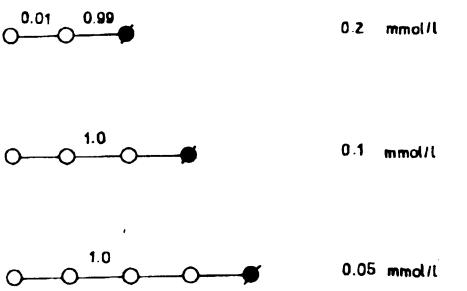


FIG. 2

Frequency of the cleavage of the bonds of tri(D-galactosiduronate), tetra(D-galactosiduronate) and penta(D-galactosiduronate) under the conditions of unimolecular hydrolysis. ○ galacturonate unit, ● [1-$^3$H] labelled galacturonate unit at the substrate reducing end

that certain binding. As the exo-poly-(α-D)-galacturonosidase is an enzyme with a typically terminal mode of action and during the hydrolysis only a disaccharide is formed from oligo(D-galactosiduronates) and sodium pectate, it is possible to presume a model of the active site presented in Fig. 3. The active site has $m = 5$ subsites. The subsites are numbered from left to right starting from the non-reducing unit of the substrate located in the productive type of binding. The catalytic site where the bond is cleaved, is located between the second and third subsites. Affinities of subsites are designated by symbols $A_i$ ($i = 1, 2 \ldots m$). From the mechanism of degradation of linear oligo(D-galactosiduronates) by this enzyme it follows that the productive type of binding j = 2 or p = 2 (Fig. 3) is negligible and the binding constant for the enzyme E and substrate $S_n$ bound by the j type is given as follows:

$$k_0/K_m = k_{int} K_{n,j} = k_{int} K_{n,1} , \qquad (2)$$

$k_0/K_m$ representing only the expressions for the productive type of binding.

In accordance with the theory of subsites[1,12], the reaction rate constants $k_0/K_m$ for $n$-dimensional substrate are equal to the association constant for the productive type of binding which is determined by the affinity of subsites occupied by the substrate. This relationship can be expressed by the equation

$$(k_0/K_m) = k_{int} K_{n,p} = 0.0186\, k_{int} \exp\left(\sum_{1}^{cov} A_i/R\,T\right), \qquad (3)$$

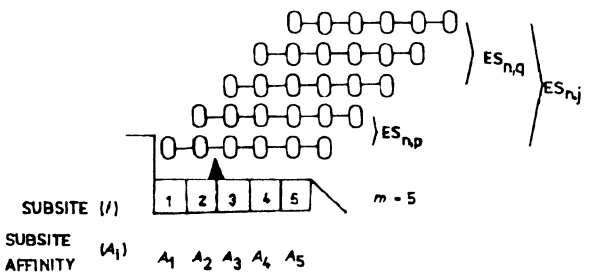


FIG. 3

Schematic model of the active site of exo-poly-(α-D)-galacturonosidase and binding of hexa(D-galactosiduronate). The active site is composed of $m = 5$ subsites. Subsites are numbered from left to right, starting from the non-reducing substrate unit situated in the productive type of binding. The catalytic centre, where the substrate bond is cleaved, is marked by a gusset. Affinities of subsites are marked by symbols $A_i$ ($i = 1, 2, 3 \ldots m$). $ES_{n,j}$ is the enzyme–substrate complex of the $n$-dimensional substrate in the j-type of binding which represents either the productive ($ES_{n,p}$) or the non-productive ($ES_{n,q}$) complex. Indexes j, p, q are whole numbers which specify the type of binding and are equal to the subsite number at which the substrate non-reducing end is situated in the appropriate binding type. O represents the galacturonate unit

where $k_{int}$ is the actual reaction rate constant for the hydrolysis of the substrate bond in the productive complex, $K_{n,p}$ represents the association constant for an $n$-dimensional substrate which forms the productive complex ES. The expression $\sum_{1}^{cov} A_i$ is a sum of all subsites occupied by the $n$-dimensional substrate and bind specifically. Factor 0.0186 represents the correlation for the free energy change of the reaction solution after the binding of the enzyme and the substrate at 30 °C. Affinities of subsites $A_4$ and $A_5$ can be then calculated similarly as in the case of β-amylase[27] using $k_0/K_m$ values as follows:

$$A_{n+1} = R\,T\,[\ln (k_0/K_m)_{n+1} - \ln (k_0/K_m)_n \,. \qquad (4)$$

After the substitution with experimental data $k_0/K_m$ from Table I into the Eq. (4), we obtained values for affinities of subsites $i = 5$ and $i = 4$ equal to:

$$A_5 = 3.98 \text{ kJ/mol}$$
$$A_4 = 8.1 \text{ kJ/mol} \,.$$

*Mode of Action of the exo-Poly-α-D-galacturonosidase and Calculation of the Approximate Affinity of the First Subsite of the Active Centre $A_1$*

The mode of action of the exo-poly-α-D-galacturonosidase was studied on the basis of the formation of products during the interaction of the enzyme with oligo(D-galactosiduronates) with the degree of polymerization between 2 and 8. We found that di(D-galactosiduronate) is not degraded within the concentration range of 0.3 to 4 mmol/l. The prevailing product of the degradation of higher oligo(D-galactosiduronates) was a disaccharide, in the whole course of the followed reaction. During the interaction of exo-poly-α-D-galacturonosidase with oligo(D-galactosiduronates) labelled with [1-$^3$H] at the reducing end and with $n = 2 - 5$ (substrate concentrations lower than the $K_m$ value, at which biomolecule and multiple reactions do not take place), di(D-galactosiduronate) was not degraded by the enzyme under the conditions of first order reaction kinetics. The second bond from the non-reducing end of the degraded molecule is dominant, as was ascertained on the basis of the frequency of cleavage (Fig. 2).

During action of the enzyme on the [1-$^3$H] labelled tri(D-galactosiduronate) under the conditions of unimolecular hydrolysis, monomer (*GalpA)$_1$*, [1-$^3$H] labelled at the reducing end formed the major product of the reaction, accompanied with a minimum amount of a [1-$^3$H] labelled disaccharide (*GalpA)$_2$. The ratio (*GalpA)$_1$ : (*GalpA)$_2$

* (*GalpA)$_n$ represents [1-$^3$H] labelling of the reducing unit of the oligosaccharide with a degree of polymerization $n$.

determined from the frequency of cleavage of bonds in [1-$^3$H] tri(D-galactosiduronate) is 99 : 1. Therefore, it is evident that the trisaccharide can be bound to the active centre of exo-poly-α-D-galacturonosidase in a productive manner j = 1, or p = 1 and j = 2, or p = 2 (Fig. 3). From the experimental data it follows that the type of binding j = 1 or p = 1 is the prevailing one. The relative rate of the formation of products in $(*GalpA)_1$ and $(*GalpA)_2$ is given by the ratios between the probabilities of binding types p = 1 and p = 2 to enzymes with the terminal mode of action. According to Suganuma et al.[28] it can be exressed by the relation

$$\frac{v_{(*GalpA)_1}}{v_{(*GalpA)_2}} = \frac{d[*GalpA]_1\, dt}{d[*GalpA]_2\, dt} = \frac{P_1}{P_2} . \qquad (5)$$

In accordance with the theory of subsites, the ratio of the products can be determined from the difference of affinities $A_1$ of two subsites which do not participate simultaneously in the bond formation in both possible types[1,12]. In the case of exo-poly-α-D-galacturonosidase subsites $A_1$ and $A_4$ come into the consideration (Fig. 4), resulting in a relation

$$\frac{P_1}{P_2} = \exp\left[(A_1 - A_4)/RT\right] . \qquad (6)$$

The real reaction rate can be determined on the basis of the formation of [1-$^3$H] products under conditions of the unimolecular hydrolysis, by establishing the remaining substrate which did not participate in the reaction and using the relation[28] (which is valid for $[S] << K_m$):

$$-\frac{d[S]}{dt} = \frac{V[S]}{K_m + [S]} = (V/K_m)[S] = \frac{k_0}{K_m} e_0 [S] . \qquad (7)$$

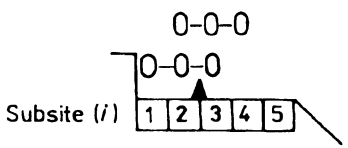


FIG. 4
Schematic design of the binding of two molecules of tri(D-galactosiduronate) to the active site of exo-poly-α-D-galacturonosidase. It shows two possible productive types of binding p = 1 and p = 2

Taking into account the Eq. (7) and the relative rate of product formation during unimolecular hydrolysis, we obtain the following:

$$\frac{v_{(*\mathrm{GalpA})_1}}{v_{(*\mathrm{GalpA})_2}} = \frac{\mathrm{d}[*\mathrm{GalpA}]_1\,\mathrm{d}t}{\mathrm{d}[*\mathrm{GalpA}]_2\,\mathrm{d}t} = \frac{k\,[\mathrm{S}]}{k'\,[\mathrm{S}]}\,, \tag{8}$$

where $k$ and $k'$ represent the reaction rate constants of first order of formation of $(*\mathrm{GalpA})_1$ and $(*\mathrm{GalpA})_2$, and thus

$$\frac{\mathrm{d}[*\mathrm{GalpA}]_1}{\mathrm{d}[*\mathrm{GalpA}]_2} = K\,. \tag{9}$$

At $t = 0$, the concentration of both products is zero and the form of the Eq. (9) is

$$[*\mathrm{GalpA}]_1 = K\,[*\mathrm{GalpA}]_2\,, \tag{10}$$

from which it follows:

$$\frac{v_{(*\mathrm{GalpA})_1}}{v_{(*\mathrm{GalpA})_2}} = \frac{[*\mathrm{GalpA}]_1}{[*\mathrm{GalpA}]_2}\,. \tag{11}$$

The relation for $(A_1 - A_4)$ can be obtained from Eqs (5) and (6) as follows:

$$(A_1 - A_4) = \boldsymbol{R}\,T\,\ln\left[v_{(*\mathrm{GalpA})_1}/v_{(*\mathrm{GalpA})_2}\right] =$$

$$= \boldsymbol{R}\,T\,\ln\left([*\mathrm{GalpA}]_1/[*\mathrm{GalpA}]_2\right) = \boldsymbol{R}\,T\,\ln(90/1)\,, \tag{12}$$

$$(A_1 - A_4) = 11.58\ \mathrm{kJ/mol}\,. \tag{13}$$

After substitution of the value for $A_4$ ($A_4$ = 8.10 kJ/mol) we obtain the result

$$A_1 = 19.68\ \mathrm{kJ/mol}\,. \tag{14}$$

The calculated values $A_1$, approximate affinity $A_1$ and values $A_4$ and $A_5$ are presented in the histogram of the active site of the exo-poly-α-D-galacturonosidase in Fig. 5.

## DISCUSSION

The mode of action of exo-poly-α-D-galacturonosidase from *Selenomonas ruminantium* was followed on the basis of the analysis of products during the interaction of the enzyme with linear oligo(D-galactosiduronates) with a degree of polymerization be-

tween 2 and 8, and with oligo(D-galactosiduronates) labelled with [1-$^3$H] at the reducing end (degree of polymerization 2 to 5). It was found that di(D-galactosiduronate) is not degraded by the enzyme and higher oligosaccharides are cleaved only at the second bond from the non-reducing end of the molecule. The substrate binding site of exo-poly-α-D-galacturonosidase was studied on the basis of the kinetics of degradation of linear oligo(D-galactosiduronates) with degrees of polymerization $n$ = 3 to 6 and 8, under the conditions of unimolecular hydrolysis. With this aim we determined experimentally Michaelis constants and limiting reaction rate constants for individual substrates. We employed these values for calculations of the rate constant $k_0$ and parameters $k_0/K_m$. From the logarithmic dependences of $k_0$ and $k_0/K_m$ on the degree of polymerization of substrates it was found that the active centre of exo-poly-α-D-galacturonosidase from *Selenomonas ruminantium* is formed of five subsites, the catalytic site being situated between the second and third subsites. The affinity of subsites at the right side from the catalytic site was determined by means of the kinetic theory of Hiromi et al.[1,12] using the method published for the calculation of the affinity of subsites of β-amylase from corn bran[27].

It was ascertained that the affinity of the fourth subsite (counted from the non-reducing end) is $A_4$ = 8.10 kJ/mol, affinity of the fifth subsite $A_5$ = 3.98 kJ/mol. The approximate value of the affinity of the first subsite (second subsite to the left from the catalytic site), $A_1$ = 19.68 kJ/mol, was calculated from the distribution of products of degradation of [1-$^3$H] trisaccharide, derived from the frequency of bond cleavage. Affinities of the two subsites in the vicinity of the catalytic site could not be ascertained by means of the presented procedures. The product analysis of [1-$^3$H] labelled and non-labelled oligo(D-galactosiduronates) enabled to express a hypothesis on a relative affi-

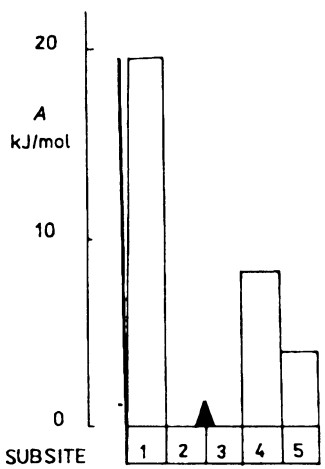


FIG. 5

Histogram of the affinity $A$ of the active site of exo-poly-α-D-galacturonosidase. The active site is composed of five subsites. The catalytic site is situated between the second and third subsite (marked by a gusset). Subsites are numbered from the non-reducing end (from the left). The affinity of the first subsite $A_1$ = 19.68 kJ/mol, $A_4$ = 8.1 kJ/mol, $A_5$ = 3.98 kJ/mol

nity of these two subsites. Facts that di(D-galactosiduronate) is not degraded by the enzyme and that in each phase of the hydrolysis, the primary product of the hydrolysis is a disaccharide regardless the chain length, indicate that the affinity of the second subsite (first subsite from the catalytic site at the non-reducing end) is negligible. Contributions of $A_1$ and $A_3$ are essential for the productive binding of the substrate. Therefore, we presume a substantially higher affinity of the third subsite $A_3$ (subsite at the reducing end from the catalytic site) in comparison to $A_2$. This presumption is also supported by the finding that tri(D-galactosiduronate) is a relatively good substrate for exo-poly-α-D-galacturonosidase. The reaction rate constant $(k_0)_3 = 0.029/s$ for the hydrolysis of the trisaccharide is only 13 times lower than that for the tetrasaccharide $(k_0)_4 = 0.146/s$.

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