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EFFECT OF IMMOBILIZATION OF *Aspergillus Niger* EXTRACELLULAR ENDO-D-GALACTURONANASE ON KINETICS AND ACTION PATTERN

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ABSTRACT

The extracellular endo-D-galacturonanase of *Aspergillus niger* was covalently bound to (hydroxyalkyl) methacrylate gel by using glycine, β-alanine, 4-aminobutanoic acid, glycyl-glycine, and 6-aminohexanoic acid, respectively, as spacers. Immobilization of the enzyme invariably led to decreased activity, the extent of the decrease being inversely proportional to the chain length of the spacer. This fact, as well as apparent kinetic parameters of the immobilized enzyme indicated that steric hindrance and, probably, diffusion effects are responsible for the decrease in activity. The covalent binding also caused an alteration of the action pattern of the enzyme on polymeric and oligomeric substrates. In the case of the polymers the randomness in degradation was lowered because of restriction of the enzyme action to peripheral areas of the substrate molecule. Among oligomeric substrates, the most important change was observed in tetra(D-galactosiduronic acid), in which (2 + 2) degradation occurred as well as the (1+3) degradation characteristic of the soluble enzyme.

INTRODUCTION

Endo-D-galacturonanase [poly(1→4)-α-D-galactosiduronate glycanohydrolase, EC 3.2.1.15] catalyzes random hydrolysis of internal α-(1→4) glycosidic linkages in D-galacturonans, resulting in pronounced diminution in viscosity of the substrate solution at a low degree of degradation. Recently[1], we have shown that the covalent immobilization of endo-D-galacturonanase of *Aspergillus niger* on a cyanogen bromide-activated (hydroxyalkyl)methacrylate gel(Spheron[2]) leads to a lowering of enzyme activity by 45–80%. The extent of the inactivation depends on the pore size of the carrier, the amount of enzyme bound onto the support, and the pH at which the coupling reaction is performed. The decrease in activity is connected with a slight increase in the K_m value and a pronounced decrease in the V value, this

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indicating that steric effects, together with another factor related to the mechanism of the catalytic reaction, are responsible for the decrease in enzyme activity.

It is known that steric hindrance, rendering certain parts of an enzyme molecule less accessible to the substrate molecule, may, in some instances, bring about an alteration in action pattern, particularly with polymer-degrading enzymes[3–8]. This effect should be decreased by the insertion of a spacer between the enzyme molecule and the matrix. In order to characterize the factors that play a role in the changes mentioned in the activity and kinetics of immobilized endo-D-galacturonanase, in the present study the enzyme was covalently bound to the same carrier as was previously employed, using spacers of different chain length, namely glycine, β-alanine, 4-aminobutanoic acid, glycyl-glycine, and 6-aminohexanoic acid. The effect of spacer size on the kinetics and action pattern of the enzyme was investigated by using polymeric and oligomeric substrates.

EXPERIMENTAL

Enzyme. — Endo-D-galacturonanase was purified from the filtrate of a surface culture of *A. niger* growing on Czapek–Dox nutrient medium containing 1.5% citrus pectin as the source of carbon. The purification procedure included salting-out of the protein fraction by ammonium sulfate (0.9 saturation), precipitation with 76% ethanol, and affinity chromatography on cross-linked pectic acid[9]. The specific activity of the preparation on sodium pectate at pH 4.2 (0.1M acetate buffer) and 30° was 0.95 μmol of reducing groups.s^{-1}.mg $protein^{-1}$.

Substrates. — Sodium pectate (*D*-galacturonan content 89.8%, average molecular weight determined viscometrically 27,000) was prepared from citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) by repeated alkaline deesterification with 0.1M sodium hydroxide, followed by precipitation by hydrochloric acid at pH 2.5 and by neutralization with sodium hydroxide. Oligo-D-galactosiduronic acids, degree of polymerization (d.p.) 4–6, were isolated from a partial acid hydrolyzate of pectic acid by gel chromatography on Sephadex G-25 (Fine)[10]. The purity of the preparation was tested by t.l.c. on silica gel[11], and also on the basis of content of reducing groups, as determined spectrophotometrically[12], and carboxyl groups determined by titrimetry.

Carriers. — The (hydroxyalkyl)methacrylate gel Spheron 500 (particle size 40–80 μm, molecular mass exclusion-limit 500,000) was a product of Lachema n.p., Czechoslovakia. After activation with cyanogen bromide[13] glycine, glycyl-glycine (both Lachema, n.p., Czechoslovakia), β-alanine (Koch–Light, Ltd., England), and 4-aminobutyric acid, and 6-aminohexanoic acid (both from Calbiochem, Switzerland), respectively, were bound by the procedure of Cuatrecasas[14]. Spheron 1000 (particle size 100–200 μm, molecular mass exclusion-limit 10^6) was kindly donated by Dr. J. Čoupek (Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague). The carrier was activated by cyanogen bromide[13].

Enzyme immobilization. — The enzyme was attached to the amino groups of the

Spheron derivatives by use of *N'*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide[15]: 3 g of the Spheron derivative, 20 mg of enzyme, and 50 mg of the carbodiimide derivative (Fluke, Switzerland) were incubated under constant stirring (at 4° for 20 h in water suspension adjusted to pH 4.7 by hydrochloric acid. The unreacted enzyme was eluted by 0.1M acetate buffer, pH 4.2. The amount of bound enzyme was determined by amino acid analysis[16] of an acid hydrolyzate of the dried gel, the amino acid used as spacer being excluded from the calculation. The spacerless immobilization on CNBr-activated Spheron was performed as described previously[1].

Enzyme assay. — Enzyme activity was assayed at pH 4.2 (0.1M acetate buffer) and 30° by spectrophotometrically measuring[12] the increase of reducing groups. The mixture containing immobilized enzyme was incubated with constant stirring in a constant-temperature, double-jacketed vessel. Sodium pectate (0.5% solution) and oligo-D-galactosiduronic acids (mM solutions) were used as substrates. Activity is expressed in μmol of reducing groups liberated within 1 s, either by 1 mg of enzyme (free or bound) or by 1 g of enzyme gel. The relative activity of the immobilized enzyme is the ratio of the activities of bound and free enzymes, expressed as a percentage. The kinetic constants K_m and V were calculated on a computer by the least-squares procedure[17] on the basis of initial velocities determined at five substrate concentrations, ranging from 0.1–1mM and computed by a polynomial procedure[18].

For viscometric determination of enzyme activity, mixtures containing 18 mL of 0.5% solution of sodium pectate, (pH 4.2) and 2 mL enzyme (soluble or gel suspension) were incubated under constant stirring at 30°. The reaction time varied from 0 to 30 min. The reaction was stopped by removing the enzyme gel by fast filtration through a sintered-glass filter. The viscosity of the filtrate was measured at 30° with an Ubbelohde viscometer. The viscosity was correlated with reducing groups determined in the same filtrate.

Analysis of reaction products. — T.l.c. of products was performed on silica sheets of Silufol (Kavalier, Czechoslovakia) in 2:3:1 1-butanol–formic acid–water[11]. The compounds were detected with aniline hydrogenphthalate.

Gel-permeation chromatography (*g.p.c.*). — The molecular-size distribution of products was examined on Sephadex G-25 (Fine)[10]. The content of product in column eluates was determined by the method of Somogyi[12].

RESULTS AND DISCUSSION

The preparations of immobilized endo-D-galacturonanases and their activities towards substrate of high molecular weight are listed in Table I. Immobilization led in all instances to a lowering of enzyme activity, as found previously for the direct (spacer-less) coupling onto a CNBr-activated carrier[1]. The extent of activity decrease in spacer-mediated immobilization, however, is affected by the distance between the matrix and the enzyme. The relative activities increase with increasing size of the spacer, obviously because of progressively decreased steric hindrance. Enzyme bound through the longest spacers (6-aminohexanoic acid and glycyl-glycine) showed the

TABLE I

AMOUNTS AND ACTIVITIES OF ENDO-D-GALACTURONANASE COVALENTLY BOUND TO SPHERON 500 THROUGH DIFFERENT SPACERS

Spacer	*Enzyme bound (mg/g gel)*	*Activity (μmol red. groups.s^{-1} per mg enzyme bound)*	*(per g gel)*	*Relative activity (%)*
Glycine	7.3	0.217	1.582	22.9
β-Alanine	4.5	0.262	1.118	27.6
4-Aminobutanoic acid	3.7	0.263	0.976	27.9
Glycyl-glycine	6.8	0.382	2.595	40.4
6-Aminohexanoic acid	5.9	0.575	3.392	60.7

TABLE II

KINETIC CONSTANTS OF SOLUBLE AND IMMOBILIZED ENDO-D-GALACTURONANASE

Enzyme/spacer	K_m; $\overline{K_{m,app}}$ *($M \times 10^5$)*	V; $\overline{V_{app}}$ *(μmol red.g./mg.s)*
Soluble	5.38	1.663
Glycine	5.11 ±0.63	0.340 ±0.024
β-Alanine	5.68 ±0.76	0.378 ±0.045
4-Aminobutanoic acid	6.34 ±0.61	0.340 ±0.070
Glycyl-glycine	5.38 ±0.49	0.587 ±0.041
6-Aminohexanoic acid	5.89 ±0.48	1.180 ±0.038

Substrate: sodium pectate d.p. 153.

highest relative activities. The effects of β-alanine and 4-aminobutyric acid seem to be similar, although the sizes of the spacers are different. It is, however, possible that the enzyme activities, which are also controlled by the amount of enzyme attached to the matrix[1], would differ more if the amounts of enzyme bound per unit quantity of the gel were the same.

Apparent kinetic constants of immobilized-enzyme preparations, averaged from three parallel experiments, are summarized in Table II. The positive effect of increasing the size of the spacers is reflected only in the $\overline{V_{app}}$ values, whereas for $\overline{K_{m,app}}$ the chain length of the spacer seems not to be crucial.

The role of steric hindrance in these changes in activity was further studied by investigating the relation between size of the substrate molecule and enzyme kinetics, with glycyl-Spheron as the carrier. Sodium pectate, d.p. 153, and oligo-D-galactosiduronic acids, d.p. 4–6 served as substrates. Kinetic constants of soluble and immobilized enzyme are shown in Table III. For oligo-D-galactosiduronic acids, the values of both kinetic constants for the immobilized enzyme are substantially lower than those for the soluble one. The ratios of V for immobilized enzyme and soluble enzyme

TABLE III

KINETIC CONSTANTS OF SOLUBLE AND IMMOBILIZED ENDO-D-GALACTURONANASE WITH SUBSTRATES OF DIFFERENT D.P.

D.p. of substrate	*Soluble enzyme* K_m *($M \times 10^5$)*	V *(μmol red.g./mg.s)*	*Immobilized enzyme* $\overline{K_{m,app}}$ *($M \times 10^5$)*	$\overline{V_{app}}$ *(μmol red.g./mg.s)*	$V_{rel.}$ *(%)*
153	5.38	1.663	5.11 ±0.63	0.340 ±0.024	20.4
6	6.10	1.608	1.51 ±0.19	0.390 ±0.042	24.2
5	12.11	0.982	2.20 ±0.18	0.287 ±0.022	29.2
4	21.60	0.990	5.30 ±0.31	0.278 ±0.029	28.1

TABLE IV

DEGREE OF DEGRADATION OF SODIUM PECTATE CORRESPONDING TO 50% DECREASE IN VISCOSITY

Enzyme (spacer)	*Degradation (%)*
Soluble	2.05
(CNBr-activated)	16.00
Glycine	12.61
Glycyl-glycine	7.35
6-Aminohexanoic acid	6.61

(V_{rel}) show that at the same spacer length, the activity is inversely proportional to the substrate size, obviously because of limited access of bulkier molecules to the structure of the gel. The decrease in K_m values indicates that, as well as steric hindrance, diffusion effects could also be responsible for the changes in enzyme activity. However, over the concentration range used, Lineweaver–Burk plots of data for individual substrates did not indicate departure from Michaelis–Menten kinetics.

The effect of immobilization of endo-D-galacturonanase on the pattern of degradation of the high-molecular substrate was investigated (*a*) by determining the degree of randomness of splitting of glycosidic linkages, and (*b*) by characterizing the products released. The first parameter was expressed as percentage of glycosidic bonds cleaved at 50% decrease in viscosity (Table IV). As the splitting of each glycosidic linkage releases one reducing group, and the viscosity is an inverse function of the chain length of the D-galacturonan, it follows that an enzyme exhibiting a high degree of randomness of degradation will show a smaller increase in reducing power, for a given decrease in viscosity, than will an enzyme exhibiting a lower degree of randomness of degradation. The results, summarized in Table IV, show in all instances that the immobilized enzyme, bound either directly onto CNBr-activated carrier, or through a spacer, exhibited degradation that was less random than that

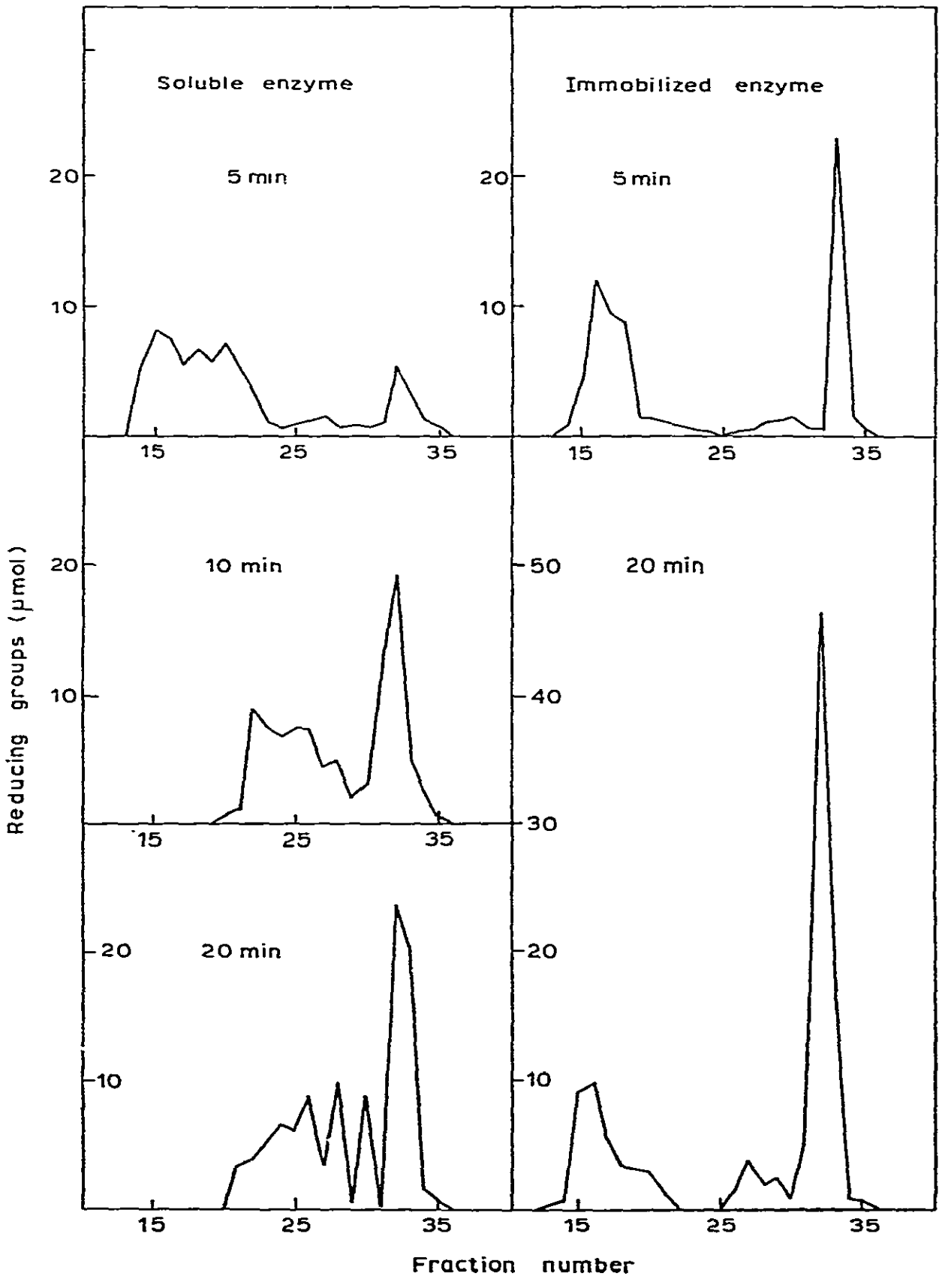

Fig. 1. G.p.c. on Sephadex G-25 Fine of products of degradation of sodium pectate by soluble and immobilized endo-D-galacturonanase. The carrier was CNBr-activated Spheron 500.

exhibited by the soluble enzyme. The lowest randomness of degradation is seen with the enzyme bound directly onto the carrier, where 50% decrease in viscosity corresponds to 16% degradation. An increase in the enzyme–matrix distance by insertion of a spacer leads to an increased randomness of degradation. However, not even the enzyme bound through the longest spacer, (6-aminohexanoic acid) reached the parameters shown by the free enzyme. These results indicate that endo-D-galacturonanase, covalently bound through amino groups onto (hydroxyalkyl)methacrylate

gel, does not cause random cleavage of glycosidic linkages of a high-molecular substrate, but its action is restricted to external, accessible areas of the molecule, the length of which increases with increasing distance between the active site of the enzyme and the matrix of the carrier.

Reaction products analyzed by t.l.c. and g.p.c. (Fig. 1) indicated a similar difference in the action pattern of soluble and immobilized enzyme. The soluble enzyme liberated higher oligo-D-galactosiduronic acids as primary products from the high-molecular-weight substrate, through random splitting of internal glycosidic linkages. In the course of further degradation, the d.p. of the products gradually decreased, as manifested by increasing R_F values in t.l.c. and elution volumes in g.p.c. On the other hand, enzyme immobilized onto CNBr-activated carrier released mainly D-galactopyranuronic acid and lower oligomers right from the beginning of the reaction, and during the course of further degradation their content increased much more than in the case of the degradation by soluble enzyme.

Immobilized endo-D-galacturonanase differed from the soluble enzyme also in its action pattern on oligo-D-galactosiduronic acids. The most important difference was observed in the degradation of tetra(D-galactosiduronic acid) (Fig. 2), which is degraded by the soluble enzyme specifically by a (1+3) action pattern. The immobilized enzyme produced, mono- and tri-saccharide, plus a lower amount of di(D-galactosiduronic acid), regardless of the pore size of the carrier (Spheron 500, Spheron

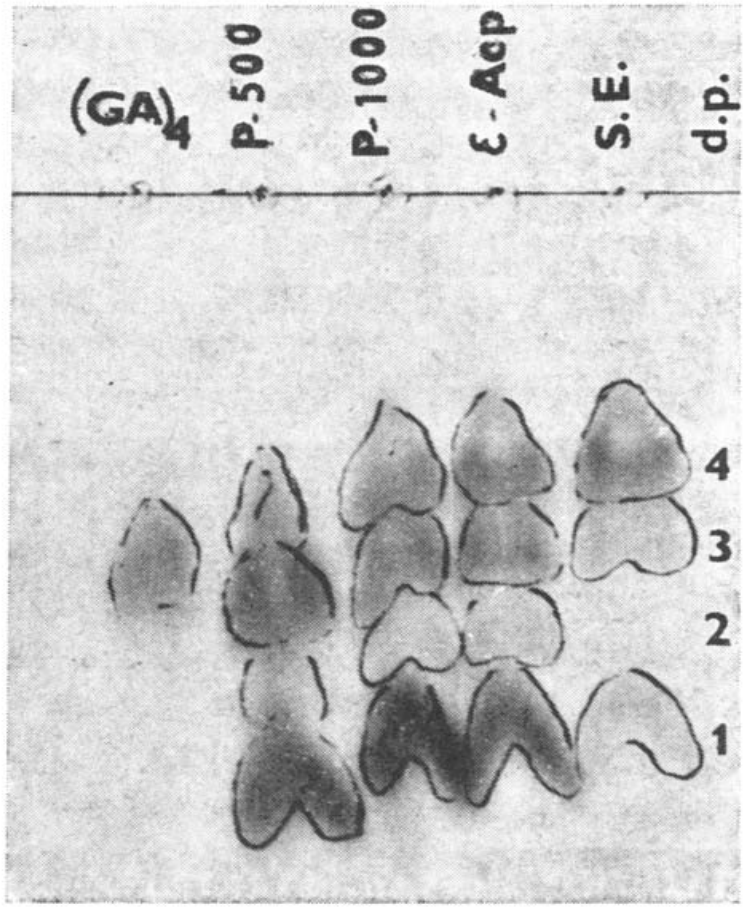

Fig. 2. T.l.c. of products of degradation of tetra (D-galactosiduronic acid) (GA_4) by soluble and immobilized endo-D-galacturonanase. S.e. denotes products of degradation by soluble enzyme; P-500 and P-1000 are products of degradation by enzyme immobilized on CNBr-activated Spherons 500 and 1000; ε-Apc denotes products of degradation by enzyme immobilized on Spheron 500 through 6-aminohexanoic acid. Solvent system[12]: 3:2:1 1-butanol–formic acid–water.

1000), on whether the enzyme was bound directly onto the carrier or through the longest spacer. The degradation of penta(D-galactosiduronic acid) catalyzed by the immobilized enzyme led to a higher production of disaccharide than in the reaction catalyzed by the soluble enzyme, where (4 + 1) degradation prevails[20]. The degradation of hexasaccharide differed in that there was higher production of disaccharide and tetrasaccharide by the immobilized enzyme than by the soluble one.

It is supposed that the specific (3 + 1) degradation of tetra(D-galactosiduronic acid) by soluble *A. niger* extracellular endo-D-galacturonanase is determined by its active site, which has four binding subsites and has catalytic groups located between the subsites interacting with the first and second sugar residues (counted from the reducing end of the substrate molecule)[19]. The altered degradation of this substrate by immobilized enzyme might be connected with substantially decreased affinity of the subsite that binds the sugar residue at the non-reducing end of the substrate molecule. This change could arise either from blocking of this subsite by a covalent linkage to the carrier, or because of a perturbation of the three-dimensional structure of the active site of the enzyme. Under such conditions, tetra(D-galactosiduronic acid) could interact with the remaining three subsites in two ways leading either to (3 + 1) or (2 + 2) degradation, in a manner similar to that supposed with tomato endo-D-galacturonanase[21].

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