THE ROLE OF SECONDARY ALCOHOLIC GROUPS OF D-GALACTURONAN IN ITS DEGRADATION WITH exo-D-GALACTURONANASE

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The effect of exo-D-galacturonanase from carrot on O-acetyl derivatives of pectic acid of various acetylation degree was studied. Substitution of hydroxyl groups at $C_{(2)}$ and $C_{(3)}$ of D-galactopyranuronic acid units influences the initial rate of degradation, degree of degradation and its maximum rate, the differences being found also in the time of limit degradations of the individual O-acetyl derivatives. Value of the apparent Michaelis constant increases with increase of substitution and value of V_{max} changes. O-Acetyl derivatives act as a competitive inhibitor of degradation of D-galacturonan. The extent of the inhibition effect depends on the degree of substitution. The only product of enzymic reaction is D-galactopyranuronic acid, what indicates that no degradation of the terminal substituted unit of O-acetyl derivative of pectic acid takes place. Substitution of hydroxyl groups influences the affinity of the enzyme towards the modified substrate. The results let us presume that hydroxyl groups at $C_{(2)}$ and $C_{(3)}$ of the galacturonic unit of pectic acid are essential for formation of the enzyme–substrate complex.

Experimental technique in enzyme research, employing modified substrates, was exploited by several authors. It has been found that exo-D-galacturonanases of plant and microbial origin, which catalyze the cleavage of pectic substances by a terminal hydrolytic splitting of glycosidic $\alpha(1\rightarrow4)$ bonds between D-galactopyranuronic acid units, favourize the deesterified substrates before the esterified ones. Experiments with oxidized and reduced oligomeric substrates showed that exo-D-galacturonanases catalyze the cleavage of glycosidic $\alpha(1\rightarrow4)$ bonds from the nonreducing end of the molecule[1,2]. D-Galacturonanases with a terminal way of action can be divided into two groups according to their effect on substrates containing 4-deoxy-L-*threo*--4-enopyranosyluronate unit at the nonreducing end. The OH group at $C_{(4)}$ of the D-galactopyranuronic acid unit at the nonreducing end is essential for some enzymes, whereas it does not play any role either in formation of the enzyme–substrate complex, or at catalytic reaction[3–6] with other enzymes. Some exo-D-galacturonanases favour a polymeric substrate, other low-molecular one and for further exo-D-galacturonanases the chain length of the molecule is not decisive[7–10]. Another exo--D-galacturonanases cleave the synthetic substrate, 4-nitrophenyl-α-D-galactosiduronate[6,8].

So far, the role of secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ of D-galactopyranuronic acid units in degradation of D-galacturonans by exo-D-galacturonanases was not investigated. Function of these groups was examined only when studying the action of endo-D-galacturonanase on O-acetyl derivatives of pectic acid[11-13]; more or less different opinion was achieved. Koller and Neukom[11] excluded the role of secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ in a catalytic reaction of endo-D-galacturonanase *Aspergillus niger*; on the other hand, Solms and Deuel[12] found that the degree of substitution of secondary alcoholic groups by acetyl group at $C_{(2)}$ and $C_{(3)}$ in pectic acid influences its degradation by microbial preparation Pectinase[12]. Rexová-Benková and coworkers[13] proved that acetyl groups introduced into the molecule of D-galacturonan resulted in the affinity decrease of endo-D-galacturonanase *A. niger* towards acetylated substrates; they presume that some hydroxyl groups of D-galactopyranuronic acid units take part in formation of the enzyme–substrate complex. It seems that secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ of D-galactopyranuronic acid units are essential for degradation catalyzed by endo-D-galacturonanase and therefore, examination of this problem with exo-D-galacturonanase was worth studying.

EXPERIMENTAL

Material and Methods

Exo-D-Galacturonanase was isolated from the extract of the residue of pressed carrot (*Daucus carota* L.). The press cake was extracted with NaCl solution so that the final concentration of NaCl in solution was 5% at pH 5·0. The isolation process involved salting-out of proteins with ammonium sulfate to 90% saturation, separation of proteins by chromatography on DEAE-cellulose, repeated gel filtration on Sephadex G-100, desalting of the enzyme on Sephadex G-25 Medium and freeze drying[8]. This way prepared enzyme is a specific exo-D-galacturonanase (EC 3.2.1 67), seventyfold purified, of 0·579 μmol min^{-1} mg^{-1} activity on the optimum substrate — oligogalacturonic acid of polymerization degree $DP \approx 10$.

Acetyl derivatives of pectic acid were prepared according to Carson and Maclay[14] with acetic anhydride in formamide-pyridine. Characteristic data of the starting pectic acid, prepared from the commercially available citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark), the content of D-galacturonan, composition of neutral saccharides *etc.* were reported in the previous paper[13]. Preparation of O-acetyl derivatives and analytical determination of acetyl groups were reported in more detail[15]. Acetyl derivatives of pectic acid were prepared as sodium salts.

The mean degree of acetylation is expressed in two ways (Table I): $\overline{DAc}$ represents the ratio between the total content of acetyl groups and D-galactopyranuronic acid units regardless of the content of neutral saccharide units in the pectin molecule; $\overline{DAc(p)}$ expresses the probable acetylation degree of D-galactopyranuronic acid units. The $\overline{DAc}$(p) values were calculated on the basis of chemical composition of pectic acid under a simplifying presumption that acetylation of uronic acid units and neutral saccharide units proceeds to the same degree.

Enzymic Hydrolysis

The enzymic hydrolysis was investigated under optimum conditions for the action of the enzyme, at pH 5·1 and 30°C by measuring the increase of reducing groups per time interval. Concentration of the substrate was adjusted so that the uronate content of all samples was equal. The reaction mixture contained a 0·5% solution of the uronate and 0·5—1 mg of the enzyme in 1 ml. Reducing groups were determined by means of a calibration graph for D-galactopyranuronic acid by the Nelson and Somogyi method[16]. The initial rate of reaction (v_i) was calculated by a graphic extrapolation from experimental data describing the change of the reaction rate with time; it was expressed in micromoles of reducing groups liberated by 1 mg of protein within 1 min. The protein was estimated according to Lowry and coworkers[17], the degree of maximum enzymic degradation on the basis of the maximum value of reducing groups liberated by the enzyme within 4 h; it is expressed in % of cleaved glycosidic bonds. The limit time of degradation was such a time after which no increase of reducing groups during a long-term hydrolysis was observed. Michaelis constants and maximum rates of degradation by exo-D-galacturonanase were determined according to Lineweaver and Burk[18] employing pectic acid and its acetyl derivatives in a concentration range 0·1% and 1% of uronate units and 0·25—1·0 mg of the enzyme in 1 ml of the reaction mixture. The incubation time at 30°C was 10—30 min. The V_{max} and apparent K_m values were calculated always from 18 experimental data, v_i and S by a linear regression (6 various concentrations of the substrate in 3 parallel experiments). The Michaelis constant is given in mol l^{-1} of uronate units, the maximum rate V_{max} is expressed by the number of micromoles of reducing groups per 1 mg of the enzyme within 1 min. The extent and mode of inhibition were determined at two constant concentrations of the native substrate (0·4 and 0·6% solutions of pectic acid) in relation to concentration of the acetyl derivative (0·1 to 0·6%). Graphic treatment of the measured data according to Dixon[19] determined the type of inhibition. The inhibition constant K_i was estimated directly from the intersection point of lines.

Products of the enzymic cleavage of D-galacturonans were identified by paper chromatography after separation in the solvent system ethyl acetate–acetic acid–water (18 : 7 : 8) according to[20]. D-Galactopyranuronic acid was detected with anilinium phthalate, O-acetyl derivatives of saccharides with hydroxylamine; acetylhydroxamic acid thus formed was visualized as an iron complex, 6-O-acetyl-D-glucose being the standard[21].

RESULTS AND DISCUSSION

Five samples of O-acetyl derivatives of pectic acid of mean degree of acetylation $\overline{DAc}$(p) 0·41, 0·63, 0·85, 1·05 and 1·54 (Table I) were used when investigating the role of secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ of D-galactopyranuronic acid units during degradation by carrot exo-D-galacturonanase; the change of initial rate of degradation, time of limit degradation and the maximum degree of degradation in relation to the native substrate (unsubstituted pectic acid) were examined. The initial rate of cleavage of glycosidic bonds (v_i, Table II) was considerably influenced by the substitution degree of the secondary alcoholic groups. Blocking of already 20% of hydroxyl groups (sample 2) resulted in a decrease of the initial rate of cleavage by 63% and the substrate with a 77% substitution (sample 6) showed a 96% decrease of the initial degradation rate (v_i). The degree of maximum degradation of substrates decreased proportionally with the increase of the substitution

degree. The maximum degree of degradation dropped from 1·74 (found with the native substrate) to 0·23 with substrate having a 42% substitution, what means lowering of the degree of degradation up to 87%. Also the limit time of cleavage of the individual acetyl derivatives is different. Time, in which still a measurable degradation of glycosidic bonds occurs is shortened proportionally with the increasing degree of substitution (Table II). Even these results, agreeing with work of Solms and Deuel[12] and Rexová-Benková and coworkers[13] indicate that some secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ take part in the enzymic reaction. Relatively regular decrease of degradation rate of acetyl derivatives of by exo-D-galacturonanase in the whole region of substitution indicates that substitution of hydroxyl groups is not block-wise, but random with a relatively uniform distribution of acetyl groups. The fact that exo-D-galacturonanase catalyses cleavage of glycosidic bonds even with substrate of the highest degree of acetylation $\overline{DAc}$(p) 1·54 supports the con-

TABLE I

Characteristic Data of O-Acetyl Derivatives of Pectic Acid (Na-salt)

Sample	$\overline{DAc}$	$\overline{DAc}$(p)	[COOH] mmol g^{-1}
1	0·46 ± 0·02	0·41 ± 0·02	3·88
2	0·71 ± 0·02	0·63 ± 0·02	3·80
3	0·96 ± 0·03	0·85 ± 0·03	3·65
4	1·19 ± 0·01	1·05 ± 0·01	3·33
5	1·74 ± 0·02	1·53 ± 0·02	3·26

TABLE II

Rate and Degree of Degradation of Pectic Acid and Its Acetylated Derivatives by Exo-D-galacturonanase

Sample	$\overline{DAc}$(p)	v_i μmol min^{-1} mg^{-1}	Relat. v_i, %	Maximum degradation %	Time of limit degradation min
1	0·00	0·380	100	1·74	720
2	0·41	0·142	37·3	0·68	480
3	0·63	0·075	19·7	0·35	180
4	0·85	0·050	13·1	0·23	90
5	1·05	0·035	9·2	0·14	30
6	1·54	0·015	3·9	0·05	30

ception of Rexová and coworkers[13] that acetylation of pectic acid leads highly preferentially or exclusively to diacetyl derivatives of uronic units. Under other circumstances no interaction of the enzyme with the molecule of this substrate could take place, since unsubstituted D-galactopyranuronic acid unit would be no more present in its molecule[13].

The dependence of values of initial rate of enzymic reaction (v_i) on concentration of the individual acetyl derivatives of pectic acid (S), graphically plotted according to Lineweaver and Burk[18], was linear for all substrates with $\overline{DAc}$(p) 0, 0·41, 0·63, 0·85 and 1·05 in agreement with the mechanism proposed by Michaelis and Menten (Fig. 1). The K_m value (Table III) rose with the increasing substitution of hydroxyl groups. Increase of the degree of substitution resulted in a relatively regular decrease of V_{max}, the considerable decrease being observed during transition from the native substrate to that of acetylation degree 0·41. Decrease of the initial rate of degradation with the increasing degree of acetylation and the concurrent increase of the ap-

TABLE III
Kinetic Constants of Exo-D-galacturonanase

Sample	$\overline{DAc}$(p)	K_m mol l^{-1}	V_{max} μmol min^{-1} . mg^{-1}
1	0·00	$5{\cdot}71 \cdot 10^{-2}$	1·776
2	0·41	$7{\cdot}54 \cdot 10^{-2}$	0·441
3	0·63	$7{\cdot}83 \cdot 10^{-2}$	0·261
4	0·85	$9{\cdot}17 \cdot 10^{-2}$	0·098
5	1·05	$1{\cdot}78 \cdot 10^{-1}$	0·056

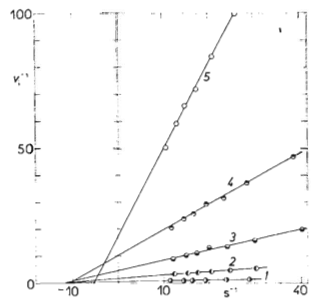

FIG. 1
Dependence of the Initial Rate of Degradation (v_i) of Pectic Acid *1* and its Acetylated Derivatives of $\overline{DAc}$(p) 0·41 *2*; 0·63 *3*; 0·85 *4*; 1·05 *5* on Concentration Plotted According to Lineweaver and Burk
v_i Micromol of reducing groups min^{-1} mg^{-1}; S concentration of uronic acid units of pectic acid and its O-acetyl derivative, mol l^{-1} (0·1—1% in 1 ml of reaction mixture).

parent Michaelis constant with the increasing substitution indicate that formation of the complex enzyme–substrate was influenced, what might be due to an altered affinity of the enzyme resulting from the blockade of hydroxyl groups. Interpretation of these results let us suggest that exo-D-galacturonanase does not form with acetyl derivatives of pectic acid such complexes, in which the binding sites are maximally occupied.

The effect of substitution of hydroxyl groups on the affinity of the enzyme to the substrate was also seen when studying the degree of interference of the native substrate and acetylated substrates with $\overline{DAc}$(p) 0·85 and 1·54 during degradation by exo-D-galacturonanase. The effect of increasing concentration of acetyl derivative on the rate of enzymic degradation of the native substrate at two constant concentrations was investigated. Figs 2 and 3 show this dependence in a plot according to Dixon[19]. The graphic relation reveals that both substrates under investigation behave as a competitive inhibitor and are, therefore, bound to the same site of the enzyme. The rate of degradation of D-galacturonan decreases in the presence of acetyl derivatives and this effect becomes lower with the increase of the acetylation degree. The

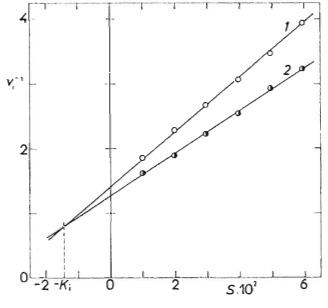

FIG. 2

Dependence of Decrease of the Degradation Rate (v_i) of Pectic Acid on Concentration (S) of the O-Acetyl Derivative of $\overline{DAc}$(p) 0·85 Plotted According to Dixon

Concentration of pectic acid 0·4% *1* and 0·6% *2*; v_i micromol of reducing groups $min^{-1} . mg^{-1}$; S concentration of uronic acid units of O-acetyl derivative, mol l^{-1} (0·1—0·6% in 1 ml of reaction mixture).

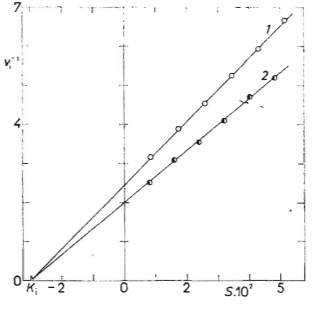

FIG. 3

Dependence of Decrease of the Degradation Rate (v_i) of Pectic Acid on Concentration (S) of O-Acetyl Derivative of $\overline{DAc}$(p) 1·54

For lettering see Fig. 2.

constant of inhibition (K_i) for a sample having $\overline{DAc}$(p) 0·85 is $2{\cdot}95 \cdot 10^{-2}$ mol and for sample of $\overline{DAc}$(p) 1·54 $K_i = 1{\cdot}45 \cdot 10^{-2}$ mol. In connection with the fact that acetylated substrates as competitive inhibitors lower the rate of degradation of pectic acid one can deduce that exo-D-galacturonanase and acetylated derivatives of pectic acid form a productive complex with a lowered affinity. Further, acetylated derivatives are not in a full contact with the catalytic centre of the enzyme, but due to the blockade of a part of the binding centre, they act as a competitive inhibitor.

Since the inhibition effect considerably decreases with increase of the degree of substitution one can ask a question whether the full acetylation of substrate could be the reason for its unability to be attacked by the enzyme. As found (Table II), acetyl derivatives of pectic acid of various degree of substitution are cleaved by the enzyme in a certain time interval only; this time interval is shorter with the increasing degree of acetylation. Basing upon this finding and the above-mentioned results we concluded that the lowered affinity of the enzyme is associated with the hindrance of acetyl group which, not only lowers the possibility of interaction of the enzyme with the substrate, but makes this interaction impossible in the case when substitution of the hydroxyl group of the terminal unit of D-galacturonan was involved. This question was investigated by studying the products of cleavage formed after 100 h of action of the enzyme on substrates of $\overline{DAc}$(p) 0·41, 1·05, 1·54 by chromatography under simultaneous detection of D-galactopyranuronic acid and O-acetyl derivatives with anilinium phthalate and hydroxylamine, respectively. The only detectable product was D-galactopyranuronic acid what indicates that the secondary alcoholic groups at $C_{(2)}$ and $C_{(3)}$ of the terminal uronate unit of galacturonan in the pectic acid macromolecule are essential for a catalytic reaction of the enzyme under investigation.

It is quite difficult to explain the mechanism of action of exo-D-galacturonanase from carrot on O-acetyl derivatives of pectic acid, since there is nothing known about its active centre. Our interpretation is based upon a suggestion that the active centre of exo-D-galacturonanase consists of several subcentres. This suggestion followed also from results of our preceding paper[8], in which it was proved that values of maximum rates of degradation increase with the length of the oligosaccharide chain (the optimally degradable substrate being the oligosaccharide of $DP \approx 10$), even though exo-D-galacturonanase from carrot degrades digalacturonic acid. A like conclusion has been reported also by Pressey and Avants[22] when investigating the mechanism of action of exo-D-galacturonanase from carrot on the homologous series of oligomeric and polymeric substrates.

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