THE ROLE OF sec-ALCOHOLIC GROUPS OF D-GALACTURONAN IN ITS DEGRADATION BY ENDO-D-GALACTURONANASE

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The action of extracellular endo-D-galacturonanase of *Aspergillus niger* on the O-acetyl derivatives of pectic acid was investigated. The initial rate and the degree of degradation of a substrate are dependent on the content of free hydroxyl groups at $C_{(2)}$ and $C_{(3)}$ of D-galactopyranuronic acid units. The substitution of these groups resulted in an enhanced value of apparent Michaelis constant, the V_{max} value being unaltered. The rate of degradation of D-galacturonan decreased in the presence of acetyl derivatives according to relationship for competitive inhibition. The extent of inhibition effect decreases inversely with the degree of substitution of the interfering substrate. The Hill's logarithmic plot of the dependence of the degradation rate on the concentration of substituted units of D-galactopyranuronic acid was linear throughout the overall range of the degree of substitution; the appropriate coefficient n_H equals 3·04. It is assumed that the lowered rate and the degradation degree of acetylated D-galacturonans is associated with the lowered affinity of the enzyme towards substrates, due to modification of groups essential for the formation of the enzyme–substrate complex. The decrease of the degradation degree, proportional to the degree of acetylation throughout the whole substitution range of acetyl groups indicates, at the same time, that the acetylation leads exclusively or highly preferentially to diacetyl derivatives of D-galactopyranuronic acid units.

Endo-D-galacturonanase* (poly-1,4-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.15) catalyses the degradation of pectic substances proceeding by a random hydrolytic cleavage of glycosidic α-1,4 bonds between D-galactopyranuronic acid units. The degree and rate of degradation of pectic substrates depend both on the degree of esterification and the distribution of free and esterified sugar units regardless the character of ester (methyl, hydroxyethyl) groups[3–5]. The optimum substrate of endo-D-galacturonanase is a polymeric, fully de-esterified D-galacturonan. The rate and degree of enzymic degradation decrease with increasing degree of esterification. It is, therefore, presumed the free carboxyl group of D-galactopyranuronate to be essential for the enzyme-substrate complex formation[6,7].

Basing upon both the degradation mode of pectic acid by *A. niger* endo-D-galacturonanase purified from the Swiss commercial preparation Pectinase, and degree of

* Hitherto designated endopolygalacturonase. The new name[1] has been proposed by Horton[2] according to nomenclature of the preferred substrate.

polymerization (DP) of prevalent products (DP 3 an 6) Koller and Neukom[6,8] presumed that the substrate is bound in the complex with enzyme by two carboxyl groups belonging to sugar units separated by three or six D-galactopyranuronic acid units. The configuration at $C_{(1)}$, $C_{(4)}$, $C_{(5)}$ and $C_{(6)}$ is regarded determining for the specificity of the enzyme. The role of sec-alcoholic groups at $C_{(2)}$ and $C_{(3)}$ in the catalytic reaction was excluded by the above-mentioned authors on the basis of a finding that even substrates containing 34% and 69% of these groups substitued by acetyl groups were degraded by endo-D-galacturonanase by 35–40%. Contrary to this finding, according to other results[9], the degree of degradation by microbial pectinase considerably decreases with the increasing degree of acetylation of pectic acid. It is therefore considered that the sec-alcoholic groups at $C_{(2)}$ and $C_{(3)}$ are essential for enzymic reaction.

These differences led us to verify the action of extracellular endo-D-galacturonanase of *A. niger* on acetyl derivatives of pectic acid. We examined the effect of degree of acetylation of D-galacturonan on the kinetics of its degradation, on the affinity towards the enzyme, as well as on the extent of interference of the acetylated substrate with the native one in the interaction with the enzyme.

EXPERIMENTAL

Materials and Methods

The endo-D-galacturonanase was obtained from the filtrate of a 10-day surface culture of *A. niger* growing on Czapek-Dox nutrient medium containing 1·5% of citrus pectin as a carbon source. The purification process involved the salting-out of proteins with ammonium sulphate at a saturation degree of 0·9, precipitation with 76% ethanol[10], affinity chromatography on a cross-linked pectic acid[11], and desalting by gel filtration through a column of Sephadex G-25 (Medium).

Acetylated substrates were prepared from pectic acid, this being obtained by alkaline deesterification of citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) in 60% ethanol[12] and purified as reported earlier[13]. Pectic acid contained 89·8% of D-galacturonan, and 10% of neutral saccharides: D-galactose, L-arabinose, D-glucose, D-xylose, and L-rhamnose in molar ratios 9 : 3 : 2 : 1 : 0·4. Its average molecular weight $M\eta$, determined viscometrically, was 16 500, the sulphate ash content 0·1%. Pectic acid was analysed as before[13]. Potassium pectate was prepared by neutralization of pectic acid by potassium hydroxide.

Acetyl derivatives of pectic acid were prepared according to Carson and Maclay[14] using acetic anhydride in formamide–pyridine[15]. The content of acetyl groups was determined alkalimetrically, after steam distillation, by a procedure described in the previous paper[15]. The average degree of acetylation is expressed in two ways (Table I): *a)* $\overline{DAc}$ represents the ratio of the total content of acetyl groups and D-galactopyranuronic acid units, irrespective the content of neutral saccharides in the molecule of pectic acid. *b)* $\overline{DAc}(p)$ expresses the probable degree of acetylation of D-galactopyranuronic acid units. The $\overline{DAc}(p)$ values were calculated on the basis of chemical composition of pectic acid under a simplifying assumption that the acetylation of uronic acid units and neutral saccharide units proceeds to the same degree[15]. Acetyl derivatives of pectic acid were prepared as potassium salts.

The enzymic hydrolysis was investigated at pH 4·2 (0·1M acetate buffer) at 30°C, by measuring the increment of reducing groups at various time intervals by spectrophotometric method using Nelson–Somogyi reagent[16]. The reaction mixture contained 5 mg of the appropriate D-galacturonan and 0·015 mg of the enzyme per ml. Reducing groups were determined by means of calibration graph for D-galactopyranuronic acid. The initial velocity calculated on a computer, using a program based on the polynomial procedure of Booman and Niemann[17], was expressed in micromoles of reducing groups, liberated by 1 mg of protein. The protein was determined according to Lowry and co-workers[18] with human serum albumin as standard. For determination of kinetic constants the substrate solutions were diluted with a buffer to 5 various concentrations. Kinetic constants K_m and V_{max} were calculated with a computer using a program for Wilkinson's method of least squares[19]. The K_m value is given in molar concentration of D-galactopyranuronic acid units[20]. The degree of maximum enzyme degradation of the substrate was estimated on the basis of the maximum value of reducing groups liberated by the enzyme, constant within 3 h. The products of enzymic degradation of D-galacturonans were identified by TLC chromatography on silica gel (Silufol, Kavalier) using solvent system n-butanol–formic acid–water (2 : 3 : 1) (ref.[21]).

RESULTS

For investigation of degradation of D-galacturonan catalyzed by endo-D-galacturonanase six substrates of acetylation degree $\overline{DAc(p)}$ 0 to 1·6 (Table I) were used. The initial velocity of glycosidic bond splitting was measured and the degree of the maximum enzymic degradation of individual substrates was determined. The concentration of all substrate solutions was identical with respect to the content of D-galacturonan, or glycosidic bonds. Results listed in Table II show the initial velocity of the degradation and the maximum number of cleaved glycosidic bonds (the degree of degradation) to be dependent on the content of free hydroxyl groups of the substrate. The blocking of 15% of these groups (sample 2) resulted in a decrease of the

TABLE I
Characteristic Data of Acetyl Derivatives of Pectic Acid

Sample	$\overline{DAc}^a$	$\overline{DAc(p)}^b$	[COOH] mequiv./g
1	0·00	0·00	4·71
2	0·35 ± 0·03	0·31 ± 0·03	3·66
3	0·73 ± 0·03	0·65 ± 0·03	3·41
4	1·14 ± 0·06	1·01 ± 0·05	3·26
5	1·38 ± 0·01	1·22 ± 0·01	3·08
6	1·81 ± 0·02	1·60 ± 0·02	3·04

[a] Number of acetyl groups per number of D-galactopyranuronic acid units; [b] the probable degree of acetylation of D-galactopyranuronic acid.

initial velocity by 56%. The substrate with 61% of acetylated hydroxyl groups (sample 5) was cleaved by a 0·9% rate, whereas the enhancement of the degree of acetylation to 80% (sample 6) was associated with a 0·45% initial velocity of cleavage. It has been found that the degree of maximum degradation of the substrate decreases inversely with the increase of the degree of acetylation. The blocking of 61% of hydroxyl groups, unlike the results reported by Koller and Neukom[6], caused a decrease of the maximum degree of degradation approximately by one forth.

The average DP of degradation products calculated on the basis of degree of degradation of the respective substrates (Table II) corresponded to results of chromatographic analysis. The substrates of $\overline{DAc}(p)$ 0·31 and 0·65 were degraded to oligogalactosiduronates of DP 1–7 (mostly 1–4) and DP 1–9 (maximum 4–6), resp. The substrate of $\overline{DAc}(p)$ 1·02 afforded products of mobility identical with that of oligogalactosiduronates of DP $\geqq$ 4. All products of the last two substrates had DP higher than 9.

To ascertain the mechanism responsible for the decrease of the degradation rate of acetylated substrates, the kinetics of the enzyme reaction with substrates of $\overline{DAc}(p)$ 0, 0·31, and 0·65 was examined. The dependence of the initial velocity upon the substrate concentration plotted according to Lineweaver and Burk[22] was found to be linear in all three cases in accordance with kinetics of Michaelis and Menten (Fig. 1). The results show that the decrease of the degradation rate of acetylated substrates is associated with the formation of enzyme–substrate complex; blocking of hydroxyl groups resulted in an increase of the apparent Michaelis constant from $1{\cdot}11 \cdot 10^{-2}$ M of D-galactopyranuronic acid units, found with the unsubstituted substrate, by $4{\cdot}15 \cdot 10^{-2}$M with substrate of $\overline{DAc}(p)$ 0·31 and to $2{\cdot}45 \cdot 10^{-1}$M with substrate of $\overline{DAc}(p)$

TABLE II

Rate and Degradation Degree of Pectic Acid and Its Acetyl Derivatives by Endo-D-galacturonanase

Sample	$\overline{DAc}(p)$	v_i μmol . min^{-1} . mg^{-1}	Relat. v_i, %	Degradation degree, %	Products ~DP
1	0·0	100·67	100	37·75	2·6
2	0·31	44·05	43·76	31·16	3·2
3	0·65	7·27	7·22	28·65	3·5
4	1·02	2·21	2·10	15·58	6·3
5	1·22	0·91	0·90	9·47	11·1
6	1·60	0·46	0·45	5·67	17·6

0·65. On the other hand, the V_{max} value of all three substrates is equal and corresponds to 156 ± 9 micromoles of reducing groups . min^{-1} . mg^{-1}.

The effect of substitution of hydroxyl groups on the affinity of the enzyme to substrate was also manifested when examining the degree of interference of the native substrate and acetylated substrates of $\overline{DAc(p)}$ 0·65 – 1·6 upon the cleavage by the enzyme. The effect of 0·3% of the acetylated substrates on the degradation rate in 0·5% solution of unacetylated pectic acid was examined. The degradation rate of D-galacturonan decreases in the presence of acetyl derivatives. The extent of decrease depends on the degree of substitution of the interfering acetylated substrate. The higher is the degree of acetylation, the lower is its inhibitory effect (Table III).

Consonant with this finding are also results obtained when studying the effect of concentration of the native D-galacturonan on the decrease of its degradation rate in the presence of acetyl derivatives of $\overline{DAc(p)}$ 0·65 – 1·02 (Fig. 2). It was simultaneously shown that both types of substrates (acetylated and unacetylated) are bound to the same locus of the enzyme molecule, since the obtained values fit the relationship for competitive inhibition, where the ratio of rates of the competitive cleavage of

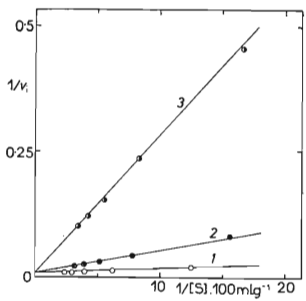

FIG. 1

Plot of the Initial Velocity of Pectic Acid *1* and Its Acetyl Derivatives of $\overline{DAc(p)}$ 0·31 *2* and 0·65 *3* on the Concentration of the Substrate Plotted According to Lineweaver and Burk[22]

v_i Micromol of reducing groups . min^{-1} . . mg^{-1}; [S] g/100 ml of the reaction mixture.

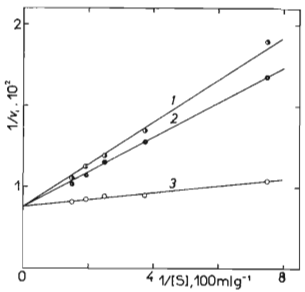

FIG. 2

Dependence of the Decrease of Degradation Rate of Pectic Acid in the Presence of Acetyl Derivatives of $\overline{DAc(p)}$ 0·65 *1* and 1·02 *2* on Concentration Plotted According to Lineweaver and Burk[22], *3* Pectic Acid without addition of Acetyl Derivative

Concentration of both acetyl derivatives in the reaction mixture = 0·8 mg/ml.

TABLE III

Decrease of the Rate of Degradation of Pectic Acid in the Presence of Its Acetyl Derivatives

Sample	$\overline{DAc(p)}$	v_i Decrease %
3	0·65	20·7
4	1·02	16·3
5	1·22	9·8
6	1·60	1·8

D-galacturonan (v_A) and of its acetyl derivatives (v_B) calculated according to the equation for competitively degraded substrates[23], is linearly proportional to the concentration of the unacetylated substrate (Fig. 3). The presented results indicate that the blocking of hydroxyl groups lowers the ability of the substrate to form the catalytic complex with the enzyme.

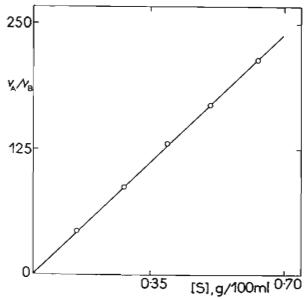

FIG. 3

Dependence of the Ratio of the Rates of Cleavage of Competitively Degraded Pectic Acid and Its Acetyl Derivative of $\overline{DAc(p)}$ 0·65 on the Concentration of Pectic Acid

v_A Initial degradation rate of pectic acid, v_B initial degradation rate of acetyl derivative, [S] concentration of pectic acid in g/100 ml.

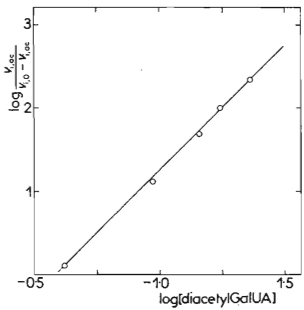

FIG. 4

Hill's Logarithmic Plot of the Degradation Rate of Decrease of Pectic Acid $v_{i,ac}/(v_{i,0} - v_{i,ac})$ *versus* Concentration of Substituted D-galactopyranuronic Acid Units

To determine the quantitative relation between the rate of cleavage of glycosidic bonds and the substitution of hydroxyl groups, the rate parameter $v_{i,ac}/(v_{i,0} - v_{i,ac})$ was plotted as a function of the concentration of substituted uronic acid units in the form of Hill's logarithmic plot (Fig. 4); ($v_{i,ac}$ is the initial velocity of degradation of acetylated substrates, $v_{i,0}$ – the initial velocity of degradation of unacetylated D--galacturonan). Basing upon arguments given in following discussion it was assumed, when calculating this function, the diacetyl derivatives of D-galactopyranuronic acid to be preferentially formed by acetylation of D-galacturonan. The values of initial velocity given in Table II were used. The plot is linear in the whole concentration range of modified uronic acid units (Fig. 4), the Hill's coefficient n_H equals 3·04.

DISCUSSION

The effect of substitution of hydroxyl groups both on kinetic constants and the interference of acetyl derivatives with pectic acid upon the reaction with endo-D-galacturonanase show that the lowering of the rate and degradation degree due to the introduction of acetyl groups into the molecule of D-galacturonan is associated with the decrease of the enzyme affinity to acetylated substrates. This might be caused *a*) by the blocking of groups essential for the formation of the enzyme–substrate complex; *b*) by steric hindrance, due to the introduction of a bulky group into the molecule of the substrate; *c*) by a conformation change of the substrate molecule, due to acetylation and the inability of the enzyme and the modified substrate to form a required catalytic conformation.

The molecule of D-galacturonan is a *trans*-1,4-polysaccharide the chain of which has a three-fold screw symmetry with a period of identity 1·31 nm (ref.[15]). It is a relatively rigid linear chain with a restricted flexibility where the shortest possible distance between two carboxyl groups amounts 0·55–0·56 nm (ref.[15]). Pectic acid looses, already at a low degree of acetylation, the ability to form gels, due to a steric hindrance which exerts the acetyl group against the mutual contact of molecules[25]. It is, however, presumed that the acetylation alters the conformation of the D-galacturonan chain. The enhanced viscosity found with aqueous solutions of partially acetylated D-galacturonans, was ascribed by Solms and Deuel[9] to a certain extension or unfolding of the molecule. Palmer and Ballantyne[26] reported, with fully acetylated derivative of pectic acid, a transition of the D-galacturonan chain to a conformation possessing a two-fold screw symmetry with a period of identity 0·87 nm. In such a modified molecule the carboxyl groups of uronic acid units are alternatively situated at one or the other side of the chain and consequently, their actual distance is greater than in an unsubstituted substrate. Considering the function, which is ascribed to carboxyl groups of a substrate in formation of the complex with endo-D-galacturonanase[6,7] this change of conformation could exhibit an unfavourable effect on the catalytic reaction. With a fully acetylated substrate it could be one of the reasons of its stability

towards endo-D-galacturonanase. So far, it is neither known how deep si the conformation change of the macromolecule of a partly acetylated D-galacturonans, nor their distribution of acetyl groups.

The acetylation of D-galacturonan is regarded by Schweiger[27] a two-stage reaction in which monoacetyl derivatives of uronic acid are formed first, whilst in the second, slower step the second hydroxyl group of the uronic acid unit undergoes acetylation. Substrates of DAc > 1 are supposed to have every sugar unit substituted at least by one acetyl group. Koller and Neukom[6] consider a random acetylation, during which $C_{(2)}$ or $C_{(3)}$ monoacetyl derivatives together with diacetyl derivatives were formed at the same time.

The results of investigation of the action of endo-D-galacturonanase on acetyl derivatives of D-galacturonan do not favour any of the considered distributions of acetyl groups. In the case of the primary formation of monoacetyl derivatives, the $\overline{DAc(p)} = 1$ would be critical for enzymic degradation, regardless which of the considered factors affecting the formation of the complex enzyme–substrate was involved. If one acetylated hydroxyl group in the uronic acid unit hinders the formation of the enzyme–substrate complex, no enzymic reaction would take place at $\overline{DAc(p)} > 1$. Providing the formation of the complex was prevented by two acetylated hydroxyl groups at the uronic acid unit, no lowering of the enzyme reaction would occur with substrates of $\overline{DAc(p)} < 1$. In case that the distribution of acetyl groups is consonant with that considerd by Koller and Neukom[6], the critical $\overline{DAc(p)}$ would be about 1·3. At this degree of acetylation no unacetylated uronic acid unit could appear in the substrate molecule.

The relatively regular decrease of the degradation degree in the entire substitution range indicates that the acetylation leads highly preferentially or exclusively to diacetyl derivatives of uronic acid units. This presumption is confirmed also by the linearity of Hill's plot for the whole range of the degree of acetylation, which may be obtained only under condition that diacetyl derivatives are preferentially formed. In such a case the substrate molecules of $\overline{DAc(p)} < 2$ possess unsubstituted D-galactopyranuronic acid units enabling their interaction with the enzyme. The character of the degradation products of the respective acetylated substrates indicates at the same time that the distribution of substituted and unsubstituted units is not blockwise, but rather random. The blockwise distribution would produce in all acetylated substrates lower oligogalacturonates as degradation products (in addition to a high molecular residue) formed by a repeated attack of the enzyme in the block of unsubstituted uronic acid units.

It is supposed that extracellular endo-D-galacturonanase of *A. niger* interacts in the catalytic reaction with a substrate by means of four binding subsites which bind a segment of the substrate composed of four units of D-galactopyranuronic acid[28]. In addition to this binding there are further sites of contact of lower affinity, between

the enzyme and a substrate[29] supposed in the complex. In the case of the considered distribution of diacetyl derivatives of uronic acid units in the substrates of $\overline{DAc(p)} > 1$ at least two sugar units of the bound segment are substituted. Had the decrease of activity be caused by steric hindrance, no of this substrates would form a complex with the enzyme at such a high frequency of substitution. The same effect would also have the change of the conformation of substrate macromolecule. Since even the substrate of acetylation degree $\overline{DAc(p)}$ 1·6 undergoes an enzymic degradation, we presume the cause of the lowered affinity of the enzyme to acetylated substrates to be the blocking of some groups essential for the enzyme–substrate complex formation. We therefore assume that, in addition to carboxyl groups, also some hydroxyl groups of D-galactopyranuronic acid units are involved in the formation of this complex, forming hydrogen bonds with groups of the binding site of the enzyme, similarly, as is the case with lysozyme[30], or pancreatic α-amylase[31]. As it follows from both the linear course of the Hill's plot and the value of the association coefficient n_H, hydroxyl groups of some three uronic acid units contribute to the affinity of the substrate towards the enzyme. Their blocking results in the loss of substrate ability to form complex with the enzyme. As it follows from the effect of acetylation on the interference of acetylated and unacetylated D-galacturonans in the enzymic reaction, the affinity of endo-D-galacturonanase to acetylated substrates is the greater, the more D-galactopyranuronic acid units are unsubstituted.

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