EVIDENCE FOR THE ROLE OF CARBOXYL GROUPS IN ACTIVITY OF ENDOPOLYGALACTURONASE OF *Aspergillus niger*. CHEMICAL MODIFICATION BY CARBODIIMIDE REAGENT

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Endopolygalacturonase (E.C. 3.2.1.15) of *Aspergillus niger* was modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and glycine ethyl ester. The modification resulted in total irreversible inactivation of the enzyme and derivatization of carboxyl acid residues and tyrosine residues. The treatment of the modified enzyme with hydroxylamine led to a restoration of modified tyrosine residues but not to reactivation of the enzyme. The inactivation with carbodiimide was pH dependent, the rate of inactivation increased with decreasing pH. Tri(D-galactosiduronic acid), a competitive inhibitor, or crosslinked pectic acid protected the enzyme against the inactivation. In bioaffinity chromatography of partially inactivated endopolygalacturonase, all residual enzyme activity was retained on the adsorbent while all inactive fraction passed without retardation through the column. On the basis of these results, as well as proximity of the rate constants for enzyme inactivation and the carboxyl group modification it is suggested that the loss of endopolygalacturonase activity is due to the modification of carboxylic acid residues and that at least one is essential for enzyme activity.

Endopolygalacturonase [poly(1,4-α-D-galacturonide) glycanohydrolase, E.C. 3.2.1.15] catalyzes a random hydrolytic cleavage of internal glycosidic linkages of D-galacturonan chains of pectic substances. At present, an enhanced interest is paid to immobilization of the enzyme on insoluble supports. In connection with this problem, characterization of active groups of the enzyme, essential for its activity is important.

Inhibitory effect of some compounds on endopolygacturonases of different origin suggested amino groups[1], tyrosyl residues[2] and histidine residues[3,4] to be essential for enzyme activity. On the basis of pH effect on kinetic parameters K_m, k_{cat} and k_{cat}/K_m of *Aspergillus niger* endopolygalacturonase a carboxyl group having apparent pK 3·0 and imidazole group dissociating in the free enzyme at pH 5·0 and in catalytic complex at pH 5·7 resp., have been considered catalytically essential[5]. Other evidence of the role of carboxylic acid residues in action of the enzyme has not been presented so far.

The method most commonly used for the examination of the role of carboxylic acid residues in enzyme catalysis is based on the modification of the enzyme with

a water-soluble carbodiimide[6]. The same reaction can be used for the coupling of enzyme via carboxyl groups to insoluble supports[7]. In the present work extracellular endopolygalcturonase of *A. niger* was modified with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and [^{14}C]labelled glycine ethyl ester. The effect of the modification on enzyme activity, kinetics and biospecific interaction was studied.

EXPERIMENTAL

Material and Methods

Endopolygalacturonase was isolated from the filtrate of a surface culture of *A. niger* growing on Czapek-Dox nutrient medium containing 1·5% citrus pectin as carbon source. The isolation procedure included salting-out by ammonium sulphate, precipitation with 76% ethanol, affinity chromatography on crosslinked pectic acid[8] and affinity chromatography on tri(D-galactosiduronic acid)-Separon (ref.[9]). Products obtained after individual purification steps were desalted on Sephadex G-25 (Medium) and freeze-dried. Purity of the final product was estimated by discontinuous polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8·3 and by gel chromatography on Superose 12^{TM}, FPLC System Pharmacia (Sweden).

Sodium pectate (M_r 27 000, determined viscometrically, D-galacturonan content 89·8%) was prepared by alkaline deesterification of citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark), followed by precipitation with hydrochloric acid at pH 2·5 and neutralization with sodium hydroxide. Hepta(D-galactosiduronic acid) and tri(D-galactosiduronic acid) were isolated from a partial acid hydrolysate of pectic acid (ref.[10]) by gel chromatography on Sephadex G-25 Fine). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Fluka AG, Switzerland), glycine ethyl ester hydrochloride (Serva, F.R.G.) and [^{14}C]glycine ethyl ester (Isocommerz GmbH, Berlin-Buch, G.D.R.) were used without further purification. Tri(D-galactosiduronic acid)-Separon used as biospecific adsorbent was prepared as described previously[9].

Enzyme assay: Endopolygalacturonase was assayed at pH 4·2 (0·1M acetate buffer) by determining the increment of reducing groups at different time intervals by spectrophotometric method of Somogyi[11]. The initial velocities, defined in micromol reducing groups liberated by 1 mg protein per second, were calculated using a program based on polynomial procedure[12]. K_m and V were computed using the least-square procedure[13]. In kinetic measurements hepta-(D-galactosiduronic acid) was used as the substrate. K_m is expressed in mol l^{-1} D-galacto-pyranuronic acid units. Protein was determined by the method of Lowry et al.[14] using human serum albumin as standard.

Modification of endopolygalacturonase: The reaction procedure employed for the modification of the enzyme by carbodiimide and glycine ethyl ester was essentially that described by Hoare and Koshland[6]. The reaction was carried-out at pH 4·7 and room temperature. Endopolygalacturonase (10 mg) was incubated in 10 ml water with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (50 mg) and a mixture of unlabelled and [^{14}C]-labelled glycine ethyl ester (300 mg). Constant pH (controlled with Radiometer PHM 28 instrument, Denmark) was maintained by adding 0·1M-HCl. Aliquots for enzyme assay, radioactivity measurement and amino acid analysis were withdrawn at appropriate intervals. Enzyme activity was determined in aliquots diluted by 10 volumes of 0·1M acetate buffer pH 4·2. The samples for radioactivity measurements and amino acid analysis were added to equal volumes of 0·4M acetate buffer pH 4·2 to stop the reaction, then desalted on Sephadex G-25M, PD-10 column (Pharmacia, Sweden). Radioactivity was

measured on Liquid Scintillation Counter 1214 Rackbeta (LKB, Sweden). Amino acid analysis was carried out on Amino Acid Analyzer T 339, Mikrotechna, Prague (Czechoslovakia) after 24 h hydrolysis of samples in 6M-HCl at 106°C. UV spectrum was measured on the registration spectrophotometer Beckman DB-GT in 1 cm cells. Hydroxyaminolysis of modified endopolygalacturonase was carried out according to Carraway and Koshland[15] by treating the modified enzyme with 0·5M hydroxylamine for 7 h at pH 6·8 and room temperature. Before further examination the samples were desalted by gel filtration on PD-10 column of Sephadex G-25 M.

Bioaffinity chromatography of endopolygalacturonase modified with [^{14}C]glycine ethyl ester on tri(D-galactosiduronic acid)-Separon was carried out in 0·1M acetate buffer, pH 4·2. The retained fraction was eluted by 0·1M acetate pH 6·0 (ref.[9]). Radioactivity was measured in aliquots of fractions eluted from the adsorbent.

RESULTS

Endopolygalacturonase used for the modification by carbodiimide was homogeneous in discontinuous polyacrylamide gel electrophoresis[9] and gave a single symmetrical peak by FPLC on Superose 12 column. Its relative molecular mass determined using bovine serum albumin, ovalbumin, chymotrypsin, and ribonuclease as standards was found to be ~35 000. This value is identical with the value previously found by ultracentrifugation and on the basis of amino acid content, resp.[16].

The reaction of the enzyme with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and glycine ethyl ester resulted in total loss of enzyme activity. At used concentrations of the reagents, until about 90% inactivation the decrease in activity displayed pseudo-first-order kinetics (Fig. 1) with the rate constant $k_A = 2{\cdot}04 \pm 0{\cdot}19 \,.\, 10^{-3}\ s^{-1}$. The inactivation was irreversible since gel filtration or dialysis of the inactivated enzyme did not restore enzymic activity. The decrease in activity was accompanied with an increase in radioactivity and glycine content, and derivatization of some tyrosine residues (Table I). All other amino acids remained unchanged. A radioactivity increase of about 86% occured within first 15 min, the rate constant $k_{RA} = 1{\cdot}89 \pm 0{\cdot}54 \,.\, 10^{-3}\ s^{-1}$. The remaining radioactivity increased at lower rate, $k'_{RA} = 2{\cdot}14 \,.\, 10^{-5}\ s^{-1}$. The total radioactivity increase corresponded to an incorporation of 3 glycine residues. The rate constant of the incorporation measured within first 15 min of the reaction $K_I = 2{\cdot}25 \pm 0{\cdot}22 \,.\, 10^{-3}\ s^{-1}$. As carboxyl groups are the only ones which react with the nucleophile giving a stable derivative[6] the incorporation of glycine residues was considered a measure of their modification. The content of tyrosine residues decreased in the course of the first 30 min by about 18% and then remained unchanged, i.e. some 3 out of 15 residues present in the molecule of the native enzyme[16] have undergone reaction with carbodiimide. The treatment of the fully inactivated enzyme with hydroxylamine led to a reincrease of tyrosine residue content, it failed, however, to restore enzymic activity. The modification of tyrosine residues by carbodiimide and deacetylation with hydroxylamine were manifested also in differential absorption spectra of the native enzyme measured against the modified enzyme and the deacetylated one, resp. (Fig. 2).

TABLE I

Radioactivity and glycine and tyrosine content of endopolygalacturonase in the course of the reaction with carbodiimide and [^{14}C]glycine ethyl ester

Rection time min	Radioactivity d.p.m.	Glycine		Tyrosine	
		g/100 g prot.	mol residue per M_r 35 000	g/100 g prot.	mol residue per M_r 35 000
0		5·10	31·29	6·76	14·50
5	680	5·37	32·95	6·46	14·30
15	1 991	5·52	33·89	6·27	13·45
30	2 160	5·56	34·11	5·54	11·88
60	2 250	5·58	34·24	5·52	11·84

The modification of the enzyme with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide without the nucleophile proceeded at the rate by one order lower and was pH and concentration dependent. The inactivation process obeyed in all cases pseudo-first-order kinetics. The rate of inactivation decreased with increasing pH (Fig. 3). The logarithmic plot of pseudo-first-order rate constants of inactivation measured at 540-, 770-, and 1 150-fold molar excess of the modifier vs carbodiimide concentration[17] gave a stright line with a slope of 0·98 suggesting that at least 1 mol carbodiime binds an equivalent mol of endopolygalacturonase for inactivation. The treatment of the enzyme in the presence of 22 molar excess of tri(D-galactosiduronic acid), a competitive inhibitor[18] or 20-fold amount of crosslinked pectic acid, a bioaffinity adsorbent[19], caused the lowering of the inactivation rate by about 30% and 65%, resp. ($k_A = 1{\cdot}48 \cdot 10^{-3}\ s^{-1}$ and $7{\cdot}13 \cdot 10^{-4}\ s^{-1}$).

The effect of the modification of endopolygalacturonase on kinetics of action was examined using the enzyme preparation with 78% residual activity. The $K_{m,app}$ found with hepta(D-galactosiduronic acid) as the substrate $3{\cdot}65 \cdot 10^{-3}$ mol l^{-1} was very close to K_m value of the native enzyme $3{\cdot}48 \cdot 10^{-3}$ mol l^{-1}. On the other hand, V value decreased due to modification from original 0·67 μkat to 0·52 μkat. In view

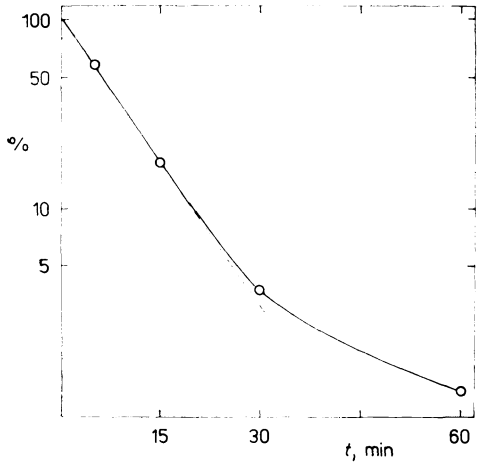

FIG. 1

Semilogarithmic plot of time-course of inactivation of endopolygalacturonase by carbodiimide and glycine ethyl ester at pH 4·7. The enzyme (10 mg) was incubated with 50 mg carbodiimide and 300 mg glycine ethyl ester. Activity is given in %

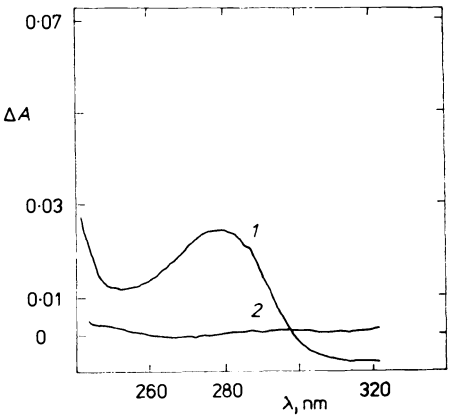

FIG. 2

Differential absorption spectra of native endopolygalacturonase measured against the enzyme fully inactivated with carbodiimide and glycine ethyl ester (*1*) and the product of subsequent treatment with hydroxylamine (2). Wavelength λ is given in nm

of the substantial drop of V value and the similarity in K_m values it seems likely that the activity of partially inactivated enzyme corresponds to residual unmodified enzyme rather than to altered enzyme with distinct kinetic characteristics and affinity. The same conclusion resulted also from bioaffinity chromatography of the enzyme partially modified (68% original activity) with labelled glycine ethyl ester (Fig. 4). All labelled enzyme was found in the inactive fraction passing through the adsorbent, tri(D-galactosiduronic acid)-Separon, without retardation while all residual activity was retained on the column and subsequently released at pH 6·0.

DISCUSSION

On the basis of the obtained results it seems likely that the loss in enzyme activity occuring in the course of the reaction of endopolygalacturonase with carbodiimide

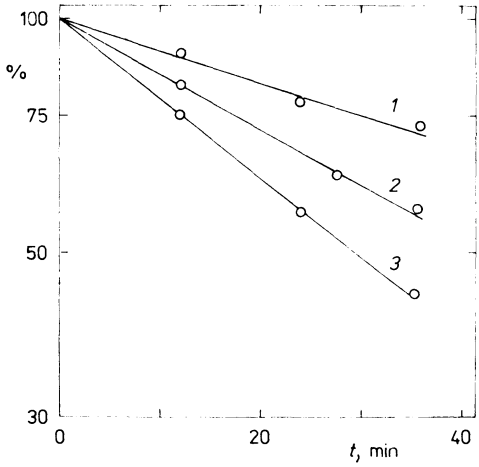

FIG. 3

Semilogarithmic plot of time-course of inactivation of endopolygalacturonase by carbodiimide at pH 5·8 (*1*), pH 4·9 (2), and pH 4·2 (*3*). The enzyme was incubated with 770-molar excess of carbodiimide, pH was maintained constant by 0·05M-HCl. Activity is given in %

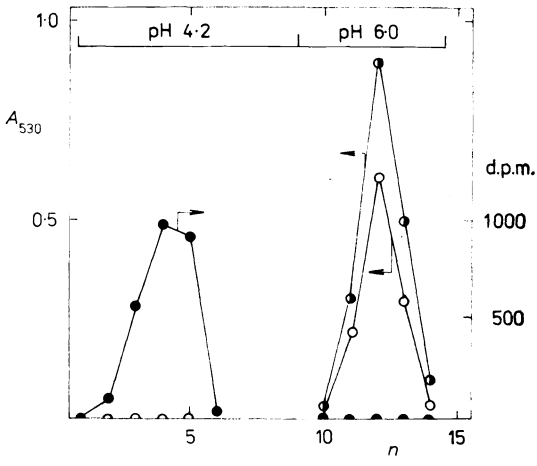

FIG. 4

Bioaffinity chromatography of endopolygalacturonase partially inactivated (68% residual activity) with carbodiimide and [^{14}C]labelled glycine ethyl ester on tri(D-galactosiduronic acid)-Separon. The enzyme sample was applied on the column at pH 4·2 (0·1M acetate buffer), the adsorbed fraction was eluted with 0·1M acetate, pH 6·0. Radioactivity (●); enzyme activity of modified enzyme (○), of native enzyme (◐). A_{530} Enzyme activity measured at 530 nm; n fraction number

is caused by the modification of carboxyl groups. This is consistent with the possibility that at least one carboxyl group participated in endopolygalacturonase action. Although under the conditions used tyrosine residues were modified by carbodiimide too, it is not probable that the inactivation is connected with this process. This conclusion is based on the following experimental evidence: *a*) the irreversibility of the inactivation with carbodiimide and glycine ethyl ester shown when the enzyme was treated with hydroxylamine, *b*) the decreasing trend of inactivation with increasing pH, typical for the modification of ionizable carboxyl group[20], *c*) difference in the rates of inactivation by carbodiimide alone and in the presence of the nucleophile, *d*) the observation that the incorporation of [^{14}C]glycine ethyl ester into endopolygalacturonase closely parallels the loss of enzyme activity (the proximity of the rate constants K_A, k_I and k_{RA}). The slope of the logarithmic plot of the rate constant of inactivation vs carbodiimide concentration near to 1 indicates that at least one carboxylic acid residue modified by carbodiimide is essential for enzyme activity. The protection of endopolygalacturonase against inactivation by tri(D-galactosiduronic acid) or crosslinked pectic acid is in favour of the presence of carboxylic acid residue in the active site of the enzyme.

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