IMMOBILIZATION OF EXO-D-GALACTURONANASE BY COUPLING TO A POLYACRYLAMIDE TYPE SUPPORT

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Exo-D-galacturonanase (E.C. 3.2.1.67) from carrot was immobilized by covalent bonding to a polyacrylamide type support (with free carboxyl groups) activated by water-soluble carbodiimides. The activity of the immobilized enzyme (under optimal reaction conditions of the immobilization) was around 43% of the activity of the free enzyme. The pH-optimum of activity was shifted from 5·1 to 5·3. The immobilization of the enzyme did not change its temperature optimum and the thermal stability of the enzyme was slightly increased after its immobilization. No change in the mode of action of the immobilized enzyme on a polymeric substrate or digalacturonic acid was observed. When sodium pectate was digested with the immobilized enzyme the value of K_{mapp} was substantially increased and the V_{app}-value dropped to 40% of that observed with the free enzyme.

The presence of exo-D-galacturonanase has been observed in many higher plants[1-9] even though very little is known[7,9] about the physiological function of these enzymes. One of the most probable hypotheses of the function of exo-D-galacturonanase in the process of degradation of pectic substances in higher plants postulates a concerted action of the whole complex of pectic enzymes[7]. The explanation of the function of exo-D-galacturonanases is more complicated in cases where these enzymes are present in plants alone, *i.e.* without endo-D-galacturonanase[1-4]. Enzymes in the natural milieu of living cells are often bound to insoluble biostructures; hence, enzymes coupled to insoluble supports may serve as very simplified systems for the investigation of microenvironmental effects on the mechanism of enzyme action. In one of our preceding studies[10] we stabilized purified exo-D-galacturonanase from carrot by irreversible adsorption to polyethylene terephthalate–sorsilen. This method of immobilization did not affect any substantially the properties of the enzyme and the mode of its action on a polymeric substrate. We observed, however, differences in the kinetics of the degradation. Covalent binding of enzymes is often paralleled by changes in some of the properties of the enzyme and in the mechanism of its action; we investigated therefore the effect of immobilization by covalent binding also on the properties of exo-D-galacturonanase. The binding to an appropriately chosen support permits us not only to utilize to advantage the specific catalytic action of the enzyme but also to modify the properties of the covalently immobilized catalyst; this is very important for studies on the mechanism of enzyme action[11]. Covalent attachment to polyacrylamide activated with water-soluble carbodiimide has been used successfully for the immobilization of many enzymes[12-14]. This method has been also employed by Young[15] who bound a complex of pectic enzymes present in a commercial preparation (Pectinol)

to polyacrylic acid by using water-soluble carbodiimide and obtained a preparation with all three pectolytic activities constant even after a 16-fold use.

This study has been designed to investigate the optimal conditions of covalent binding of exo-D-galacturonanase, the catalytic properties and the stability of the immobilized enzyme and to compare its characteristics with those of the free enzyme.

EXPERIMENTAL

Material and Methods

Bio-Gel, a macroporous polyacrylamide polymer (100—200 mesh) with carboxyl groups (6·0 ± ± 0·3 meq/g dry support), was a product of BIO-RAD Laboratories, Richmond, CA, U.S.A.. Water-soluble carbodiimides: 1-cyclohexyl-3[2-(4N-methylmorpholinum)ethyl] carbodiimide tosylate and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from Serva Feinbiochemica GmbH, Heidelberg, F.R.G.. Exo-D-glacturonanase was isolated from an extract (1·0 mol l^{-1} NaCl) of carrot debris (var. *Daucus carota* L.) by chromatography on DEAE-cellulose and repeated gel chromatography on Sephadex G-100. The enzyme was desalted on Sephadex G-25 medium and lyophilized[2]. The specific activity of the enzyme at pH 5·1 (0·1 mol l^{-1} acetate buffer) and 30°C was 0·0129 catal kg^{-1}. Sodium pectate (containing 82·9% of D-galacturonate, 10% of neutral saccharides, molecular weight determined viscosimetrically 27 000) was prepared by repeated alkaline deesterification from citrus pectin (Genu Pectin Københavns Pectinfabric Denmark), followed by precipitation with hydrochloric acid at pH 2·5 and neutralization with sodium hydroxide. Digalacturonic acid was prepared from an enzymic digest of D-galacturonate by separation by gel chromatography on Sephadex G-25 Fine and desalting on Sephadex G-10 (refs[16,17]).

Preparation of Immobilized Exo-D-galacturonanase

Bio gel CM-100 (100 mg) was allowed to swell in water (acidified to pH 5·0 by HCl) 30 min at room temperature. The support was activated in the same solution using a stoichiometric quantity (with respect to its number of carboxyl groups) of CMC or EDAPC. After 60-min activation the support was washed several times with acetate buffer at pH 4·8. The enzyme (16—30 mg) dissolved in 10 ml of 0·05 mol l^{-1} acetate buffer at pH 4·8 was incubated with the gel for 24 h at 4°C with two 6-h periods of shaking. The gel slurry was filtered off, washed 3 times with 100 ml of 0·1 mol . . l^{-1} acetate buffer at pH 5·1, 3 times with 100 ml of the same buffer containing 0·5 mol l^{-1} NaCl and again with 3 100-ml portions of buffer. The gel slurry with the immobilized enzyme was kept at 4°C. The quantity of the immobilized enzyme was determined indirectly from the difference between the activity of the enzyme (and the quantity of the enzyme protein) entering the reaction and the activity (and the quantity of protein) present in the supernatant after centrifugation and in the washings.

Enzyme Assays

The activity of free and immobilized exo-D-galacturonanase was determined at pH 5·1 or 5·3, resp. (0·1 mol l^{-1} acetate buffer) at 30°C by measuring the increase in reducing groups at time intervals during the degradation of sodium pectate; the spectrophotometric method of Somogyi[18] and the calibration curve prepared with D-galacturonic acid were used. The activity of the im-

mobilized enzyme was determined under constant stirring of the reaction mixture in a thermostated jacketed vessel. The activity of the immobilized enzyme was expressed in micromol of reducing groups and based on 1 mg of immobilized enzyme or on 100 mg of support and second. The relative activity of the immobilized enzyme is defined as the ratio of the activity of the immobilized enzyme to the activity of an equal quantity of the free enzyme, expressed in percent. The kinetic constants of the free and the immobilized enzyme, K_m and K_{mapp} and V and V_{app}, were determined from the initial reaction rate v, measured at six substrate concentrations c (0·1—1% sodium pectate) and were calculated by nonlinear regression analysis from $v = V.c/(c + K_m)$. The calculations were made in a Spectrum ZX minicomputer using BASIC, version Sinclair ZX. The reliability of the calculation was evaluated in terms of standard deviation of experimental rate values from regression values and was correlated with the index of regression analysis (i). The dependence of activity of the immobilized enzyme on pH was determined in 0·1 mol l^{-1} acetate buffers over the pH-range 3·6—5·6 after equilibration of the gel with the corresponding buffer. The thermal stability of the free and immobilized enzyme was examined in terms of activity determined after 2-h incubation of the enzyme at 30, 40, 50, 55, and 60°C followed by cooling the solution down to 30°C. For viscosimetric determination of the activity of immobilized exo-D--galacturonanase 5 ml of the enzyme gel slurry was incubated with 10 ml of 1% solution of sodium pectate and 5 ml of acetate buffer at pH 5·3 in a thermostated vessel at 30°C. The reaction was terminated by filtering off the gel slurry on a glass filter. The viscosity of the filtrate was determined in an Ubbelohde viscosimeter at 1-h intervals over the period of 0—24 h. The viscosity measurement was paralleled by the determination of the reducing groups liberated. The percent of viscosity decrease was correlated with the percent of glycosidic bonds cleaved. The products of enzymatic degradation of sodium pectate and digalacturonic acid were analysed by thin-layer chromatography on silica gel (Silufol sheets, a product of Kavalier, Czechoslovakia) in the system 1-butanol–-formic acid–water (2 : 3 : 1) (ref.[19]). Protein content was determined according to Bradford[20].

RESULTS AND DISCUSSION

Biogel CM 100 activated in soluble carbodiimides is a suitable support for covalent immobilization of exo-D-galacturonanase. Judging by the relative activity of the immobilized enzyme, EDAPC was more suitable for the activation of the enzyme than CMC. The reaction conditions for the preparation of the insoluble exo-D-galacturonanase derivative were chosen with respect to the required properties of the immobilized enzyme. The quantity of the enzyme attached and its relative activity were dependent on the pH of the solution used for immobilization. The latter was effected at pH 4·0, 4·4, 4·8, and 5·2. Optimal results were obtained when the enzyme was immobilized at pH 4·8 (Table I). The quantity of the enzyme attached to the support and its relative activity were found to depend also on the ionic strength of the reaction medium. As shown in Table II best results were obtained when 0·05 mol l^{-1} acetate buffer was used: 99% of enzyme protein with a relative activity of 43·2% was attached. The optimal ratio of enzyme quantity to the quantity of the support was examined in three experiments in which 16, 20, and 30 mg of enzyme were used per 100 g of dry support (Table III). The highest relative activity, 44·2, showed a preparation obtained by coupling 16·0 mg of enzyme to 100 mg of the support. The application of larger enzyme concentrations resulted in a drop of relative activity, obviously as a result

of steric effects connected with the high enzyme density in the gel matrix and of the interaction of the enzyme with the polymeric substrate. The bond between the enzyme and the support was very stable. Trace amounts only of the free enzyme were found in the first washings, the activity of the preparation did not change during subsequent washing and remained constant 6 months when the preparation was stored at 4°C and 3 weeks when it was stored at room temperature and used 20 times. The activity of exo-D-galacturonanase dropped during covalent immobilization in all cases yet this decrease of activity was compensated by the high stability of the covalently immobilized enzyme compared to the free enzyme. Covalent immobilization obviously

TABLE I

Effect of pH on covalent binding and activity of exo-D-galacturonanase

pH	% of enzyme protein bound[a]	Relative activity of immobilized enzyme in %[b]
4·0	78·2	26·8
4·4	80·2	31·5
4·8	99·0	43·2
5·2	80·6	38·3

[a] Determined from the quantity of protein used for the reaction (8 mg per 50 mg of support) and its quantity found in the supernatant after centrifugation of the gel slurry; [b] relative activity expressed with respect to the activity of the soluble enzyme at pH 5·1 and 30°C (0·0129 mol s^{-1} . . mg^{-1}) and calculated for the quantity of immobilized enzyme protein.

TABLE II

Effect of ionic strength on covalent binding and activity of exo-D-galacturonanase

Ionic strength mol l^{-1}	% of enzyme protein bound[a]	Relative activity of immobilized enzyme in %[b]
0·05	99·0	43·2
0·10	80·9	38·6
0·15	76·4	27·6

[a] Determined from the difference in the quantity of protein used for the reaction (8 mg of enzyme per 50 mg of gel) and its quantity found in the supernatant after centrifugation of the gel slurry; [b] relative activity expressed with respect to the activity of the soluble enzyme in 0·1 mol l^{-1} acetate buffer at pH 5·1 and 30°C, calculated for the quantity of immobilized enzyme protein.

stabilized the enzyme by the attachment of many sites of its molecule to the support, as observed with numerous other covalently immobilized enzymes[21-23].

The effect of immobilization on the properties of the enzyme was examined with the preparation showing the highest relative activity. The pH-optimum of catalytic activity, which is 5·1 for the free enzyme, was shifted to pH 5·3 because of the anionic medium surrounding the enzyme (Fig. 1). The temperature optimum (50°C) was practically the same as for the free enzyme (Fig. 2). The thermal stability of the enzyme was slightly increased after its immobilization (Fig. 3). The mode of action of the immobilized enzyme was investigated by assaying the products of sodium pectate and digalacturonic acid degradation and also by measuring the viscosity decrease in correlation to the increase of reducing groups liberated (Fig. 4). Immobilized exo-D-galacturonanase degrades substrates *via* a terminal mechanism. This is evidenced by the following facts: the single product of 24-h action of the enzyme on the polymer is D-galacturonic acid, a 54% drop of viscosity of the sodium pectate solution is paralleled by cleavage of 40% of glycosidic bonds and digalacturonic acid is degraded by the immobilized enzyme. The immobilization of exo-D-galacturonic acid by covalent coupling to polyacrylamide did not change its mode of action on the polymeric substrate as follows from the above results.

In our preceding study[10] purified exo-D-galacturonanase from carrot was immobilized by irreversible adsorption to polyethylene terephthalate–sorsilen. We observed differences in the properties of exo-D-galacturonanase immobilized by adsorption and by covalent bonding. The latter resulted in a shift of the pH-optimum of catalytic activity, in an increase of the thermal stability of the enzyme and affected the kinetics of degradation of the polymeric substrate more than the immobilization by adsorp-

TABLE III

Effect of enzyme concentration on the process of covalent attachment of exo-D-galacturonanase

mg of enzyme applied to 100 mg of support	Quantity of protein bound[a]	mg of enzyme bound to 100 mg of support[b]	Activity μmol s^{-1}		Relative activity %
			per mg of bound enzyme	per g of support	
16·0	99·0	15·85	0·0054	0·856	44·2
20·0	85·6	16·15	0·0052	0·826	40·3
30·0	78·0	21·15	0·0039	0·825	30·3

[a] Determined indirectly from the difference in the quantity of enzyme used for the reaction and its quantity in the supernatant after centrifugation of the gel slurry; [b] determined from the difference in the activity of the enzyme applied to the support and its activity found in the supernatant and washings.

tion. The mode of action of the enzyme on the polymeric substrate was not changed any substantially either after immobilization by adsorption or by covalent bonding.

The most active preparation was also used for the measurement of the kinetics of degradation of the polymeric substrate. The degradation of sodium pectate follows

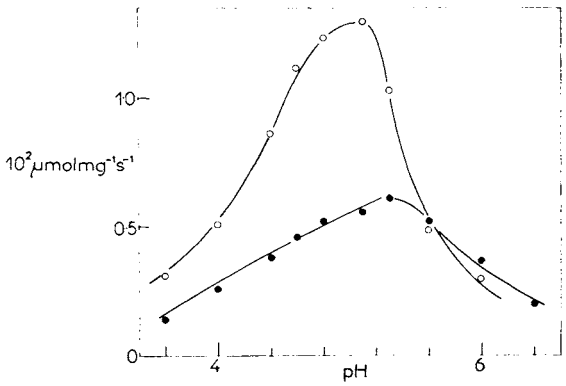

FIG. 1

pH-profile of activity of soluble and immobilized exo-D-galacturonanase; ○ free enzyme, ● immobilized enzyme

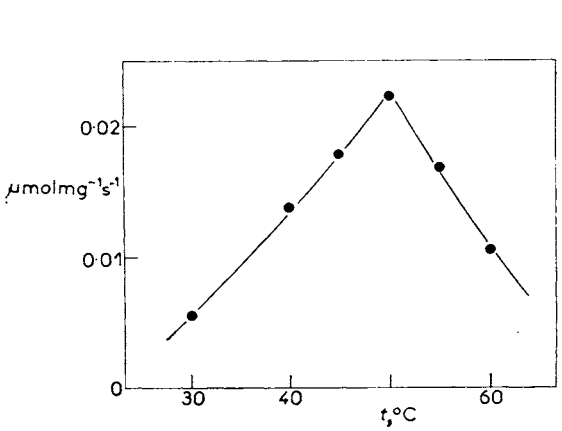

FIG. 2

Temperature profile of activity of immobilized exo-D-galacturonanase

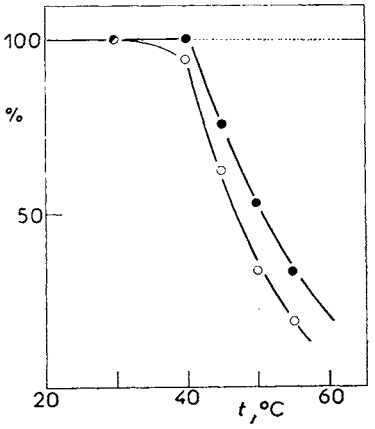

FIG. 3

Thermal stability of exo-D-galacturonanase free and immobilized on polyacrylamide. Activity of free enzyme at 30°C 0·0129 μmol . . s^{-1} mg^{-1}, activity of immobilized enzyme under identical conditions 0·0059 μmol s^{-1} . . mg^{-1} ○ free enzyme, ● immobilized enzyme; % relative enzyme activity

the Michaelis–Menten kinetics. The immobilization resulted in a drop of V_{app} to $1{\cdot}057 \cdot 10^{-2}$ μmol s^{-1} mg^{-1} from a value of $V = 1{\cdot}596 \cdot 10^{-2}$ μmol s^{-1} mg^{-1}. The value of K_{mapp} markedly increased from the value of $1{\cdot}1550 \cdot 10^{-5}$ mol l^{-1}, observed with the free enzyme, to the value of $8{\cdot}7427 \cdot 10^{-5}$ mol l^{-1} (dispersion of $V = 0{\cdot}462 \cdot 10^{-4}$, correlation index $i = 0{\cdot}9868$; dispersion of $V_{app} = 0{\cdot}352 \cdot 10^{-4}$, correlation index $i = 0{\cdot}9868$). An almost eight-fold increase of the value of the Michaelis constant of covalently immobilized exo-D-galacturonanase is the most marked difference between the enzyme immobilized covalently and by adsorption. The process of immobilization of exo-D-galacturonanase by irreversible adsorption[10] has a much smaller effect on the change in the Michaelis constant. The increase of the value of K_{mapp} to $2{\cdot}139 \cdot 10^{-5}$ mol l^{-1} from $K_m = 1{\cdot}233 \cdot 10^{-5}$ mol^{-1} l^{-1} observed with the free enzyme, is a result of the hydrophobic character of the support which contributes to the decreased ability of the hydrophilic substrate to approach the enzyme and of the steric hindrance which the support represents for the polymeric substrate. A substantial increase of the K_{mapp} value observed with covalently immobilized exo-D-galacturonanase can be a result of decreased attractiveness between the polyanionic matrix of the support and the substrate of acidic character. Even though this assumption is probable and is also in agreement with the concept of Goldstein and coworkers[24] and of Kovacs and coworkers[14] our opinion is that it represents merely one of the possible explanations of the problem. It is possible that the marked increase in the Michaelis constant also results from the fact that the enzyme is bound to the support during covalent immobilization *via* certain amino acids which are localized in the neighborhood of the active site; this contributes to an increased steric effect. Another explanation could postulate a change in the mechanism of action of the covalently immobilized enzyme, a change which has been observed,

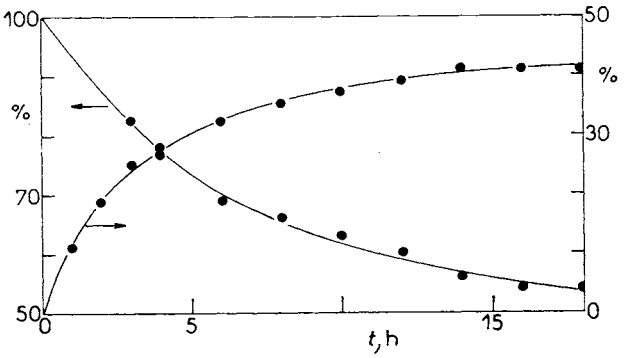

FIG. 4

Effect of immobilized exo-D-galacturonanase on viscosity decrease in correlation with percent of glycosidic bonds cleaved. Decrease of viscosity in per cent (left); increase of reducing groups expressed in per cent of glycosidic bonds cleaved (right)

in connection with substantial changes in the K_{mapp}-value by several authors[11,24,25]. Ledingham and Hornby[25] found that the multiplicity of cleavage of the same molecule of substrate was considerably increased by α-amylase immobilized on polystyrene than by the free enzyme. Similarly, β-amylase coupled to polyaminostyrene also shows an increased multiplicity[26]. A change in the mechanism of action of β-amylase caused by covalent immobilization of the enzyme to the support which affected the distribution of net charge in the neighborhood of the enzyme has been also observed by Hornby and Morris[27]. Free exo-D-galacturonanase from carrot (under optimal conditions of its action) catalyzes the degradation of polymeric and oligomeric substrates predominantly *via* a multi-chain mechanism with prevailing single collision[28] It remains to be shown in detailed studies on the mechanism of catalytic action of covalently immobilized exo-D-galacturonanase on defined oligomeric substrates whether or not the substantially increased K_{mapp}-value observed with the covalently immobilized enzyme reflects a change in the mechanism of its action.

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