POLY(ETHYLENE TEREPHTHALATE) IMMOBILIZED EXO-D-GALACTURONANASE

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Exo-D-galacturonanase (E.C. 3.2.1.67) isolated from carrot was irreversibly adsorbed on poly(ethylene terephthalate) in a 0·1 mol l^{-1} acetate buffer solution of pH 5.1. Activity of the immobilized enzyme depended on the amount of the enzyme bound to the support, on the ionic strength, and, to a very little extent, also on the pH of the reaction medium during immobilization. Activity of the immoblized enzyme dropped; the greatest relative activity (51·8%) had the preparation composed of 9·12 mg of the enzyme per 1 g of the support. The pH optimum for catalytical activity of the immobilized enzyme was identical with that of the dissolved enzyme (5·1). Immobilization of the enzyme did not change its thermal optimum, and its thermal stability did not improve, either. The substrate specificity did not alter by immobilization, no differences were found in the mode of action on the polymeric substrate; digalacturonic acid was degraded by the immobilized enzyme like by the dissolved one. Differences in the kinetics of polymeric substrate degradation by the bonded exo-D-galacturonanase were manifested by a lowered V' app value and by an increased K'_m value. Exo-D-galacturonanase preparation obtained by adsorption on poly(ethylene terephthalate) was extraordinarily stable during storage at 4°C, and towards action of salts and pH changes; its operation stability was high.

Papers dealing with immobilized pectolytic enzymes published so far could be classified into two groups: the first one comprises those concerning immobilization of the whole complex of pectolytic enzymes aiming to apply it in the can industry[1-4], the second one concerns the immobilization of separate purified pectolytic enzymes, which were further examined for the effect of mode of immobilization on changes of their properties and mode of degradation of polymeric substrates. The most frequented immobilized enzyme was endo-D-galacturonanase[5-10], less attention has been paid to pectinlyase[11,12] and pectinesterase[13]. Commercial preparations of pectolytic enzymes and many plants contain another pectolytic enzyme – exo-D-galacturonanase, which has hitherto not been immobilized. The most frequent method for immobilization of pectolytic enzymes is a covalent binding to insoluble supports[2,4,5,8,10,13], and the adsorbtion on a solid support[1,3,9]. The adsorption method was used for the immobilization of exo-D-galacturonanase because this is the simpliest procedure for binding enzymes. The selection of a support was determined by its accessibility and properties. The employed poly(ethylene terephthalate)–sorsilen

is porous, hydrophobic adsorbent having high light, thermal and chemical stabilities, it is resistant against action of microorganisms and not toxic. This support proved quite suitable to immobilize endo-D-galacturonanase of *Aspergillus* sp.[9]. This paper describes the preparation of immobilized exo-D-galacturonanase and some of its properties.

EXPERIMENTAL

Material and Methods

Poly(ethylene terephthalate) forms spheric particles of specific surface 80 – 100 m^2/g and pore size 2 – 3 cm^3/g. This support, prepared in the Department of Polymers, Prague Institute of Chemical Technology, was washed with water to eliminate the non-setting particles. Exo-D-galacturonanase was isolated from an extract (1·0 mol l^{-1} NaCl, pH 5·0) obtained from the debris of carrot (*Daucus carota* L.). The isolation involved the salting-out of proteins with ammonium sulfate up to a 80% saturation, separation of the mixture of proteins by chromatography on DEAE cellulose and rechromatography on a Sephadex G-100 gel, desalting of the enzyme on Sephadex G-25 Medium and freeze-drying[14]. Activity of the enzyme at pH 5·1 (0·1 mol l^{-1} acetate buffer solution) and 30°C was 0·013 katals. Sodium pectate (82·9% D-galacturonan content, 10% neutral saccharides content, molecular mass 27 000 determined viscometrically) was prepared by a repeated alkaline deesterification of 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 obtained from the enzyme hydrolyzate of D-galacturonanan by separation by means of gel chromatography on Sephadex G-25 Fine and desalting on Sephadex G-10 (refs[15,16]).

Immobilization of Exo-D-galacturonanase

Poly(ethylene terephthalate) (1 g) equilibrated with 0·1 mol l^{-1} acetate buffer of pH 5·1 (30 ml) was incubated with the enzyme at 4°C and shaken for 8 h. The sample was centrigufed, washed with the buffer solution, 0·5 mol l^{-1} NaCl and repeatedly with the buffer solution. Amount of the immobilized enzyme was indirectly estimated from the difference between activity of the enzyme entering the reaction and that present in the supernatant after centrifugation and washing solutions. The immobilized enzyme was stored as a suspension in acetate buffer solution at 4°C.

The Enzyme Assay

Activity of the free and immobilized exo-D-galacturonanases was estimated at pH 5·1 (0·1 mol l^{-1} acetate buffer solution) at 30°C by measuring the increment of reducing groups at preset time intervals during degradation of sodium pectate employing the Somogyi spectrophotometric method[17] and calibration curve for D-galacturonic acid. Activity of the immobilized enzyme was determined by incubation of the reaction mixture (5 ml of the substrate – 0·5% sodium pectate and 5 ml of the enzyme suspension) while stirring in a constant temperature double-jacket vessel. Activity of the immobilized enzyme expressed in micromoles of reducing groups referred to 1 mg of the immobilized enzyme or 1 g of the support and one second. The relative activity of the immobilized enzyme defined as the ratio of bonded enzyme activity to that of the same amount of free enzyme is given in per cent. Kinetic constants of the free and immobilized enzymes K_m and K_m' app and V and V' app, respectively, were determined from the initial reaction velocity measured at six concentrations of the substrate (concentration of sodium pectate 0·1 – 1%)

and calculated by the linear regression. Dependence of the activity of the immobilized enzyme upon pH was determined in a 0·1 mol l^{-1} acetate buffer solutions in the interval 3·6–5·6 after washing the enzyme suspension with the corresponding buffer solution. Thermal stability of the free and immobilized enzymes was estimated on the basis of activity determined after a 2-h incubation of the enzyme at 30, 40, 50, 55, and 60°C followed by cooling the enzyme to 30°C. The viscometric activity determination of the immobilized exo-D-galacturonanase was performed by incubation of the enzyme gel suspension (3 ml) with 1%-sodium pectate (10 ml) and acetate pH 5·1 buffer (7 ml) in a constant-temperature double-jacket vessel at 30°C. The reaction was stopped by recovering the gel by filtration through a sintered glass filter. The viscosity of the filtrate was determined with the Ubbelohde viscometer in one-hour intervals from 0–12 h. Concurrently with viscosity measurements also freed reducing groups were determined in the same time intervals. The viscosity decrease in per cent was correlated with the per cent of cleaved glycosidic bonds. The degradation products of sodium pectate and digalacturonic acid after exposure to the immobilized enzyme were estimated by thin-layer chromatography (Silufol sheets, Kavalier, Czechoslovakia) in the solvent system 1-butanol–formic acid–water 2 : 3 : 1, (ref.[18]), D-galactopyranuronic acid being the reference. The stability of the enzyme binding to support was examined by washing the immobilized enzyme with sodium acetate solution at pH 3·8–6·0, 0·1–2·0 mol l^{-1} sodium chloride solution, 0·5% sodium pectate solution, and 0·08% digalacturonic acid solution in a 50-fold volume per that of the gel suspension. The operational stability of the enzyme was investigated by measuring the activity during a 3-month storage at 4°C with a 20-fold application of the preparation.

RESULTS

Immobilization: The optimal ratio of the enzyme amount to that of the support was examined in a series of experiments by application 3·5–15·0 mg of the enzyme on 1 g of the support. Results listed in Table I show that activity of the enzyme bound to poly(ethylene terephthalate) rised with the increasing concentration of the

TABLE I

Amount and activity (μmol s^{-1} mg^{-1}) of exo-D-galacturonanase bound by adsorption to poly(ethylene terephthalate) (conditions: 0·1 mol l^{-1} acetate buffer, pH = 5·1 (30 ml), 4°C, 8 h)

mg of enzyme applied on g of support	mg of enzyme bound by 1 g of support	Activity		
		per 1 mg of immob. enzyme	per 1 g of support	relative, %
3·52	3·52	0·00198	0·00697	17·0
6·08	6·08	0·00340	0·02067	29·4
9·12	9·12	0·00602	0·05490	51·8
10·56	10·56	0·00499	0·05269	43·0
11·85	11·85	0·00300	0·03615	25·8
15·00	14·1	0·00131	0·01847	11·2

enzyme up to certain limit. The highest relative activity revealed preparation having 9·12 mg of the enzyme bonded per 1 g of the support. Application of higher concentrations of the enzyme was associated with a decrease of relative activity. The rate and amount of the enzyme adsorbed onto the support depended on the ionic strength of the reaction medium. Table II presents results disclosing that the slowest adsorption occured in an aqueous medium and the preparation obtained had the lowest relative activity and stability. Best results were obtained by immobilizing the enzyme in a 0·1 mol l^{-1} acetate buffer solution. The pH of the medium in 4·0–6·0 range did not influence the immobilization rate, nor the activity of the enzyme after immobilization. In a strongly acidic medium of pH 3·0–3·8 the preparations after immobilization of the enzyme had lower relative activity (8–15%).

Stability of the preparation: The enzyme to poly(ethylene terephthalate)binding was found very stable. Exo-D-galacturonanase immobilized in a 0·1 mol l^{-1} acetate buffer solution was not freed from the support by acetate pH 4·0–6·0 buffer solution, 0·1–2·0 mol l^{-1} NaCl, 0·5%-sodium pectate or digalacturonic acid. Activity of the immobilized enzyme did not change by its 20-fold application, nor by its 3-month storage at 4°C. A decrease of the preparation activity (approximately 12%) was observed in the first two days of its storage, and therefore, the enzyme was used for experiments 48 h after its immobilization.

Dependence of the catalytical activity on pH and temperature: Immobilization of exo-D-galacturonanase did not change the relationship between its activity and pH; both the free and bound enzymes showed pH optimum at pH 5·1 (Fig. 1). The

TABLE II

Dependence of ionic strength (pH 5·1 acetate buffer solution) on immobilization of exo-D-galacturonanase to poly(ethylene terephthalate) in relation to incubation time at 4°C

Buffer concentration, mol l^{-1}	Amount of enzyme immobilized, %[a]			Rel. activity, %[b] after 8 h
	2 h	4 h	8 h	
0·0	58·8	65·7	66·2	21·9
0·025	77·0	80·1	82·0	36·9
0·050	90·4	92·6	94·0	46·0
0·100	96·0	100	100	51·8
0·150	89·2	91·8	93·7	–

[a] Determined indirectly from the difference between the amount of enzyme employed in reaction (9·12 mg per 1 g of the support) and that in the supernatant after immobilization; [b] calculated per the amount of enzyme immobilized.

temperature optimum of both the freed and immobilized enzymes was found to be at 50°C (Fig. 2). Thermal stability of the enzyme was not considerably influenced by immobilization. The positive deviation from the thermal stability curve was not considerably affected by immobilization. A positive deviation from the thermal stability curve of the free enzyme was observed within 45–60°C range (Fig. 3).

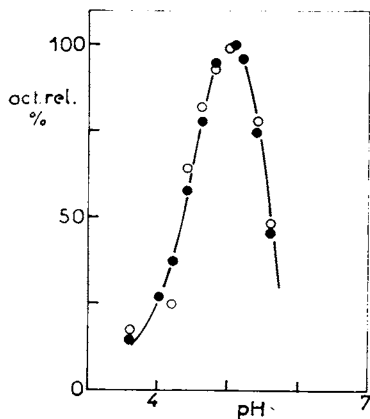

FIG. 1

Activity dependence of the soluble and immobilized exo-D-galacturonanases on pH; the 100% activity of soluble and immobilized enzymes at 30°C; ○ free enzyme, ● immobilized enzyme

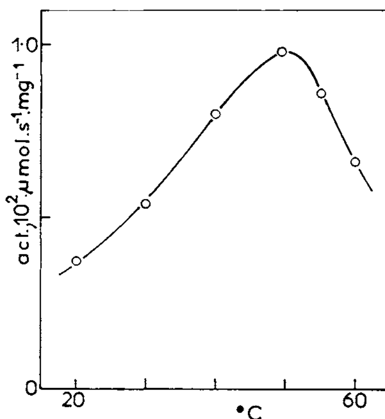

FIG. 2

Temperature dependence of the immobilized exo-D-galacturonanase

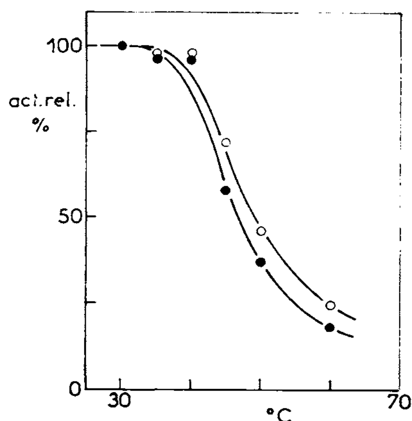

FIG. 3

Thermal stability of free and poly(ethylene terephthalate) immobilized exo-D-galacturonanases. Activities at 30°C; ○ free enzyme 0·0129 μmol s^{-1} mg^{-1}; ● immobilized enzyme 0·0054 μmol s^{-1} mg^{-1}

Kinetic constants and the mode of action: Preparation with the highest relative activity was employed for measurement of kinetics when degrading sodium pectate. The enzyme bound to poly(ethylene terephthalate) fits requirement for the Menten and Michaelis relationship for kinetics in the given concentration range. The $K_{m'}$ app value amounting $2{\cdot}159 \,.\, 10^{-5}$ mol l^{-1} was slightly higher when compared with that of the free enzyme ($K_m = 1{\cdot}233 \,.\, 10^{-5}$ mol l^{-1}). The V' app value decreased to $5{\cdot}49 \,.\, 10^{-3}$ μmol s^{-1} mg^{-1} in contrast to $V = 1{\cdot}29 \,.\, 10^{-2}$ μmol s^{-1} mg^{-1}. The mode of action of the immobilized enzyme was examined by analysis of the degradation products of sodium pectate and digalacturonic acid and by measuring the viscosity decrease in correlation with the increase of freed reducing groups. We found that the only degradation product of the polymer was D-galactopyranuronic acid, and that the immobilized enzyme degraded digalacturonic acid similarly as did the soluble one. Cleavage of 19% of the glycosidic bonds by immobilized enzyme was manifested by a 20% viscosity decrease.

DISCUSSION

The purified exo-D-galacturonanase from carrot was immobilized by adsorption on poly(ethylene terephthalate) for simplicity of the immobilization process[9] and for good properties of the sorbent selected[19,20]. This support has already been successfuly used *inter alia* for immobilization of endo-D-galacturonanases isolated from the commercial preparation Rohament[9] and of a complex of pectolytic enzymes present in this preparation[21]. Since the nature of the support, the mode of binding of the enzyme, its stability and some properties frequently depended after immobilization also on reaction conditions, we investigated the effect of concentration of the compound adsorbed, ionic strength and pH.

The amount of enzyme bound to the support and the relative activity of the enzyme depended on the enzyme concentration during immobilization. Best results, a 100% binding of the enzyme and 51·8% maximum relative activity, were attained with preparation having 9·12 mg of the enzyme immobilized on 1 g of the support. Application of higher concentrations of the enzyme was associated with a decrease of relative activity even at binding of the whole amount of the enzyme, probably due to steric effects arising from a high density of the enzyme at the gel surface. Adsorption of the preparation containing 15 mg of the enzyme on 1 g of the support was only 94% and its relative activity was 11·2%. The rate and intensity of adsorption and also the activity of the enzyme after immobilization depended on the ionic strength of the medium and did not depend on pH in the 4·0 – 6·0 range. Immobilization in a strongly acidic medium (pH 3·0 – 3·8) become evident through lowering the relative enzyme activity; this might be associated with lowered stability of the exo-D-galacturonanase from carrot in strongly acidic medium[14]. Bearing the above-mentioned findings in mind, following conditions were chosen for immobilization

of exo-D-galacturonanase from carrot: enzyme (9·12 mg) was bound to the support (1 g) suspended in 0·1 mol l^{-1} acetate buffer solution (30 ml) at pH 5·1 (pH-optimum) and 4°C for 8 h under continuous shaking. This preparation was examined for the binding strength and stability of the enzyme during its storage in acetate buffer both at an ambient temperature and at 4°C. Binding of exo-D-galacturonanase on poly(ethylene terephthalate) was very stable like that of endo-D-galacturonanase[9]. No freed enzyme was found in supernatants during a three-month storage of the enzyme at 4°C. Activity of the preparation stored at room temperature did not change for 14 days, and during storage at 4°C the activity did not drop within three months and a twentyfold reuse of the preparation. Exo-D-galacturonanase immobilized on poly(ethylene terephthalate) was not freed from the support by acetate buffer solutions of pH 4·0–6·0, 0·1–2·0 mol l^{-1} NaCl solutions, 0·5% sodium pectate and 0·08% digalacturonic acid. This high bond stability at pH changes and high concentration of salts favourize the hydrophobic interaction of the enzyme with the support as assumed for adsorption on poly(ethylene terephthalate)[9,20]. Immobilization of exo-D-galacturonanase did not change the dependence of its catalytical activity upon pH and temperature; thermal stability raised by immobilization to 45–60°C.

The immobilizaton process influenced the kinetic constants of exo-D-galacturonanase. Immobilization resulted in a slight increase in $K_{m'}$ app (2·139 . 10^{-5} mol l^{-1}) compared with the K_m for the free enzyme (1·233 . 10^{-5} mol l^{-1}) possibly explained by the hydrophobic character of the support. The nature of the support contributed towards the lower activity of the hydrophobic substrate to approach the enzyme together with the steric hindrance due to the size of the substrate polymer[22]. The immobilized exo-D-galacturonanase showed a lowered maximal degradation rate of sodium pectate by 59% when compared with that of soluble enzyme. Considering that kinetic constants were determined for preparation with the optimum enzyme to support ratio (*i.e.* with a low steric effect following from the enzyme density at the support surface), one can assume that the considerable activity decrease might be associated with a change of a part of enzyme molecules during the immobilization process. The alternative possibility that the lowered affinity of the enzyme towards substrate polymer, regarding the hydrophobicity of the support, can lower diffusion of the reaction products from the proximity of the active center of the enzyme and therefore, the substrate is more likely to undergo a smaller number of catalytic events (less catalyses)[23].

The mode of action of the immobilized enzyme was monitored both by analysis of degradation products of sodium pectate and digalacturonic acid, and by measuring the viscosity decrease in correlation with the increase of reducing groups freed.The immobilized exo-D-galacturonanase degrades substrates *via* a terminal mechanism. This is evidenced by the findings that the only product of a 24-h treatment of the enzyme on the polymer is D-galactopyranuronic acid, that the 20% viscosity decrease

of sodium pectate solution is accompanied with a 19% of cleaved glycosidic bonds, and that the immobilized enzyme degraded digalacturonic acid. It has been found that the mode of action on polymeric substrate was not considerably altered by immobilization of exo-D-galacturonanase. This study was not directed towards the effect of immobilization on the mode and mechanism of action of the enzyme during degradation of oligomeric substrates. As already known[23], even the change of $K_{m, app}$ value on the polymeric substrate can indicate changes in the mechanism of enzyme action.

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