ENDO-D-GALACTURONANASE IMMOBILIZED BY ADSORPTION ON POROUS POLYETHYLENETEREPHTHALATE

Ľubomíra Rexová-Benková[a], Jiřina Omelková[a] and Vladimír Kubánek[b]

[a] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava and*
[b] *Department of Polymers, Institute of Chemical Technology, 160 00 Prague*

Received November 3rd, 1981

Endo-D-galacturonanase of *Aspergillus* sp. was irreversibly adsorbed on polyethyleneterephthalate in an acetate 0·1 mol l^{-1} buffer solution of pH 4·2. Immobilization of the enzyme resulted in lowering of its activity, the measure of which depended on the amount of the enzyme fixed on the carrier. The highest relative activity (42·4%) had the preparation containing 5·25 mg of the enzyme per 1 g of the carrier. The velocity and intensity of the sorption of the enzyme depended on the ionic strength of the medium, whilst pH, on the other hand, was of no influence. Endo-D-galacturonanase immobilized in a 0·1 mol l^{-1} buffer was characteristic *a*) of its fixation strength in salt solutions of various ionic strength and pH, in a 3 mol l^{-1} guanidine solution, and also in sodium pectate and pectin solutions, *b*) of its high stability during a long-lasting storage at 4°C, *c*) of its operational stability. The immobilization led to a partial change of the action pattern onto the high-molecular substrate, manifested in lowering the decrease of viscosity to degradation degree ratio.

Endo-D-galacturonanase[1-4], pectinlyase[5,6], pectinesterase[7], as well as mixture enzyme preparations utilized in can industry[8-11] are those pectolytic enzymes so far immobilized. The covalent binding to insoluble carriers[1-4,7,9,11] was the most frequently used method of immobilization. Immobilization of endo-D-galacturonanase by covalent binding through amino groups led to a change in action pattern on high-molecular substrates. This change, the measure of which depends on the distance of the enzyme from the carrier surface (length of the spacer) involves the lowering of the degree of randomness of glycosidic bond cleavage and restriction of the action of the enzyme on the peripheral area of the macromolecule[2-4]. The simplest method of immobilization – adsorption – was employed for immobilization of pectolytic enzymes only rarely[8,9]. The disadvantage of this mode of immobilization are the weak binding forces between the carrier and the immobilized substance[12]. Here, a liberation of the enzyme from the carrier frequently takes place in relation with the binding forces at increased salt concentrations, change in pH, or in the presence of a substrate, particularly if it is represented by a charged macromolecule[12]. More stable immobilized enzymes, not undergoing those effects

have been obtained by adsorption on hydrophobic carriers[13,14], or on materials containing hydrophobic substituents[15,16].

These properties are encountered with porous polyethyleneterephthalate, having high light, temperature and chemical stabilities and resistance towards the action of microorganisms. It is, at the same time, not toxic and meets requirements needed for materials used in food industry. Therefore, we examined the possibility to utilize it for immobilization of endo-D-galacturonanase. This paper deals with the preparation of the immobilized enzyme and describes some of its properties.

EXPERIMENTAL

Material and Methods

Polyethyleneterephthalate[17,18] is formed by spherical particles of a pore size 2—3 $cm^3\ g^{-1}$ and specific surface 80—100 $m^2\ g^{-1}$. The fine not-sedimenting particles were removed prior to the usage by a repeated floating with water. Endo-D-galacturonanase was purified from the commercial preparation of Pectinase Rohament P (Rohm and Haas, Darmstadt, FRG) by affinity chromatography on a crosslinked pectic acid[19]. The activity of the preparation at pH 4·2 (0·1 mol l^{-1} acetate buffer solutions) and 30°C was 1·28 kat. Sodium pectate (the D-galacturonan content 89·9%, $\overline{M}_r$ 27 000 determined viscometrically) was prepared by a repeated alkaline de-esterification of the citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) followed by precipitation with hydrochloric acid at pH 2·5 and neutralization with sodium hydroxide.

Preparation of Immobilized Endo-D-galacturonanase

Polyethyleneterephthalate (1 g) suspended in a 0·1 mol l^{-1} acetate buffer of pH 4·2 (20 ml), (ref.[20]) was incubated with the enzyme at room temperature under stirring for 4 h. The adsorbate was centrifugated and repeatidly washed with the buffer. The presence of the non-adsorbed enzyme was checked in the supernatant and in washings by its activity determination. The amount of the immobilized enzyme was estimated from the difference in activity employed for immobilization and that determined in supernatants. The immobilized enzyme was stored in an acetate pH 4·2 buffer in form of a suspension.

Enzyme Assay

Activity of the free and immobilized endo-D-galacturonanase was determined at pH 4·2 (0·1 mol . l^{-1} acetate buffer) and 30°C by measuring the increase of reducing groups during degradation of sodium pectate by a spectrophotometric method[21] using the calibration graph for D-galactopyranuronic acid. The activity of the immobilized enzyme was determined *via* incubation of the reaction mixture under constant stirring in a constant-temperature double-jacketed vessel. Activity of the enzyme is expressed in micromols of reducing groups liberated by 1 mg of the enzyme (free or immobilized), or by 1 g of the adsorbate within 1 s. The relative activity is the ratio of the activity of the immobilized enzyme and that of the same amount of free enzyme expressed in per cent. Kinetic constants of the free and immobilized enzymes K_m, $K_{m'app}$ and V, V_{app} were computed by the least squares[22] method from the initial velocities of the reaction determined at five concentrations of sodium pectate (0·1—1%) and computed by means of the polynomial

programme[23]. The dependence of the endo-D-galacturonanase activity on pH was investigated in the 3·8—5·2 pH range (0·1 mol l^{-1} acetate buffer solutions). The activity of the immobilized enzyme was determined after washing the gel with the respective buffer solution. The heat stability of the free and immobilized enzyme was characterized on the basis of activities determined after a 2 h-incubation of the enzyme at the given temperature and a following cooling to 30°C. The operational stability of the immobilized enzyme was examined by a continuous elution of the column of enzyme gel (1·5 × 1·7 cm) by a sodium pectate solution (0·5%, pH 4·2) at a 1·57 ml min^{-1} flow rate and room temperature. Reducing groups in the eluate were periodically tested. Stability of immobilization of the enzyme on the carrier at various pH, ionic strength, in NaCl, or guanidine of various concentrations was monitored by elution of the column of immobilized enzyme with the respective solution of a 50—100-fold bed volume and determination of the enzyme activity after the gel was washed with a 0·1 mol l^{-1} acetate buffer.

Analysis of Reaction Products

Products of the enzyme degradation of sodium pectate were analyzed by thin-layer chromatography on silica gel (Silufol sheets, Kavalier, Czechoslovakia) using n-butanol–formic acid–water (2 : 3 : 1, ref.[24]) as an eluent. The polymerization degree of products was estimated according to its relationship to log $R_F/(1 - R_F)$, employing D-galactopyranuronic acid as a reference.

RESULTS AND DISCUSSION

Polyethyleneterephthalate was shown to be an advantageous sorbent[25] of various organic substances. The average molecular weight of this polymer of molecular formula HO—CH_2—CH_2—(OCO—C_6H_4—COO—CH_2—CH_2)$_n$—OCO—C_6H_4——COOH is approximately 20 000, so that the concentration of terminal carboxyl groups capable of ionic interaction with immobilized enzymes is very low and it is, therefore, easy to assume that the high sorption capacity is prevalently given by its hydrophobic character. This assumption was considered when searching conditions for immobilization of endo-D-galacturonanase.

Immobilized was endo-D-galacturonanase produced by *Aspergillus* sp. purified from preparation containing two forms of endo-D-galacturonanase[26] differing in their pH optima. Endo-D-galacturonanase was immobilized at the stability optimum routinely used for *Aspergillus*[27] sp. in order to exclude the possible inactivation due to the medium. The optimum ratio of the enzyme amount to the carrier was estimated in a series of five experiments in which 2·63—7·84 mg of the enzyme and 1 g of the dry carrier were employed; with 5·26 mg per 1 g of the carrier 58 and 95% of activity was retained after 5 and 30 min, respectively (Fig. 1). No residual activity of the non-adsorbed enzyme was found in any of the solution after a 4 h-incubation. The activity of all preparations was considerably lowered when compared with that of the soluble enzyme; it was, however, dependent on the amount of the enzyme adsorbed on the carrier. The activity of preparations containing various amounts of adsorbed enzyme (Table I) increased at the beginning with the increasing amount, and, at the ratio 5·25 mg per 1 g of the carrier the maximum with 42·4% relative

activity was reached. With a further increase of the enzyme concentration in the adsorbate a considerable drop in activity took place; this was probably caused by steric effects due to a too high density of the enzyme at the carrier surface, and, consequently, the lowered accessibility for the high-molecular substrate.

TABLE I

Activity of preparations containing various amounts of the immobilized enzyme

Immobilized enzyme, mg/g of the carrier	Activity, $\mu mol\ s^{-1}$		Relative activity %
	per mg of the bound enzyme	per mg of the carrier	
2·63	0·238	0·626	18·6
4·21	0·312	1·314	24·4
5·26	0·542	2·851	42·4
6·32	0·218	1·378	14·0
7·89	0·144	1·136	11·25

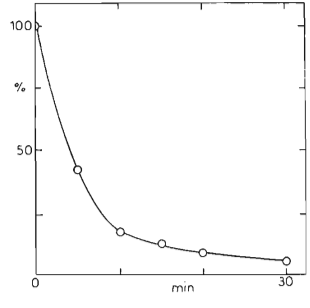

FIG. 1

Adsorption of endo-D-galacturonanase to polyethyleneterephthalate. The amount of the immobilized enzyme 5·26 mg/g of the carrier

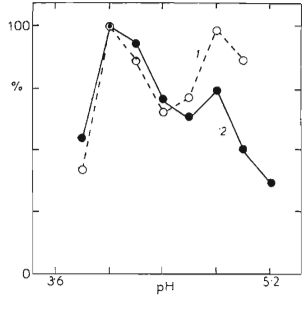

FIG. 2

Relation of the activity of the soluble and immobilized endo-D-galacturonanases on pH. 1 Soluble enzyme; 2 immobilized enzyme. 100% = activity of the free and immobilized enzymes at pH 4·0

Sorption of the enzyme was not pH dependent and in the pH 4·2–7·2 range of the medium the immobilization of the enzyme proceeded at the same velocity, and, at the given conditions the enzyme was irreversibly immobilized after a 4 h-incubation. The velocity and intensity of sorption of the enzyme on the carrier depended on the ionic strength. As follows from data presented in Table II, the immobilization rate increased at pH 4·2 with the increasing ionic strength of the solution. In an aqueous medium, where sorption of the enzyme proceeded at a lowest rate, no irreversible immobilization occurred; elution of the sorbent with water was associated with a gradual liberation of the enzyme. The optimum medium also from the standpoint of immobilization course was the acetate 0·1 mol l^{-1} buffer of pH 4·2. Under these conditions the activity of preparations thus obtained and stored at 4°C decreased during the first 1–2 days by 12–15%, but, nonetheless, it was constant during further storage for several months. The initial decrease in activity of the adadsorbate was not associated with the liberation of the enzyme from the carrier.

Binding of the enzyme to polyethyleneterephthalate was extraordinarily stable. Endo-D-galacturonanase immobilized in an acetate 0·1 mol l^{-1} buffer was not liberated from the carrier in water, acetate buffers of pH 4–7, 0·1–1 mol l^{-1} NaCl solutions, 3 mol l^{-1} guanidine, 0·5% pectine and sodium pectate solutions. The stability of immobilization in salt media of high concentration and its dependence on pH indicate that in immobilization of endo-D-galacturonanase to polyethyleneterephthalate hydrophobic interactions are involved either preferentially or even exclusively.

The immobilized enzyme was significantly stabilized in the presence of the substrate or its degradation products up to 40°C. Activity of the preparation suspended in solution of sodium pectate was unchanged during a 3-week storage at an ambient temperature. This feature, together with irreversibility of binding was also reflected

TABLE II

Activity of the soluble fraction of endo-D-galacturonanase during immobilization to polyethyleneterephthalate in water and buffers of various concentration

Concentration mol l^{-1}	Activity after incubation[a] 15 min	30 min
0 (water)	52·6	24·3
0·025	38·1	17·9
0·050	21·5	9·4
0·100	12·3	6·0

[a] In per cent of the original activity of the soluble enzyme.

in a high operational stability verified by a continuous elution of the adsorbate by a 6 000-fold bed volume of sodium pectate at room temperature. The activity of the immobilized enzyme monitored by determination of reducing groups in the column effluent had a constant value throughout the whole experiment lasting 20 days.

Immobilization of endo-D-galacturonanase did not change the dependence of its activity on pH; like curves with two pH optima at 4·0 and 4·8 showed the soluble and also the immobilized enzyme; the activity of the enzyme of higher pH optimum was noticeably lowered by immobilization (Fig. 2). Similarly, the temperature stability of the enzyme did not undergo any considerable alteration (Fig. 3). Temperature activity optimum of the immobilized endo-D-galacturonanase was found to be at 40°C (Fig. 4).

The most active preparation was subjected to measurement of degradation kinetics of the polymeric substrate and the mode of action was examined by analysis of products of cleavage and by measurement of the viscosity decrease and reducing group increase. Degradation of sodium pectate obeys the kinetics of Michaelis and Menten;

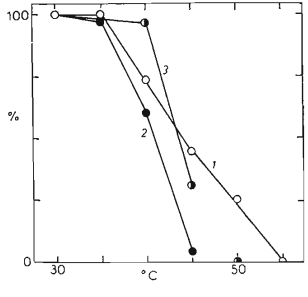

FIG. 3

Temperature stability of the soluble and immobilized endo-D-galacturonanases. The activity of the enzyme was determined at 30°C after a 2 h incubation at the given temperature. *1* Soluble enzyme; *2* immobilized enzyme; *3* immobilized enzyme incubated in 0·5% sodium pectate at the given temperature

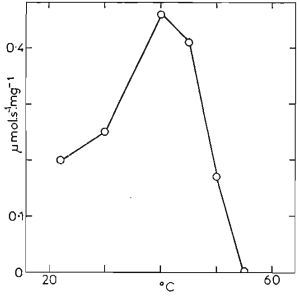

FIG. 4

Relation of the activity of the immobilized endo-D-galacturonanases on temperature

immobilization resulted in a substantial drop of V_{max} from 2·08 μmol/mg s of the free enzyme to 0·35 μmol/mg s, and also the value K_m partly decreased from the original one $1{\cdot}95 \cdot 10^{-3}$ mol l^{-1} to $1{\cdot}57 \cdot 10^{-3}$ mol l^{-1} of D-galactopyranuronic acid units. At the same time, adsorption to the carrier also changed the mode of action of the enzyme on the polymeric substrate but, nevertheless, this change is not as deep as with the covalently immobilized enzyme[2,4]. Oligogalacturonic acids of polymerization degree 1–8 were the degradation products from the beginning of the reaction, and during the degradation process the ratio of lower oligosaccharides raised more fastly than during degradation with the soluble enzyme. The viscosity decrease of sodium pectate solution by 60%, due to the action of the immobilized enzyme, was associated with the cleavage of 6% glycosidic bonds. These results show that action of the immobilized enzyme is not restricted to such an extent to peripheric spheres of the substrate macromolecule as encountered with endo-D-galacturonanase covalently immobilized through amino groups[2,4]. Endo-D-galacturonanase immobilized by sorption to polyethyleneterephthalate revealed an action to high-molecular substrate close to that of the soluble enzyme.

REFERENCES

1. Van Houndenhoven F., de Witt P., Visser J.: Carbohyd. Res. *34*, 233 (1974).
2. Bock W., Krause M., Göbel H., Anger H., Schwaller H. J., Flemming C., Gabert A.: Nahrung *22*, 185 (1978).
3. Rexová-Benková Ľ., Mračková M., Babor K.: This Journal *45*, 163 (1980).
4. Rexová-Benková Ľ., Mračková-Dobrotová M.: Carbohyd. Res. *98*, 115 (1981).
5. Sheiman M. J.: *Thesis*. Rutgers University, New Brunswick, U.S.A. 1974.
6. Hanish W. H., Rickard P. A. D., Nyo S.: Biotechnol. Bioeng. *20*, 95 (1978).
7. Weibel M. K., Barrios R., Delotto R., Humphrey A. E.: Biotechnol. Bioeng. *17*, 85 (1975).
8. Datunashvili E., Pavlenko N., Gaina B., Petraczenok V.: Vinodelie Vinogradstvo SSSR *6*, 54 (1975).
9. Young L. S.: *Thesis*. Cornell University Ithaca, U.S.A. 1976.
10. Bogatskii A. V., Davidenko T. L., Areshidze I. R., Greni T. A., Sevastyanov O. V.: Prikl. Biochim. Microbiol. *15*, 747 (1979).
11. Bogatskii A. V., Davidenko T. L., Greni T. A.: Prikl. Biokhim. Mikrobiol. *16*, 226 (1980).
12. Silman I. H., Katchalski E.: Annu. Rev. Biochem. *35*, 873 (1966).
13. Caldwell K. D., Axén A., Bergwall M., Porath J.: Biotechnol. Bioeng. *18*, 1573 (1976).
14. Caldwell K. D., Axén R., Bergwall M., Porath J.: Biotechnol. Bioeng. *18*, 1589 (1976).
15. Hofstee B. H. J.: Biochem. Biophys. Res. Commun. *50*, 751 (1973).
16. Hofstee B. H. J.: Biochem. Biophys. Res. Commun. *53*, 1127 (1973).
17. Kubánek V., Budín J.: Chem. Prům. *26*, 599 (1976); Chem. Abstr. *88*, 62989 (1977).
18. Budín J., Kubánek V.: Chem. Prům. *27*, 568 (1977);; Chem. Abstr. *88*, 23527 (1977).
19. Rexová-Benková Ľ., Tibenský V.: Biochim. Biophys. Acta *268*, 187 (1972).
20. Rexová-Benková Ľ., Omelková J., Kubánek V.: Czech. 211 694.
21. Somogyi M.: J. Biol. Chem. *196*, 19 (1952).
22. Wilkinson G. N.: Biochem. J. *80*, 324 (1961).
23. Booman K. A., Niemann C.: J. Amer. Chem. Soc. *78*, 3642 (1956).

24. Koller A., Neukom H.: Biochim. Biophys. Acta *83*, 366 (1969).
25. Čoupek J., Gemeiner P., Jirků V., Kálal J., Kubánek V., Kuniak Ľ., Peška J., Rexová Ľ., Štamberk J., Švec F., Turková J., Veruovič B., Zemek J.: Chem. Listy *75*, 512 (1981).
26. Mikeš O., Sedláčková J., Rexová-Benková Ľ., Omelková J.: J. Chromatogr. *207*, 99 (1981).
27. Heinrichová K., Rexová-Benková Ľ.: This Journal *42*, 2569 (1977).

Translated by Z. Votický.