ENDOPOLYGALACTURONASE IMMOBILIZED ON CERAMIC MATERIALS SUBSTITUTED WITH AMINOPROPYL GROUPS. SYNTHESIS, KINETICS, AND MODE OF ACTION

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Endopolygalacturonase was immobilized on ground porous tiles and on ceramic rings functionalized with 3-(aminopropyl)triethoxysilane and glutaraldehyde. The relative activity of the immobilized enzymes depended on loading, attaining 32·4% for porous tiles and 44·0% for ceramic rings. The immobilization resulted in the shift of the optimum of enzyme performance to lower pH values, the temperature optimum increasing from 40 to 60°C, along with thermostability. Immobilized enzymes showed constant activity during 8-months (porous tiles) and 3-months continuous percolation with 0·5% sodium pectate solution. In both cases, the immobilization resulted in preferential cleavage of glycosidic bonds at the end of the chain of high molecular substrate and in nonspecific cleavage of tetra(D-galactosiduronic acid).

In recent years, immobilization of pectic enzymes has attracted increasing attention. Endopolygalacturonase [poly(1,4-α-D-galacturonide)glycanohydrolase, E.C. 3.2.1.15] has been immobilized on polymer supports mostly by covalent binding *via* amino groups[1-5] or by adsorption[6]. However, for some applications of immobilized pectinases, the preparations based on rigid, mechanically stable supports with good hydrodynamic properties would be more suitable, enabling the application in flow columns even under higher pressures. These requirements are met by some inorganic materials such as glass, silica gel, ceramic materials and the like. These are most frequently modified with organosilicon compounds, introducing thus a variety of functional groups on support surface[7]. In the case of microporous supports, immobilization is realized only on their surface, resulting in a marked reduction of internal diffusion restrictions taking place in porous materials.

In the present work we report on immobilization of endopolygalacturonase on two ceramic materials of similar chemical nature, containing covalently bound 3-aminopropyl groups and prepared by the reaction of 3-aminopropyltriethoxysilane. Two materials chosen were *a*) ground porous unglazed tiles (earthenware) used in gas chromatography and *b*) ceramic rings used in distillation columns or as insulating material for electric equipments. We have examined in detail properties of the immobilized enzymes and the effect of immobilization on the kinetics and mode of enzymic action.

EXPERIMENTAL

Material and Methods

Enzyme. Endopolygalacturonase was purified from a pectinase preparation Rohament P (Röhm GmbH, F.R.G.) by affinity chromatography on pectic acid crosslinked by epichlorhydrin[9]. The specific activity of the preparation at pH 4·8 (pH optimum) and 30°C was 2·99 μkat . mg^{-1}.

Supports. Porous, unglazed tiles, ground to particles 0·2—0·3 mm in diameter (Lachema Brno) (pore volume 0·21 $cm^3 g^{-1}$; medium pore diameter calculated on the basis of surface 5 nm and the volume 650 nm; specific surface 30 $m^2 g^{-1}$) were treated in the following way[8]: A mixture of 250 ml of toluene and 50 ml of 3-aminopropyltriethoxysilane (1 mol l^{-1}) was added to 50 g of support material predried at 130°C for 3 h. The mixture was refluxed for 3 h, then half of the toluene volume was distilled off, the activated support was filtered and washed successively three times with toluene, ones with methanol and ones with water. Then it was dried under reduced pressure at 15°C for 4 h. The modification of the surface by amino groups did not change textural properties of the support.

Ceramic rings (Elektroporcelán Works, Louny, Czechoslovakia) in the form of hollow unglazed cylinders (length 4 mm, e.d. 4 mm, i.d. 2 mm) were first activated by 5 h-boiling with 4% HCl or by standing for 1 h in hydrogen fluoride, then washed several times with water and dried at 120°C. The activated rings were suspended in 40 ml toluene containing 2 g of 3-aminopropyltriethoxysilane and further worked-up similarly as the ground porous tiles.

Prior to enzyme immobilization, the supports were activated with 6·25% solution of glutaraldehyde prepared by diluting its 25% aqueous solution (Serva, F.R.G.) with 0·1M acetate buffer pH 6·0. The activated support was washed several times with the same buffer until the washings contained the reducing agent. The content of the active aldehyde groups was determined by the method for reducing groups[10], using calibration graph for glutaraldehyde.

Substrates. Sodium pectate (D-galacturonan content 89·8%, average molecular mass, determined viscosimetrically 27 000) was prepared by repeated alkaline deesterification of citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) followed by precipitation with hydrochloric acid at pH 2·5 and neutralization with NaOH.

Tetra(D-galactosiduronic acid) was isolated by gel chromatography on Sephadex G-25 (Fine)[11] from the enzymic hydrolysate of sodium pectate produced in a column of endopolygalacturonase immobilized on poly(ethyleneterephthalate)[6].

Immobilization of Endopolygalacturonase

The activated support was incubated with endopolygalacturonase in 0·1M acetate buffer (pH 6·0) under shaking for 4—6 h at 4°C. The immobilized enzyme was washed with 0·1M acetate buffer, pH 4·2, until the washings showed enzyme activity. The amount of endopolygalacturonase immobilized on the support was estimated from the difference in activity taken for immobilization and that of non-linked enzyme. The immobilized enzyme was stored in acetate buffer, pH 4·2.

Endopolygalacturonase Assay

The activity of endopolygalacturonase was determined in 0·1M acetate buffer of a given pH, by measuring the increase in reducing groups[10] in the reaction mixture containing 0·5% solution

of sodium pectate as the substrate. The immobilized enzyme was applied either as a suspension continuously shaken at constant rate or packed in a constant temperature (30°C) double-jacketted column. The activity of endopolygalacturonase is expressed in micromol reducing groups liberated *per* s (1 μkat) by 1 mg of enzyme free or bound or by 1 g of preparation and determined by means of standard graph for D-galactopyranuronic acid. The relative activity is the ratio of the activities of bound and free enzyme (in per cent). The kinetic constants for the free endopolygalacturonase and immobilized enzyme applied in suspension, K_m, $K_{m,app}$, V, and V_{app} were computed by the least square method on the basis of initial velocities determined at five substrate concentrations in the range from 0·1 to 0·5%. For the enzyme applied in the column, the kinetic constants were determined by means of the graphical method of Lilly and coworkers[12] and calculated by the least square method.

The dependence of the activity on pH was examined in 0·1M acetate buffers of pH 3·6–5·6 after washing the immobilized enzyme with the respective buffer. The reducing groups were determined by means of calibration graph for D-galactopyranuronic acid constructed for each pH.

Thermal stability was characterized by the activity determined at 30°C after 2 h-incubation of the enzyme at the given temperature. The operational stability was examined by a continuous percolation of the column of the immobilized enzyme with 0·5% solution of sodium pectate at pH 4·2 and room temperature. The activity of the preparation was evaluated on the basis of reducing groups periodically determined in the effluent. Viscometric assay of endopolygalacturonase was accomplished in reaction mixture containing 297 ml of sodium pectate (0·5% solution) and 3 ml of the immobilized enzyme. The 20 ml-aliquots were withdrawn at fixed time intervals and their viscosity was measured by Ubbelohde viscometer.

Analysis of Reaction Products

The products of enzymic degradation were analysed by thin-layer chromatography, using silica gel (Silufol plates, Kavalier, Czechoslovakia) and n-butanol–formic acid–water (2 : 3 : 1) mixture[13]. The polymerization degree of the products was determined from its dependence on log $R_F/(1 - R_F)$, using D-galactopyranuronic acid as the standard.

RESULTS AND DISCUSSION

Ceramic materials used to immobilize endopolygalacturonase represent cheap, common products based on calcium-sodium or potassium silicates. Compared with the most organic polymer supports they exhibit the higher chemical, thermal and mechanical stability. The shape of particles and its high rigidity make it possible to use these materials in flow columns even at extremely high flow rates without changing column height and density. The reaction with 3-aminopropyltriethoxysilane resulted in introduction of free amino groups on support surface enabling covalent attachement of proteins *via* their amino or carboxyl groups. Due to the microporous character of the supports, proteins are bound predominantly on the surface, being thus in immediate contact with surrounding medium and accessible to interaction even with high-molecular substrates. The binding capacity of the supports, however, is low. Endopolygalacturonase was attached to both supports *via* amino groups by means of glutaraldehyde.

Properties of Immobilized Enzymes

The activated ground porous tiles contained 40·86 μmol of aldehyde groups *per* 1 g of material. Because of instability of endopolygalacturonase in alkaline medium, the immobilization was carried out at pH 6·0. Under these conditions, 0·198 mg of the enzyme was bound *per* 1 g of the activated support. The non-activated ground porous tiles partially sorbed endopolygalacturonase, but the enzyme was released again by elution with 0·1M acetate buffer.

The activity of the preparation with covalently immobilized endopolygalacturonase towards sodium pectate at 30°C and pH 4·2 was 0·22 μkat mg^{-1} bound enzyme (relative activity 32·4%). Immobilization has changed some properties of endopolygalacturonase: *a*) pH region of activity was shifted to lower values with simultaneous change *in optima* from 4·8 and 5·4 of the soluble enzyme to pH 3·8 and 4·4, resp. (Fig. 1); *b*) temperature optimum of the activity determined on the basis of initial velocities increased from 40·0 to 60·0°C (Fig. 2); *c*) within the temperature region >30°C – <60°C and under the conditions used, the immobilized enzyme exhibited significantly increased thermostability (Fig. 3). The shift in the pH range and optimum of activity of immobilized endopolygalacturonase results from the effect of microenvironment of the enzyme formed by basic groups of the support.

The preparation was stable when stored at 4°C in 0·1M acetate buffer (pH 4·2); in the course of 8 months no release or decrease in activity of the enzyme was de-

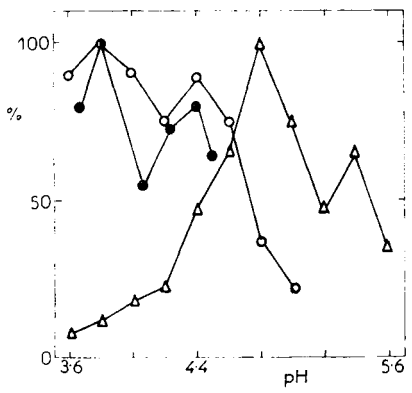

FIG. 1

pH dependence of the activity of soluble and immobilized endopolygalacturonase. The soluble enzyme (△), the enzyme immobilized on ground porous tiles (○) and on ceramic rings (●). 100% — maximum activity of both soluble and immobilized enzyme

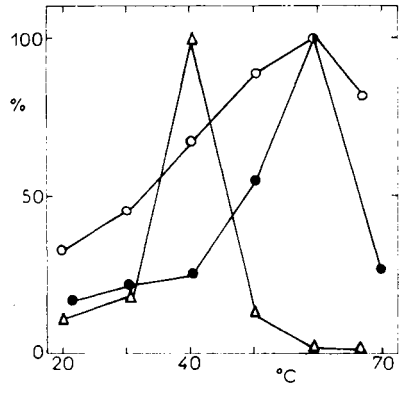

FIG. 2

Temperature dependence of the activity of soluble and immobilized endopolygalacturonase. For designation see Fig. 1

tected. However, the enzyme was inactivated by drying in air at ambient temperature or by lyophilization. The strength of binding to the support was proved also during 3-months-percolation of a column of the immobilized preparation with 0·5% solution of sodium pectate at room temperature. An additional increase in the content of reducing groups in the effluent has not been observed, thus indicating that the enzyme was not released from the support. During first days in which c. 55-fold bed volume passed through the column, the support slightly adsorbed the substrate and the activity of the preparation decreased to 48·6% of the initial value. After this period the enzyme activity remained constant.

The activated ceramic rings contained 1·48 μmol of aldehyde groups *per* g material. The activity of the immobilized endopolygalacturonase depended on the amount of the enzyme bound to the support. The highest relative activity at pH optimum, 43·99% was observed with the preparation containing 0·063 mg enzyme *per* g support. When the enzyme content was decreased to 0·025 mg g^{-1}, the preparation showed the relative activity 22·8%. On the other hand, the increase in the content of the enzyme to 0·147 mg g^{-1} resulted in the relative activity of 0·09%. Non-activated support did not adsorb endopolygalacturonase. The activity of the preparations decreased during their storage at 4°C in 0·1M acetate buffer, pH 4·2. After 3 months, the activity decreased to 70%, a release of the enzyme from the support not being, however, detected. The immobilized endopolygalacturonase was markedly stabilized in the presence of substrate and its degradation products, as well as in an apple juice. Under these conditions, the activity of the enzyme has not been changed for 8 months at room temperature. This was reflected in a high operational stability of the preparations, demonstrated by constant enzyme activity during 3-months examination at room temperature. The effect of immobilization on temperature optimum of the activity, the activity versus pH dependence (Figs 1 and 2), as well as the behaviour of the preparation after drying were the same as observed with the enzyme immobilized on ground porous tiles. Thermostability of the enzyme immobilized on ceramic rings was greater than that observed for the soluble enzyme. Within the temperature region of $>40°C - <70°C$ the thermostability of the enzyme immobilized on ceramic rings was greater than that observed with the soluble endopolygalacturonase (Fig. 3).

Mode and Kinetics of Action of Immobilized Endopolygalacturonase

The action pattern of both preparations of immobilized endopolygalacturonase was examined in stirred suspension as well as in column by measuring the viscosity of substrate solution during enzyme reaction and by correlating these data with degree of glycosidic bond cleavage (in per cent). As found earlier, in some cases the action pattern and kinetics of immobilized endopolygalacturonase may be affected by substrate transfer[14]; in the column application of both immobilized preparations,

the measurements were thus performed at different flow rates of substrate. The reaction time (the time of enzyme–substrate contact) was regulated by changing the column height.

Similarly as in previous cases of covalent immobilization of endopolygalacturonase *via* amino groups to porous polymer supports[2,4,5,14,15], the binding to both ceramic materials led to a substantial change in action pattern on high-molecular substrate. It consisted in a decrease in randomness of glycosidic linkage splitting – typical of soluble endopolygalacturonase, and the restriction of enzyme action to accessible, peripheric parts of substrate molecule. This change was manifested as the slower decrease in viscosity in the course of substrate degradation compared with the action of free enzyme (Fig. 4) and by the production of mono- through tetragalactosiduronic acid already at the beginning of the reaction. In both cases this change was more distinct when the enzyme was applied in a stirred suspension (Fig. 4, curve *1*) than in the column at whichever flow rate and similarly as with endopolygalacturonase immobilized on porous supports[4,5,14] it was observed up to the cleavage of 7–8% glycosidic bonds. Then the degradation continued by random action pattern. In both cases, immobilization led to a change in action pattern also on low-molecular

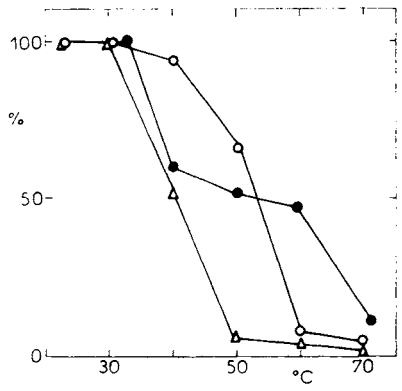

FIG. 3

Thermostability of soluble (△) and immobilized endopolygalacturonase — ground porous tiles (○), ceramic rings (●). The enzyme activity was determined at 30°C after 2 h-incubation at given temperature

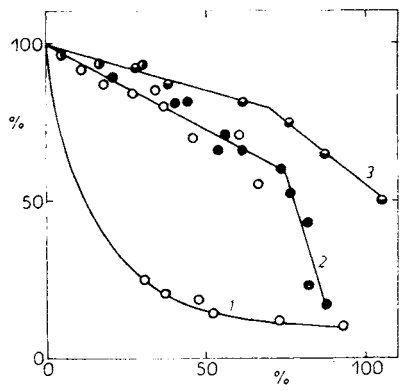

FIG. 4

Correlation of viscosity decrease with the degree of sodium pectate degradation for soluble endopolygalacturonase and immobilized preparations applied in column and in stirred suspension. *1* Soluble enzyme, *2* column-packed enzyme: ○ porous ground tiles, ● ceramic rings; *3* immobilized enzyme applied in stirred suspension: ◑ porous ground tiles, ◒ ceramic rings

substrates. The most significant effect was observed with tetra(D-galactosiduronic acid) which is cleaved by the free enzyme specifically at the reducing end, giving tri- and monosaccharide[16]. By contrast, the immobilized enzyme produced also a small amount of di(D-galactosiduronic acid), which speaks for an alternative cleavage of the medium glycosidic bond of the substrate molecule.

Kinetics of the action of immobilized preparations was measured at pH 3·8 in the column at different flow rates. The values of kinetic constants were compared with those for the free enzyme, determined at pH optimum. In stirred suspension at concentrations of sodium pectate of $1-5\ g\ l^{-1}$, in both cases the reaction obeyed the kinetics of Michaelis–Menten; both Lineweaver–Burk plot and Eadie–Hofstee plot[17] yielded straight lines. Both kinetic constants, however, were significantly changed due to enzyme immobilization: K_m increased from $3{\cdot}60 \cdot 10^{-3}$ mol l^{-1} units D-galactopyranuronic acid of the free enzyme to $2{\cdot}77 \cdot 10^{-2}$ mol l^{-1} of endopolygalacturonase bound to ground porous tiles and to $8{\cdot}21 \cdot 10^{-3}$ mol l^{-1} of the enzyme bound to ceramic rings. On the other hand, V decreased from 4·14 μkat mg^{-1} to 0·42 μkat mg^{-1} (ground porous tiles) and to 0·146 μkat mg^{-1} (ceramic rings).

The results of measurements made with the enzyme-packed column at different flow rates of the substrate were treated according to Lilly and coworkers[12], using the integrated Michaelis–Menten equation

$$(S_0 - S_\tau) - K_{m,app} \ln S_\tau/S_0 = k_3 EQ\beta\,,$$

where $(S_0 - S_\tau)$ is the difference between substrate concentrations at column inlet and outlet resp.; Q is the flow rate; β is the voidage of the column, defined as the ratio of the void volume to the total bed volume; k_3 is the rate constant of decomposition of E–S complex; E is the total enzyme concentration. Experimental data are presented graphically in Figs 5 and 6 and the values of kinetic constants are summarized in Table I. As it is seen, for all the flow rates, straight lines with similar slopes giving $K_{m,app}$ as well as similar V_{app} values were obtained for the given preparation. The markedly lower V_{app} values obtained for the column of endopolygalacturonase on ground porous tiles compared with the value determined for stirred suspension can be due to an inaccessibility of the substrate to some particles of the support in tightly packed column.

The increased concentration of the substrate corresponding to the half of the saturation rate and the lower limiting first order rate constant of the immobilized enzyme compared with the V/K_m value of the soluble enzyme are commonly ascribed to the effect of external diffusional limitations[17]. The independence of the kinetics of enzyme reaction on the rate of substrate transfer indicates, however, that the decrease in enzyme activity as well as changed kinetic constants resulting from immobilization are likely due to sterical limitations rendering only a part of the

substrate molecule accessible for the active site of the bound enzyme. The actual concentration of substrate bonds to be attacked is thus lower than in the case of the action of free enzyme.

TABLE I

Kinetic constants $K_{m,app}$(mmol l^{-1}) and V_{app}(μkat mg^{-1}) in column application of the endopolygalacturonase immobilized on ground porous tiles (A) and on ceramic rings (B) at different substrate flow rates Q (μl s^{-1})

Flow rate Q	Support A[a]		Flow rate Q	Support B[b]	
	$K_{m,app}$	V_{app}		$K_{m,app}$	V_{app}
3·3	9·88	0·0695	16·6	10·83	0·172
5·0	10·16	0·0709	25·0	7·76	0·151
10·0	11·55	0·073	50·0	13·78	0·211

Column characteristics: [a] $E = 0{\cdot}052$ mg, $V_r = 0{\cdot}5$ ml, $\beta = 0{\cdot}58$; [b] $E = 0{\cdot}223$ mg, $V_r = 10$ ml, β 0·65.

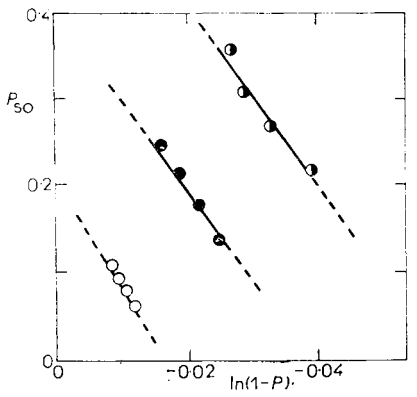

FIG. 5

Data on kinetics of enzymic reaction in the column of endopolygalacturonase immobilized on ground porous tiles at different substrate flow rates, treated according to Lilly and coworkers[14]. Flow rates: 0·01 ml . . s^{-1} (○), 0·005 ml s^{-1} (●), 0·003 ml s^{-1} (◑)

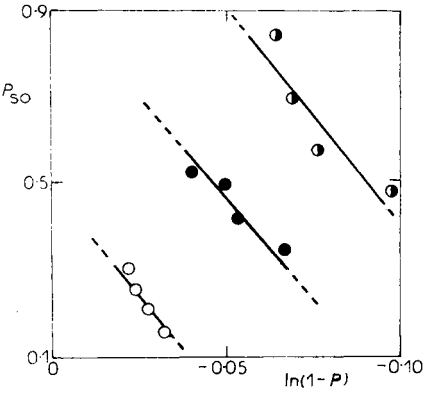

FIG. 6

Data on kinetics of enzymic reaction in the column of endopolygalacturonase immobilized on ceramic rings. Flow rates: 0·05 ml . . s^{-1} (○), 0·025 ml s^{-1} (●), 0·017 ml s^{-1} (◑)

The independence of degradation on the rate of substrate transfer observed both with porous[4,5] (micro- and macro-) and nonporous supports indicates, too, that sterical limitations resulting from the mode of enzyme binding to the support is the primary factor determining the action pattern of immobilized endopolygalacturonase. The specific cleavage of tetra(D-galactosiduronic acid) to trisaccharide and monosaccharide by the free endopolygalacturonase is determined by the size and arrangement of its active site[16]. The changed action pattern of immobilized endopolygalacturonase may relate to its binding to the support such that a part of the binding site is sterically hindered[15].

Covalent binding of endopolygalacturonase to both ceramic supports was used to synthesize the preparations suitable especially for long-term application in columns even at very high flow rates of viscous liquids. Although the immobilized enzyme operates in somewhat different way, it is possible to achieve optimum degradation of the substrate at appropriate ratio of reactor volume to flow rate.

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