A CAPILLARY GLASS REACTOR FOR POLYGALACTURONASE IMMOBILIZATION

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Received December 20, 1994
Accepted January 18, 1995

Polygalacturonase was immobilized onto a set of nonporous glass columns of the capillary reactor functionalized with aminopropyl and epoxy groups. The properties of the anchored catalysts in relation to the accessibility of the reaction sites toward polymeric and low-molecular substrates are reported.

Glass is one of the most frequently used supports for immobilization of enzymes[1,2], due to its undisputed advantage[3–5] in combining mechanical stability and good hydrodynamic properties with a variety of functional groups which could be introduced on its surface e.g. by its modification with organosilicon compounds[6].

The present work is concerned with the immobilization of *Aspergillus* sp. polygalacturonase [poly(1,4-α-D-galacturonide)glycanohydrolase, E.C.3.2.1.15] on nonporous glass capillaries of the reactor, the surfaces of which were functionalized with epoxide or 3-aminopropyl groups. Such an arrangement, which represents a novel type of bioreactors, could be useful not only in the reported polygalacturonase immobilization but is expected to find more general applications.

EXPERIMENTAL

Material and Methods

Enzyme. Polygalacturonase was purified from a pectinase Rohament P (Rohm, GmbH, Germany) by the ion-exchange and gel permeation chromatography[7]. The specific activity of the preparation at pH 4.4 (optimum pH value) and 30 °C is 0.313 μkat/mg.

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Supports. Glass capillary columns (SVUS, Hradec Kralove, The Czech Republic) were treated with 3-(2′,3′-epoxypropoxy)propyltrimethoxysilane and (3-aminopropyl)triethoxysilane in the same manner as described previously[8,9]. The reactor (7.0 mm e.d., 100 mm long) consisted of 1 200 parallel capillaries (140 μm i.d.) in the form of tight-packed bundle (total capillary volume 2 200 mm^3 and total area 61 000 mm^2).

Immobilization of Polygalacturonase

The epoxide-activated capillary reactor was percolated by 20 mg of polygalacturonase dissolved in 200 ml of 0.1 M acetate buffer of pH 3.8 for 10 days at 4 °C. Then, the immobilized enzyme was washed with the above buffer. The aminopropylated support was activated with 6.25% glutaraldehyde solution (Serva, Germany) for 12 h prior to the immobilization. The activated reactor was washed with 0.1 M acetate buffer of pH 6.0 so long as the effluent contained the reducing agent. The immobilization was carried out under the same conditions as the epoxide activation, except that the enzyme was dissolved in 0.1 M acetate buffer of pH 6.0 and the column was treated with this solution for 24 h.

Enzyme Assay

Polygalacturonase activity was assayed in 0.1 M acetate buffer of a given pH at 30 °C by measuring the increase of reducing groups[10] in the reaction mixture (free enzyme) or in the effluent (immobilized enzyme), using 0.5% sodium pectate solution or 1 μmol/ml solution of tetra(D-galactosiduronic) acid. The enzyme activity was determined by the standard correlation of D-galactopyranuronic acid. It was expressed in μmol of the reducing groups released within 1 s (μkat) by 1 mg of the enzyme or as a relative activity in per cent (the ratio of the bound enzyme activity to the free enzyme activity).

Characterization of Immobilized Enzyme

All the characteristics of the immobilized enzyme were obtained by experiments with the columns treated as described above. The required temperature was ensured by double-jacketting the columns. The dependence of the enzyme activity on pH was examined in the pH region of 3.6 – 5.4. Thermal stability of the immobilized enzyme was characterized by its activity determined at 30 °C after 2 h-incubation of the reactor at a given temperature. The working stability was tested by a continuous percolation of the reactor with the substrate solution at room temperature. Viscosimetric characteristics were made on the basis of viscosity measurements at various flow rates of the substrate solution. Data were obtained with Ubbelohde viscosimeter.

Analysis of the Reaction Products

The products of enzymatic degradation were analyzed by thin-layer chromatography, using silica gel (Silufol plates, Kavalier, The Czech Republic) and butanol–formic acid–water (2 : 3 : 1 v/v/v) mixture[11]. 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

A capillary glass reactor designed for polygalacturonase immobilization contained the enzyme chemically bound to the epoxy-functionalized glass capillary surface or to the 3-aminopropylated surface activated by glutaraldehyde. In both cases, about 2.5 mg of

the enzyme were fixed to 1 g of the carrier at optimum immobilization conditions determined for other materials[8,9]. The unactivated capillaries sorbed polygalacturonase, too, but the enzyme was easily washed out by the substrate or by 0.1 M acetate buffer. The activity of the immobilized preparations represented only 1.28% (epoxy-activated column) and 0.93% (aminopropylated column) of the free enzyme activity determined with the polymeric substrate. The reason of the unusually low activity[8,9] can be partially deduced from comparison of the above data with those determined for tetra(D-galactosiduronic) acid as the substrate: while the ratio of the free enzyme activity measured with this substrate to that obtained with pectic acid was 0.17, for the epoxide-bound enzyme it equaled to 0.78 and for the aminopropylated sample it equaled to 0.48. This documents that the interaction of oligosubstrate with the immobilized enzyme is clearly preferred. This fact indicates the existence of steric hindrance for the polymeric substrate which resulted from the matrix design and led to the lower accessibility of the reaction sites for the substrate.

On the other hand, the action pattern of the cleavage of tetra(D-galactosiduronic) acid was not influenced by the support. The epoxy-bound enzyme degradated specifically this substrate to tri(D-galactosiduronic) acid and galactopyranuronic acid as the free *Aspergillus* polygalacturonase, while the enzyme bound via amino groups yielded nonspecifically small amounts of di(D-galactosiduronic) acid, too[12]. This seems to be the consequence of steric hindrance of the subsite of the supported enzyme which interacts with the sugar unit at its nonreducing end.

The action pattern of the cleavage of the high-molecular substrate, sodium pectate, was examined by viscosity measurements of the substrate solution during the enzyme reaction, and by correlating data obtained with the degree of degradation (expressed in per cent of the cleaved glycosidic linkages; see Fig. 1). The plots of the relative

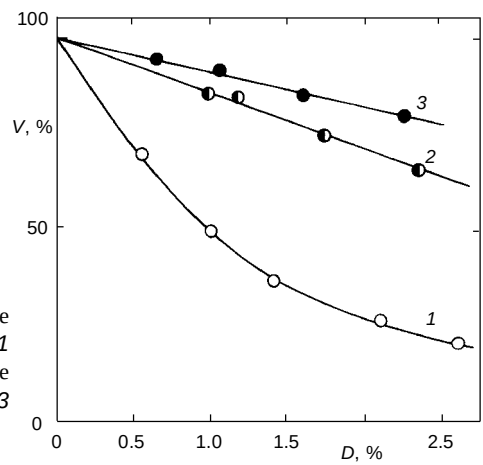

FIG. 1
Correlation of the viscosity decrease (*V*) with the degree of sodium pectate degradation (*D*) for *1* free polygalacturonase and for *2* the enzyme bound onto the epoxy-functionalized support or *3* the enzyme bound via aminopropyl groups

viscosity vs the degree of substrate degradation found for the column reactors at flow rates ranging 0.4 – 2.3 μl/s and for the free enzyme–substrate solution demonstrate the change from random splitting of the internal glycosidic linkages of D-galacturonan sequences (fast depolymerization of the substrate results in a pronounced decrease in viscosity at the low degree of degradation; 50% decrease in viscosity corresponds to the cleavage of 1% glycosidic linkages (Fig. 1, curve *1*)), to the cleavage proceeding sequentially near the chain end at intervals embracing one or more sugar units (slow decrease in viscosity observed (Fig. 1, curves *2*, *3*), mono-, di-, and trisaccharides were predominant products of the degradation). The difference between curves *2* and *3* in Fig. 1 is due to the influence of the mode of binding on the action pattern of the immobilized polygalacturonase[12].

Therefore, the immobilization has changed some properties of polygalacturonase:

a) pH region of the activity was shifted to the lower values with simultaneous change of optima from 4.4 to 4.2;

b) the temperature optimum of the activity increased from 40 °C to 50 °C;

c) within the temperature region (30 °C – 50 °C) and under conditions used, the immobilized enzyme showed significantly increased thermostability.

The shift of the pH optimum of the activity of the immobilized polygalacturonase results from the effect of the microenvironment of the enzyme formed by basic groups of the support.

The preparation was stable when stored at 4 °C in 0.1 M acetate buffer or in 0.5% substrate; in the course of 14 months no release of the enzyme was detected. The strength of its bond to the support was proved also during 3 month's-percolation of the columns with sodium pectate solution. During the first four days, the support slightly adsorbed the substrate, and the activity of the preparation decreased to 72% of the initial value. An additional increase in the content of the reducing groups in the effluent has not been observed, thus indicating that the enzyme was not released from the support. After this period the enzyme activity remained constant.

The present data show that the described arrangement has not only the attractive design (forming easily a column reactor) but that the capillary glass reactor of this type would be ideal for immobilization of enzymes for analytical or biochemical application, especially the enzymes catalyzing transformations of low-molecular substrates. Very important feature of the reactor is its extraordinary stability, nonporous surface and the absence of nonspecific interactions.

REFERENCES

1. Wingard L. B., Katchalski-Katzir E., Goldstein L.: *Applied Biochemistry and Bioengineering 1*. Academic Press, New York 1976.
2. Weetall H. H.: *Immobilized Enzymes, Antigens, Antibodies and Peptides*. Dekker, New York 1975.
3. Bosley J. A., Clayton J. C.: Biotechnol. Bioeng. *43*, 934 (1994).

4. Wilson S. A., Peek K., Daniel R. M.: Biotechnol. Bioeng. *43*, 225 (1994).
5. Hayashi S., Tubouchi M., Takasaki Y., Imada K.: Biotechnol. Lett. *6*, 227 (1994).
6. Messing R. A.: *Immobilized Enzymes for Industrial Reactors* (R. A. Messing, Ed.). Academic Press, New York 1975.
7. Stratilova E., Markovic O., Skrovinova D., Rexova-Benkova L., Jornvall H.: J. Protein Chem. *12*, 15 (1993).
8. Stratilova E., Capka M., Rexova-Benkova L.: Biotechnol. Lett. *9*, 511 (1987).
9. Rexova-Benkova L., Stratilova E., Capka M.: Collect. Czech. Chem. Commun. *51*, 2924 (1986).
10. Somogyi M.: J. Biol. Chem. *195*, 19 (1952).
11. Koller A., Neukom H.: Biochim. Biophys. Acta *83*, 366 (1964).
12. Rexova-Benkova L., Stratilova E., Capka M.: Biocatalysis *4*, 219 (1990).