ENDO-D-GALACTURONANASE IMMOBILIZED BY A COVALENT BINDING TO HYDROXYALKYL METHACRYLATE GELS. PREPARATION AND PROPERTIES

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Insoluble derivatives of endo-D-galacturonanase were prepared by a covalent binding to hydroxyalkyl methacrylate gels (Spherons) by a cyanogen bromide method. Gels of various pore size were used. The amount of the attached enzyme, its activity and kinetic constants were determined. The effect of covalent binding on the pH activity curves, heat stability, storage stability at 4°C and the operational stability of the enzyme were investigated.

Endo-D-galacturonanase[poly(1,4-α-D-galactosiduronate)glycanohydrolase, E.C. 3.2.1.15] catalyses the hydrolytic cleavage of internal α-1,4 glycosidic bonds of D-galacturonan chains of pectic substances under liberation of oligogalacturonic acids as reaction products. Its catalytic effect is being exploited together with that of other pectic enzymes when processing fruit and vegetables, where the degradation of pectic substances favourizes a better press-out, filtrability of juice and clarification properties. Recently, an enhanced interest has been paid to preparation of insoluble derivatives of pectic enzymes in connection with an industrial application, which could enable to introduce continual procedures into some industrial processes[1-5]. Endo-D-galacturonanase immobilized on Sephadex was used[6] for a continual production of oligogalacturonic acids.

This paper concerns the possibility to prepare an immobilized endo-D-galacturonanase by its covalent binding onto hydroxyalkyl methacrylate gels of Spheron type[7]. The enzyme was linked through amino groups to the hydroxyl groups of the gel by the cyanogen bromide method. The effect of covalent binding on the activity and some properties of the enzyme were examined.

EXPERIMENTAL

Material. Hydroxyalkyl methacrylate gels Spheron 300 and Spheron 500 (particle size 40—80 μm) were products of Lachema, Brno. Spheron 1000 (particle size 100—200 μm) was kindly donated by Dr J. Čoupek (Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague).

Endo-D-galacturonanase was purified from preparation Rohament P (Röhm and Haas, Darmstadt, Federal Republic of Germany) by affinity chromatography on a cross-linked pectic acid[9]. The specific activity of the preparation at pH 4·2 and 30°C was 100·9 micromoles of reducing groups min^{-1} mg^{-1}. Extracelular endo-D-galacturonanase *Aspergillus niger* prepared according to[9] was bound to Spheron 100. Its specific activity was 56·8 micromol of reducing groups min^{-1} mg^{-1} at pH 4·2 and 30°C.

Substrates. Pectic acid (D-galacturonan content 89·9%, average molecular weight 27000 — determined viscometrically) was obtained by a repeated alkaline deesterification of citrus pectin (Genu pectin Københavns Pektinfabrik, Denmark) and precipitation at pH 2·5. Its sodium salt was prepared by neutralization with sodium hydroxide. Heptagalacturonic acid was isolated from the partial acid hydrolysate of pectic acid by a repeated gel chromatography on Sephadex G-25 (Fine), ref.[10].

Preparation of the Immobilized Endo-D-galacturonanase

Hydroxyalkyl methacrylate gels were activated by cyanogen bromide and endo-D-galacturonanase was bound according to[11]. The individual experiments were carried out with the enzyme (50 mg) and the carrier (2 g), which was activated in an aqueous suspension with cyanogen bromide (3·5 g). The activated gel was incubated with the enzyme in the appropriate pH buffer solution for 20 h at 4°C under stirring. Binding at pH 8·5 was effected with a 0·1M carbonate buffer, at pH 6·5 and 5·5 with 0·1M acetate buffer solutions. The unreacted enzyme was removed by washing with 0·1M acetate buffer solution (pH 4·2) on a sintered glass filter. The immobilized enzyme was stored as a suspension in the same buffer solution at 4°C. The amount of the bound enzyme was calculated from the amino acid content determined on an automatic amino acid analyzer[12] after a 20 h acid hydrolysis of the dry gel.

Enzyme Assay

The activity of free and immobilized endo-D-galacturonanase was assayed at pH 4·2 (0·1M acetate buffer) and 30°C by measuring the increase of reducing groups at different time intervals, by spectrophotometric method of Somogyi[13]. The reaction mixture was incubated at constant stirring in a double-jacket temperated vessel when determining the activity of the immobilized enzyme. The activity is expressed in micromoles of reducing groups liberated either by 1 mg of enzyme (free or bound), or 1 g enzyme gel within 1 min. The relative activity of the immobilized enzyme is the ratio of the bound enzyme to the same amount of free enzyme activities, given in per cent.

Kinetic constants of free and immobilized enzyme K_m, $K_{m,app}$ and V were determined from the initial rate of reaction measured at five concentrations of the substrate (sodium pectate) in the 0·1—1% concentration range and calculated by the least squares method[14].

The dependence of the activity on pH was determined in 0·1M acetate buffer solutions at pH 3·2—5·4 after washing the enzyme gel with the respective buffer solution. Reducing groups were determined by means of calibration graphs for D-galactopyranuronic acid constructed for each pH.

The heat stability of free and immobilized enzyme was traced through the activity, which was determined after a 2 h-incubation of the enzyme at a given temperature and subsequent cooling to 30°C.

The operational stability of the immobilized enzyme was examined for 21 days by a continual elution of the enzyme gel packed in a column (1·5 × 3·5 cm) with a 0·5% solution of sodium pectate at pH 4·2 and room temperature. Reducing groups were determined in the eluate at given time intervals.

RESULTS AND DISCUSSION

Spherons are reported[7] to be macroporous hydrophilic gels with a high content of hydroxyl groups, prepared in form of spheric particles and having many advantages[15]. Protein can be bound to hydroxyl groups of the gel either directly, after activation with cyanogen bromide[8], or spacer mediated[16]. Spherons were proved to be good carriers for covalent binding of proteolytic enzymes[8,16].

Three types of gels – Spheron 300, Spheron 500 and Spheron 1000, differing in the pore size (Table I), were used for preparation of insoluble derivatives of endo-D-galacturonanase. The enzyme was bound, after activation of the gel by cyanogen bromide through amino groups, which are not considered primarily essential for its catalytic effect[17]. Due to instability of endo-D-galacturonanase *A. niger* in the alkaline pH (ref.[18]), the enzyme was bound at pH 5·5. The binding was also tried at pH 6·5 and at 8·5 exclusively with Spheron 300. Results of covalent binding of endo-D-galacturonanase, the amount of the enzyme bound and its activity towards sodium pectate are summarized in Table I. The greatest amount of the enzyme was bound to Spheron 300 and Spheron 500 due to their greatest specific surface. An increased pH from 5·5 to 8·5 resulted in an enhanced enzyme binding.

The covalent binding of the enzyme resulted in decrease of its activity in all cases. The extent of this decrease depended on the pore size of the carrier; an increase in the pore size was proportional to the realative activity this being likely due to a better approach of the macromolecular substrate to the gel structure. The greatest relative activity showed the preparation bound to Spheron 1000. Another factor, which might affect the activity, is the amount of the bound enzyme; the high concentration of macromolecules on the surface of the carrier can lead to an increase of steric effect and

TABLE I

Amount and Activity of Endo-D-galacturonanase Bound Covalently to Hydroxyalkyl Methacrylate Gels

Gel	Mol. weight exclusion limit	Specific[a] surface m^2/ml	pH at binding	Bound enzyme mg/g gel	Activity		Relative activity %
					per mg E. bound	per g gel	
Spheron 300	300 000	19·5	8·5	18·1	16·27	294·7	16·1
			6·5	12·6	20·23	254·9	20·0
			5·5	10·9	20·38	222·1	20·2
Spheron 500	500 000	23·0	5·5	10·8	36·93	399·4	36·6
Spheron 1 000	10^6	5·9	5·5	5·0	32·02	160·2	56·4

[a] Ref.[8]

consequently, to a decrease of activity[19]. The decrease of activity in the preparation bound at pH 8·5 might also be connected with deactivation of the enzyme due to a high pH (ref.[18]). This possibility is indicated by activity values of the bound enzyme on heptagalacturonic acid (Table II), where the presumed decrease of steric effect associated with a substantially smaller size of the substrate molecule was manifested by a higher relative activity than with polymeric substrate, exclusively with preparation formed at pH 6·5; relative activity values of the preparation formed at pH 8·5 were, on the other hand, independent on the polymerization degree of the substrate. Covalent binding of endo-D-galacturonanase (Spheron 500) was of no effect on the course of dependence of activity on pH (Fig. 1) and did not considerably influence the thermal stability of the enzyme (Fig. 2).

All preparations of immobilized endo-D-galacturonanase were stable enough during storage in suspension at 40°C. The activity of any enzyme bound to Spheron 300 and Spheron 500 did not drop under 90% of their original value during 2 years; the activity of preparation bound to Spheron 1000 did not change during 7 months of storage. The mentioned decrease of activity of preparations bound to Spheron 300 and Spheron 500 can partly be associated with the liberation of the enzyme from gel during storage, this being evidenced by the activity determined in supernatants sepa-

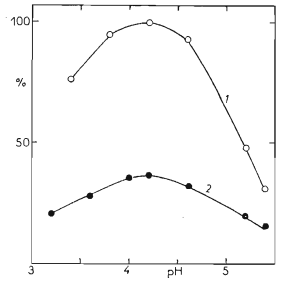

FIG. 1

Dependence of Endo-D-galacturonanase Activity (free and bound to Spheron 500) on pH.

Activity of the free enzyme at pH 4·2 was 100·9 μmol min^{-1} mg^{-1}. *1* Soluble enzyme; *2* immobilized enzyme.

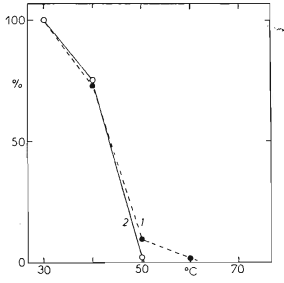

FIG. 2

Heat Stability of Endo-D-galacturonanase Free and Bound to Spheron 500

The enzyme activity was determined at 30°C after a 2 h-incubation at the appropriate temperature. Activity of the free endo-D-galacturonanase incubated at 30°C was found to be 100·9 μmol min^{-1} mg^{-1}; ○ soluble enzyme; ● immobilized enzyme.

TABLE II

Activity of Endo-D-galacturonanase (free and bound to Spheron 300) on Heptagalacturonic Acid

Enzyme	Activity μmol min^{-1} mg^{-1}	Relative activity %
Soluble	93·99	100·0
Bound at pH 6·5	35·33	37·6
Bound at pH 8·5	14·96	15·9

TABLE III

Kinetic Constants of Endo-D-galacturonanase Free and Bound

Enzyme	$K_m(K_{m,app})$ M . 10^3	V μmol min^{-1} mg^{-1}	V_{rel} %
Soluble	8·24	129·03	100·0
Bound[a]	10·41	27·16	21·05
Bound[b]	10·80	45·50	35·3

[a] Spheron 300, pH 5·5; [b] Spheron 500.

rated from the gel suspension. This liberation amounted after 2 years 6·8% of the original activity with Spheron 500 and 6·5 and 2·8% with Spheron 300 at pH 8·5 and 6·6, respectively. A continual elution of preparation bound to Spheron 500 in a column with 0·5% sodium pectate resulted in an activity decrease by 38% during 21 days.

The effect of immobilization on the reaction kinetics on sodium pectate was investigated with endo-D-galacturonanase bound to Spheron 300 at pH 5·5 and to Spheron 500. Enzyme bound to Spheron obeys the kinetics of Michaelis and Menten. The K_m values slightly increased in both cases, evidently due to a steric effect, whilst V values considerably decreased by 79% in the first and 65% in the second preparation (Table III). Changes of values of kinetic parameters indicate that, in addition to steric effects, also another factor related with the mechanism of the enzyme effect is responsible for the activity decrease.

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