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SELECTIVE PURIFICATION OF *ASPERGILLUS NIGER* ENDOPOLYGALACTURONASE BY AFFINITY CHROMATOGRAPHY ON CROSS-LINKED PECTIC ACID

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SUMMARY

Incubation of a mixture of extracellular pectolytic enzymes of *Aspergillus niger* with insoluble pectic acid cross-linked by epichlorhydrine under optimum conditions for endopolygalacturonase (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) action results in a selective adsorption of endopolygalacturonase by the insoluble support. The quantitative liberation of the enzyme into solution is achieved either in the presence of soluble sodium pectate or at pH values over pH 6. On the basis of these observations a chromatographic method for endopolygalacturonase purification was developed.

INTRODUCTION

Conventional methods for the purification of endopolygalacturonases (poly-α-1,4-D-galacturonide glycanohydrolase, EC 3.2.1.15) of microbial origin such as fractionation with $(NH_4)_2SO_4$ and ethanol, chromatography on ion exchangers such as CM-cellulose[1], DEAE-cellulose[2–4] and phosphocellulose[5,6], and molecular sieve chromatography[7,8] have been used. These procedures mostly enabled the separation of other pectolytic enzymes present in the starting material as well, but from the point of view of endopolygalacturonase purification they were complicated and time consuming.

Chromatographic techniques based on the specific reversible association of the enzyme with insoluble substrate-like compounds or inhibitors have been found useful in the purification of several enzymes. The successful application of this principle in the purification of some glycosidases where an insoluble substrate served as the specific adsorbent[4–9] led us to look for a possibility of using this principle also in the purification of *Aspergillus niger* endopolygalacturonase.

The problem of the purification lies in the separation of endopolygalacturonase from other pectolytic enzymes—endopolymethylgalacturonase, exopolygalacturonase, and pectin (methyl)esterase (pectin pectyl-hydrolase, EC 3.1.1.11) produced by the microorganism as well as from other contaminating substances present in the

starting material. The insoluble adsorbent pectic acid made insoluble by cross-linking with epichlorhydrine was used.

It was found that the incubation of the mixture of pectolytic enzymes produced by *A. niger* with cross-linked pectic acid at the optimum pH of endopolygalacturonase led to selective and tight adsorption of the enzyme to the modified substrate. The enzyme could subsequently be liberated from the insoluble support by elution with a dilute solution of sodium pectate or with a buffer of such a pH region that the endopolygalacturonase was inactive. Based upon these observations a simple procedure has been developed for the isolation of endopolygalacturonase.

MATERIALS AND METHODS

The crude preparation of pectolytic enzymes and endopolygalacturonase was prepared from the culture filtrate of *A. niger* as previously described[3]. The microorganism was grown 10 days on the Czapek–Dox nutrient medium containing apple pectin as the carbon source.

Pectic acid cross-linked by epichlorhydrine was prepared according to the method of TIBENSKÝ AND KUNIAK[15] from citrus pectin (Genu Pectin - Type B, Rapid Set, Københayns Pektinfabrik, Denmark) purified by washing with 60% ethanol containing 5% HCl and then with 60 and 96% ethanol. The purified pectin (65.1% esterified; 90.7% polygalacturonide and molecular weight 86 000) was de-esterified with 0.1 M NaOH at pH 10 and 22°. The product was precipitated with HCl at pH 2.5. The obtained solid preparation of sodium pectate was treated with equimolar quantities (related to galacturonic acid unit molar concentration) of concentrated epichlorhydrine and NaOH (15% solution) for 2 h at a temperature not exceeding 60°. The resulting gel was washed with water, dried and ground to small particles.

Sodium pectate (polygalacturonide content 75.5%, molecular weight 27 000) used as substrate for the determination of polygalacturonase activity was prepared from the apple pectin by repeated alkaline de-esterification with 0.1 M NaOH, as described with citrus pectin.

Pectinic acid (66.4% esterified) used as substrate for the determination of pectin (methyl) esterase activity was prepared by washing the apple pectin with 60% ethanol containing 5% HCl and then with 60 and 96% ethanol.

Pectinic acid (96.8% esterified) used as substrate for the determination of polymethylgalacturonase activity was prepared by esterification of pectic acid with methanolic 1 M H_2SO_4 (ref. 16).

Cellulose acetate strips for electrophoresis were purchased from The Oxoid Division of Oxo Ltd., London.

Determination of enzyme activity

Polygalacturonase activity was determined at 30° by measuring the initial rate of liberation of reducing groups by a spectrophotometric method using SOMOGYI reagent[17]. A 0.5% solution of sodium pectate in 0.1 M acetate buffer was used as substrate. Enzyme activity was determined by means of a standard graph for D-galacturonic acid.

Specific activity is defined in μequiv of reducing groups liberated by 1 mg of

protein per min. Endopolygalacturonase and exopolygalacturonase activity were distinguished by measuring the decrease in viscosity of 0.5% solution of sodium pectate in 0.1 M acetate buffer (pH 4.2) (see ref. 18) as well as on the basis of paper chromatographic analysis of reaction products in the solvent system ethylacetate–acetic acid–water (18:7:8, by vol.).

The polymethylgalacturonase activity was determined by estimating the increase of reducing groups using a photometric method with 3,5-dinitrosalicylic acid[19].

The pectin (methyl)esterase activity was determined by continuous titration of the carboxyl groups, released during the enzyme reaction from pectinic acid (66.4% esterified) with 0.01 M sodium hydroxide[20].

Adsorption of endopolygalacturonase on cross-linked pectic acid

Cross-linked pectic acid in a quantity of 100 mg/mg of the enzyme mixture was added to the solution containing the mixture of pectolytic enzymes in 0.1 M acetate buffer (pH 4.2). The suspension was incubated at room temperature with constant stirring. During the course of incubation aliquots were taken for the assay of polygalacturonase activity. After 15 min incubation the solid fraction was separated by centrifugation and washed several times with acetate buffer (pH 4.2) as long as the supernatant showed enzymatic activity. The activity of the adsorbed endopolygalacturonase was determined by suspending the complex in 0.5% solution of sodium pectate in 0.1 M acetate buffer (pH 4.2) and by measuring the increase of reducing groups as mentioned above.

The effect of pH on the formation of the endopolygalacturonase–cross-linked pectic acid complex

The experiment to determine the adsorption of endopolygalacturonase on cross-linked pectic acid was performed in acetate buffers of different pH values as indicated in Fig. 2.

The effect of pH on the dissociation of the endopolygalacturonase–cross-linked pectic acid complex

The same quantities of the endopolygalacturonase–cross-linked pectic acid complex prepared at pH 4.2 were suspended in acetate buffers of different pH values. After separation of the solid fraction by centrifugation the supernatants were adjusted to pH 4.2, made up to the same volume with 0.1 M acetate buffer (pH 4.2) and assayed for the activity of endopolygalacturonase.

Isolation of endopolygalacturonase by affinity chromatography on a cross-linked pectic acid column

Cross-linked pectic acid was suspended in 0.1 M acetate buffer (pH 4.2) and packed into a column (1.4 cm × 12 cm). 200 mg of the enzyme mixture dissolved in 5 ml of the same buffer were applied. The column was eluted with the equilibrating buffer and fractions of 10 ml per 20 min were collected. Each fraction was analysed for protein by measuring the absorbance at 280 nm. Aliquots of the fractions were analysed for polygalacturonase activity. The elution with the starting buffer was continued as long as the eluate showed adsorbance at 280 nm. The adsorbed protein

fraction was then eluted from the column with 0.1 M acetate buffer (pH 6.0). Both protein fractions were freeze dried, desalted by gel filtration through Sephadex G-25 (medium grade) and again freeze dried.

RESULTS AND DISCUSSION

Pectic acid cross-linked by epichlorhydrine is a slightly swelling, granulous material, insoluble in aqueous solutions and stable in diluted solutions of inorganic acids and bases. The degree of cross-linking has not been defined so far. It is assumed that cross-linking occurs between the hydroxyl groups at C-2 and C-3 of different sugar residues.

Incubation of the *A. niger* pectolytic enzymes mixture containing exopolygalacturonase, endopolygalacturonase, endopolymethylgalacturonase and pectin (methyl)esterase with cross-linked pectic acid under optimum conditions for endopolygalacturonase action results in a decrease in the total polygalacturonase activity in the supernatant fraction. The activity decrease depends on the quantity of the adsorbent present in the mixture, the maximum loss of activity obtained being 88% (Fig. 1). The decrease of the polygalacturonase activity in the supernatant fraction is due to the specific adsorption of endopolygalacturonase to the cross-linked pectic acid. The bound enzyme causes a rapid decrease in the viscosity of the sodium pectate solution with the simultaneous liberation of oligogalacturonides as products of the reaction. It had no effect on pectinic acid when the degree of esterification was 96.8%.

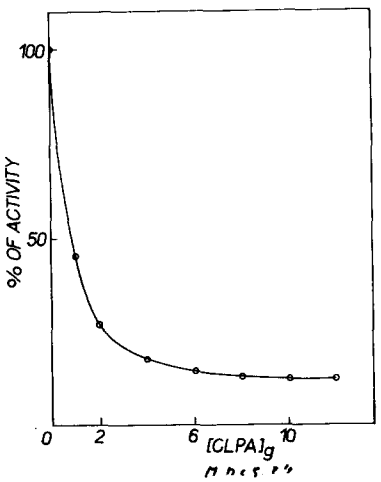

Fig. 1. Effect of adsorbent concentration on the polygalacturonase activity in the unadsorbed fraction. Series of 100-ml samples containing 100 mg crude enzyme preparation and increasing amounts of cross-linked pectic acid (CLPA) were incubated for 15 min at 23°. Polygalacturonase activity was determined in the supernatant after separation of the solid fraction.

The residual polygalacturonase activity in the supernatant fraction corresponds to exopolygalacturonase. The incubation of an aliquot of the supernatant with sodium pectate led to the liberation of D-galacturonic acid as the only product of the

reaction. Activity of pectin (methyl)esterase and endopolymethylgalacturonase was found in the supernatant fraction as well. Thus it appears that cross-linked pectic acid is specific for the adsorption of endopolygalacturonase.

In the course of the first 20–30 min incubation of pectolytic enzymes with cross-linked pectic acid, D-galacturonic acid was liberated into the supernatant. This is most probably due to exopolygalacturonase action on the periphery areas of the cross-linked pectic acid molecule. This slight degradation of the adsorbent did not have any effect on the insolubility or on the adsorption capacity of cross-linked pectic acid.

Endopolygalacturonase tightly bound to cross-linked pectic acid could not be liberated even by an excessive quantity of the incubation buffer. The liberation of the enzyme was achieved in the presence of a 0.5% solution of sodium pectate in acetate buffer (pH 4.2). Endopolygalacturonase liberated into the solution degraded the soluble substrate and after its complete degradation the enzyme could be separated from oligogalacturonides by dextran gel chromatography on Sephadex G-25 (fine grade) with 0.05 M phosphate buffer (pH 7.0) used as eluent[21]. This method of enzyme isolation from the complex is disadvantageous because of the relatively complicated separation of oligogalacturonides from the enzyme. The liberation of endopolygalacturonase from the complex in the presence of the soluble substrate, however, enables the direct determination of the activity of the bound enzyme.

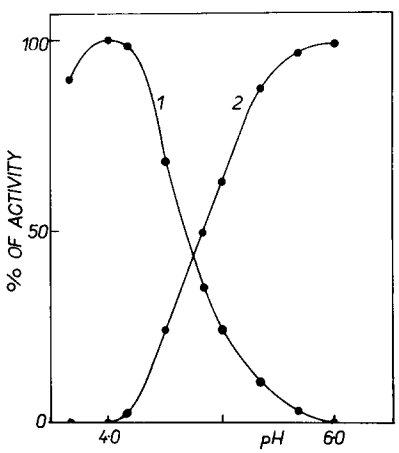

Fig. 2. Effect of pH on the formation and dissociation of the endopolygalacturonase–cross-linked pectic acid complex. Curve 1 indicates the percentage activity of bound endopolygalacturonase. Pectolytic enzymes were incubated with cross-linked pectic acid for 15 min at 23° in buffers of different pH values. Polygalacturonase activity of the solid fraction was measured after its separation by centrifugation and several washings with 0.1 M acetate buffer (pH 4.2). Curve 2 indicates the percentage activity of endopolygalacturonsae liberated from the complex at different pH values. Equal quantities of the complex were formed at pH 4.2 and were suspended in 0.1 M acetate buffer at different pHs. Polygalacturonase activity of supernatants was adjusted to pH 4.2 and the constant volume was determined.

As shown in Fig. 2 the binding of endopolygalacturonase to cross-linked pectic acid (Curve 1) as well as the dissociation of the complex (Curve 2) are effected by pH. Maximum adsorption was achieved at pH 4.0–4.2; at this pH endopolygalacturonase

is tightly bound to cross-linked pectic acid. At pH 6.0 the complex formation did not take place. The enzyme bound to the insoluble support under the optimum conditions was completely liberated at pH 6.0. This method of endopolygalacturonase liberation was used in the isolation of endopolygalacturonase from the column of cross-linked pectic acid. As the effect of pH on the complex formation shows the same profile with maximum at pH 4.0–4.2, as was seen with the pH effect on endopolygalacturonase activity[22], it can be supposed that similar groups are involved in the interaction of the enzyme with substrate and with cross-linked pectic acid.

Isolation of endopolygalacturonase by column chromatography on cross-linked pectic acid

The results obtained with the isolation of endopolygalacturonase by chromatography on the column of cross-linked pectic acid are shown in Fig. 3. About 90% of the protein and approximately 10% of polygalacturonase activity was eluted in

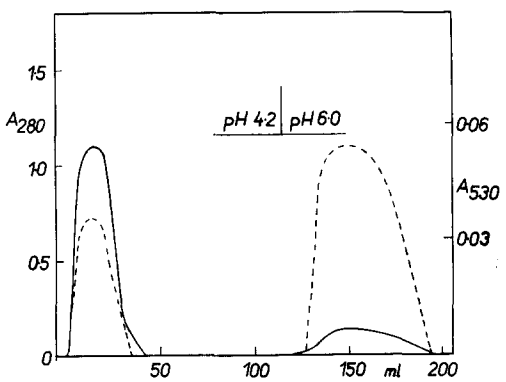

Fig. 3. Isolation of endopolygalacturonase on a column of cross-linked pectic acid. The column (1.4 cm × 12 cm) was equilibrated with 0.1 M acetate buffer (pH 4.2). The first peak was eluted with the equilibrating buffer. Endopolygalacturonase was eluted with 0.1 M acetate buffer (pH 6.0). – – –, polygalacturonase activity of 1 ml of eluate per 1 min determined by Somogyi reagent. The activity is expressed as change in absorbance at 530 nm. ———, Absorbance at 280 nm.

TABLE I

SUMMARY OF PURIFICATION OF ENDOPOLYGALACTURONASE BY AFFINITY CHROMATOGRAPHY ON CROSS-LINKED PECTIC ACID

Polygalacturonase activity was determined at 30° by measuring the initial rate of liberation of reducing groups by a spectrophotometric method using Somogyi reagent. The specific activity is defined in μequiv of reducing groups liberated per mg of protein per min. Simultaneously the endopolygalacturonase activity was characterized by measuring the time necessary for a 50% decrease in viscosity of 20 ml of 0.5% solution of sodium pectate in 0.1 M acetate buffer (pH 4.2) caused by 1 mg of protein at 30°.

Procedure	*Protein (%)*	*Specific activity of polygalacturonase (μequiv reducing groups)*	*Viscosity decrease* t_{min}
Initial culture filtrate	13.0	0.92	19.7
Crude preparation	37.5	2.40	13.5
Adsorption on cross-linked pectic acid	70.3	12.80	3.1
Unadsorbed fraction	11.6	0.60	76.5

the exclusion volume of the column with the starting buffer solution. All of the endopolygalacturonase activity was retained on the column and was readily eluted with acetate buffer (pH 6.0). The first peak did not have any detectable endopolygalacturonase activity. All polygalacturonase activity recorded corresponded to that of exopolygalacturonase.

The results of the purification are summarized in Table I. Specific activity of the purified endopolygalacturonase at pH 4.2 and 30° was found to be 12.8 μequiv of reducing groups. Specific activity of endopolygalacturonase purified nonspecifically[3] determined by the Somogyi method is 14.2 μequiv of reducing groups.

The purified endopolygalacturonase showed a single zone in electrophoresis on acetate cellulose strips in veronal buffer (pH 9.6) as well as in phosphate buffer (pH 5.4).

The described method was used with success also in the purification of endopolygalacturonase from commercially prepared Pectinase (Fy. Koch-Light Laboratories Ltd.). The possibility of the liberation of the adsorbed enzyme in the presence of soluble substrate enables the complex to be used for the specific degradation of pectic acid directly without further liberation of the enzyme.

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