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ON THE CHARACTER OF THE INTERACTION OF ENDOPOLYGALACTURONASE WITH CROSS-LINKED PECTIC ACID

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SUMMARY

The combination of cross-linked pectic acid with endopolygalacturonase (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) and its effect on the kinetics of action on sodium pectate as well as on the thermal stability of the enzyme was studied.

It was shown that endopolygalacturonase is competitively inhibited by cross-linked pectic acid. The value of the inhibitory constant K_i obtained (3.24 mg of cross-linked pectic acid per ml) was found to be very close to the value of the association constant of the complex of endopolygalacturonase with cross-linked pectic acid formed in the absence of the substrate (3.47 mg of cross-linked pectic acid per ml). The character of the inhibition as well as the similarity of the constant values indicate that the process of the complex formation is due exclusively to a functional affinity of the binding site of endopolygalacturonase to the adsorbent.

The binding of endopolygalacturonase to cross-linked pectic acid considerably increases the thermal stability of the enzyme.

INTRODUCTION

An incubation of the mixture of *Aspergillus niger* extracellular pectolytic enzymes with pectic acid cross-linked by epichlorhydrine at the pH optimum of endopolygalacturonase (poly α-1,4-D-galacturonide glycanohydrolase, EC 3.2.1.15) results in the selective adsorption of endopolygalacturonase on the insoluble adsorbent. On the basis of this phenomenon a new procedure for the purification of endopolygalacturonase has been elaborated[1]. It was of interest to investigate the character of the interaction and the principle of the selectivity of the enzyme binding.

The identity of the pH effect on the endopolygalacturonase–cross-linked pectic acid complex formation and on the activity of endopolygalacturonase indicated that similar groups take part in the enzyme–substrate and enzyme-modified substrate interaction[1]. It has been further shown that the enzyme bound to the insoluble modified substrate may be liberated in the presence of the soluble substrate, *e.g.* sodium pectate. This result can be interpreted in the light of the competition of the substrate

and cross-linked pectic acid for endopolygalacturonase as the result of the active site participation in the complex formation.

Such consideration led us to the study of the combination of cross-linked pectic acid and endopolygalacturonase and of the action kinetics of endopolygalacturonase in the presence of the adsorbent in order to characterize the factors which play a role in the interaction of the enzyme with cross-linked pectic acid.

MATERIALS AND METHODS

Endopolygalacturonase was prepared from the filtrate of *A. niger* culture growing 10 days on Czapek-Dox nutrient medium containing apple pectin as the carbon source by the procedure described elsewhere[2].

Pectic acid cross-linked by epichlorhydrine was prepared from the purified sodium pectate according to the method of Tibenský and Kuniak[3]. The procedure is described in the previous paper[1].

Sodium pectate (content of polygalacturonide 75.5%, average molecular weight determined viscosimetrically, 27 000) used as substrate for endopolygalacturonase activity determination was prepared from Czechoslovak commercial apple pectin by repeated alkaline deesterification[1].

Determination of enzyme activity

Activity of endopolygalacturonase was determined at 30 °C with a 0.5% solution of sodium pectate in 0.1 M acetate buffer (pH 4.2) used as substrate. The initial rate of reducing group liberation was measured spectrophotometrically using the Somogyi reagent[4]. The absorbance changes recorded at 530 nm were interpreted in terms of reducing sugar 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 1 min.

Kinetics of endopolygalacturonase action in the presence of cross-linked pectic acid

Activity of endopolygalacturonase was measured at various concentrations of sodium pectate and at a constant concentration of cross-linked pectic acid. Typically 0.2 mg sample of the enzyme was added to 5 ml of 0.1 M acetate buffer (pH 4.2) containing an appropriate concentration of the substrate and cross-linked pectic acid. The mixture was incubated at 23 °C for 20 min under continual stirring. In 5-min intervals 1-ml aliquots of the supernatant were taken for the assay of the reducing groups. The activity was measured in two series of experiments at concentrations of cross-linked pectic acid of 30 mg/ml and alternatively of 45 mg/ml. The concentration of the substrate varied from 1–10 mg/ml. Simultaneously the activity of endopolygalacturonase in the absence of the cross-linked pectic acid was determined. The data were treated according to the method of Lineweaver and Burk[5] and the apparent Michaelis constant and inhibitory constant were determined.

Combination of endopolygalacturonase with cross-linked pectic acid

Series of 5 ml samples containing 5 mg of endopolygalacturonase in 0.1 M acetate buffer (pH 4.2) were treated with cross-linked pectic acid at 23 °C under continual stirring. The quantity of the adsorbent varied from 5 to 50 mg/ml. After 15

min the solid fraction was separated by centrifugation and the endopolygalacturonase activity in the supernatant was determined. The difference between this and the activity of the enzyme before the addition of cross-linked pectic acid gave the activity of the enzyme bound.

Thermal stability of the bound endopolygalacturonase

The complex of 0.2 mg of endopolygalacturonase with 10 mg of cross-linked pectic acid in 5 ml of acetate buffer (pH 4.2) was maintained at 30 ± 0.2 °C and its activity after 2 and 4 h incubation was determined. Control experiments were performed using free endopolygalacturonase in the same buffer. The percentage of the original activity remaining after the incubation was calculated. The experiment was repeated at 40, 50 and 60 °C over similar heating periods. The activity of the adsorbed endopolygalacturonase was determined by the standard assay after suspending the complex separated from the suspension by centrifugation in 5 ml of 0.5% solution of sodium pectate in 0.1 M acetate buffer (pH 4.2).

RESULTS

The results of the study of the kinetics of endopolygalacturonase action on pectic acid in the presence and in the absence of cross-linked pectic acid are shown in Fig. 1. The double reciprocal plot represents the data obtained at a concentration of cross-linked pectic acid of 30 mg/ml (line 1), of 45 mg/ml (line 2) and in the absence of cross-linked pectic acid (line 3). The data indicate that endopolygalacturonase is competitively inhibited by cross-linked pectic acid. The fraction of activity inhibited is dependent on substrate concentration and the reciprocal plot of initial velocity against substrate concentration in the range where was no inhibition has in all cases a common intercept on the vertical axis. The K_m of enzyme–substrate complex derived from this plot equals 1.25 mg of pectic acid per ml. The dissociation constant K_i of the complex endopolygalacturonase–cross-linked pectic acid equals

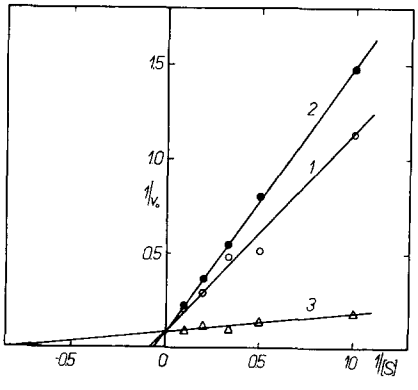

Fig. 1. A double-reciprocal plot of initial activity of endopolygalacturonase against substrate concentration measured in the presence of cross-linked pectic acid of 30 mg/ml (line 1), 45 mg/ml (line 2) and in the absence of cross-linked pectic acid (line 3). The reaction mixtures containing 0.2 mg of endopolygalacturonase and appropriate concentrations of the substrate and of cross-linked pectic acid were incubated at 23 °C. The concentration of the substrate varied from 1 to 10 mg/ml.

3.24 mg of cross-linked pectic acid per ml. K_i as well as K_m are expressed in mg of the substrate or inhibitor per ml because the cross-linked pectic acid is not sufficiently characterized.

The combination of endopolygalacturonase with cross-linked pectic acid was measured at increasing concentrations of the adsorbent. The experiments were effected under conditions which approximated those of kinetic measurements. The quantity of the enzyme bound is expressed by its initial activity on sodium pectate. The results are shown in Table I. Formation of the cross-linked pectic acid–endopolygalacturonase complex yielded hyperbolic plot of the quantity of the enzyme bound versus cross-linked pectic acid concentration identical with the Langmuir adsorption isotherm (Fig. 2A). The data were treated according to the method of

TABLE I

EFFECT OF CROSS-LINKED PECTIC ACID CONCENTRATION ON THE EXTENT OF ENDOPOLYGALACTURONASE BINDING

5-ml samples containing 5 mg of endopolygalacturonase in 0.1 M acetate buffer (pH 4.2) were treated with increasing quantity of cross-linked pectic acid at 23 °C. After 15 min the solid fraction was separated by centrifugation and the activity in the supernatant was determined. The activity of the enzyme bound was calculated from the difference between the activity of the enzyme originaly used and that of the enzyme unadsorbed.

Concentration of the adsorbent (mg/ml)	*Activity of unadsorbed enzyme (μequiv/mg per min)*	*Activity of adsorbed enzyme (μequiv/mg per min)*
0	11.2	—
5	4.5	6.7
10	2.9	8.3
20	1.7	9.5
30	1.1	10.1
40	0.9	10.3
50	0.7	10.5

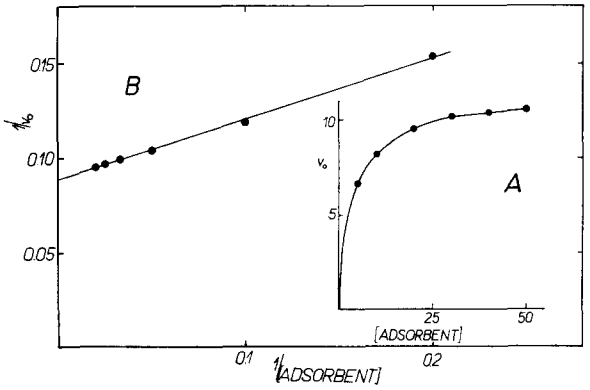

Fig. 2. A. Effect of the adsorbent concentration on the quantity of the adsorbed enzyme. B. Double-reciprocal plot of the same data. 5 mg of endopolygalacturonase in 5 ml of 0.1 M acetate buffer (pH 4.2) were treated with various quantities of cross-linked pectic acid at 23 °C. After 15 min incubation endopolygalacturonase activity in the supernatant fraction was measured. The difference between this and the activity of the enzyme in the absence of cross-linked pectic acid gave the activity of the enzyme bound. v_0 = activity corresponding to the enzyme bound. Concentration of adsorbent in mg/ml.

Lineweaver and Burk[5] on the basis of observations of Hitchcock[6], *i.e.* by plotting the reciprocal of the initial velocity of the protein bound ($1/v_0$) against the reciprocal of the cross-linked pectic acid concentration as shown in Fig. 2B. The intercept of the straight line at the ordinate is equal to the reciprocal of the maximum of the enzyme bound and the slope is equal to $k_{maximum}$ of the enzyme bound. The association constant calculated from this data was found to be 3.47 mg of cross-linked pectic acid per ml.

Thermal stability of bound endopolygalacturonase

In Table II the percentage of the original activity of the free and bound enzyme after 2 and 4 h incubation at a temperature of 30–60 °C is shown. The binding of the enzyme to cross-linked pectic acid considerably increases its thermal stability. During storage at 40 °C the activity of soluble endopolygalacturonase slightly decreased while

TABLE II

THE HEAT INACTIVATION OF SOLUBLE AND BOUND ENDOPOLYGALACTURONASE

0.2 mg of endopolygalacturonase with 10 mg of cross-linked pectic acid in 5 ml of 0.1 M acetate buffer (pH 4.2) were maintained at constant temperature and its activity after 2 and 4 h incubation was determined. The same experiments were performed with free endopolygalacturonase. The percentage of the original activity remaining after the incubation was calculated.

Temp. (°C)	*% of original activity*			
	Soluble enzyme		*Bound enzyme*	
	2 h	*4 h*	*2 h*	*4 h*
30	100	100	100	100
40	87	68	100	100
50	0	0	95	88
60	0	0	67	32

the activity of the bound enzyme remained unchanged. After 2 h heating at 50 °C the soluble enzyme was completely inactivated while the bound enzyme under the same conditions preserved 88% of the original activity. At 60 °C after 2 h heating 67% and after 4 h heating 32% of the original activity were found in the complexed enzyme.

DISCUSSION

Cross-linking of pectic acid by epichlorhydrine results in the production of an insoluble substance selectively and reversibly adsorbing endopolygalacturonase unless attacked by the enzyme. It has been demonstrated[1] that the complex of endopolygalacturonase with cross-linked pectic acid is in equilibrium with the free enzyme and adsorbent as the addition of the competing substrate to the complex resulted in the release of a large part of the enzyme from the complex. This fact indicates that the active-site directed mechanism is involved in the selective binding of the enzyme.

The character of the inhibitory effect of cross-linked pectic acid on the activity of endopolygalacturonase indicates too that the process of the adsorption of the en-

zyme is due to a functional affinity of the binding site of endopolygalacturonase to the specific adsorbent.

It was of interest whether only this form of the interaction is the factor which plays a role in the formation of the complex. Since cross-linked pectic acid contains charged groups an ion-exchange effect could be included in the interaction as well. We therefore studied the combination of cross-linked pectic acid and endopolygalacturonase in order to determine how the association constant k of the complex obtained would compare with equilibrium constant K_i obtained by kinetic methods. As seen, the value of the association constant obtained is very close to the value of K_i, *i.e.* the concentration of cross-linked pectic acid necessary for half maximum binding and inhibition is the same. This result indicates that endopolygalacturonase is bound to cross-linked pectic acid exclusively by the binding site.

The binding of endopolygalacturonase to cross-linked pectic acid results in the preservation of the tertiary structure of the enzyme especially of its active site against thermal inactivation.

The character of the interaction of endopolygalacturonase with cross-linked pectic acid gives an opportunity to use it for studying the character of the binding site of endopolygalacturonase as well as for the selective purification of the enzyme.

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