SEPARATION OF PECTIC ENZYMES FROM TOMATOES BY AFFINITY CHROMATOGRAPHY ON CROSS-LINKED PECTIC ACID

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Tomato D-galacturonanase was separated from pectinesterase, which is present both in the crude product and also in the partly purified preparation of endo-D-galacturonanase by affinity chromatography on pectic acid cross-linked by epichlorohydrine. Both enzymes were adsorbed to a column and then stepwise eluted by buffers of increasing pH. All five molecular forms of pectinesterase were eluted at pH 4·5—5·2. Starting from pH 5·2, three fractions showing D-galacturonanase activity and corresponding to three different molecular forms of the enzyme were eluted in the order of their increasing basicity. The effect of pH on the binding and displacement of enzymes indicates that in the interaction of D-galacturonanase with cross-linked pectic acid a role plays not only the active center of the enzyme but also an ion-exchange mechanism; the latter mechanism only is responsible for the binding of pectinesterase.

The common separation techniques, such as ion-exchange chromatography and chromatography based on molecular sieving effects, which have been used in various combinations for the separation of pectic enzymes from tomatoes[1–5], only rarely permit a complete separation of pectinesterase (pectin pectyl hydrolase, E. C. 3.1.1.11) from *endo*-D-galacturonanase* (poly-α-1,4-D-galacturonide glycanohydrolase, E.C. 3.2.1.15). High purification degrees obtained in multi-step processes were paralleled by extremely low yields[8].

Rexová-Benková and Tibenský[9] used affinity chromatography on pectic acid cross-linked by epichlorohydrine, a specific, insoluble adsorbent, to selectively separate *endo*-D-galacturonanase from a mixture of extracellular pectic enzymes of *Aspergillus niger*. The enzyme was first selectively bound to the cross-linked substrate at pH 4·2 (pH-optimum of *endo*-D-galacturonanase) and then eluted at pH 6·0. Foglietti and coworkers[10] have been able to purifity D-galacturonanase present in the digestive juice of *Helix pomatia* by the same method.

The aim of this study was to check on the use of affinity chromatography on cross-linked pectic acid also for the separation of pectic enzymes from tomatoes. The method was tested both with a crude product and also with a partly purified preparation of *endo*-D-galacturonanase. It was obvious from the preliminary results that the experimental conditions used for the separation of microbial pectic enzymes cannot

* So far called endopolygalacturonase. The new name[6] has been proposed by Prof. D. Horton[7] with respect to the nomenclature of the substrate preferred.

be used for pectic enzymes from tomatoes. Since pectic enzymes from tomatoes are basic proteins[3,11], their contact with cross-linked pectic acid can involve besides the interaction of the enzyme with the cross-linked substrate also an ion-exchange effect. Therefore the main difference between the two separation procedures was that the ion-exchange effect was suppressed by an increase of the ionic strength of the equilibrating buffer and of the eluting buffers; the stepwise pH-gradient of eluting buffers was replaced by a linear pH-gradient.

EXPERIMENTAL

Material

The crude preparation of pectic enzymes (M-2) was obtained by extraction of the tomato homogenate with 5% solution of sodium chloride, fractionation of the proteins with ammonium sulfate (saturation degree 0·2 and 0·9), dialysis, and desalting on Sephadex G-25 Medium as reported earlier[2]. Partly purified *endo*-D-galacturonanase M-3/II was prepared by chromatography of the crude product on a column of DEAE-Sephadex A-50 and gel chromatography on Sephadex G-75 (ref.[2]). Fractions corresponding to the second peak and containing the chief amount of *endo*-D-galacturonanase were lyophilized, desalted on Sephadex G-25 and again lyophilized.

Sodium pectate (D-galacturonan content 95·5%, average molecular weight determined viscosimetrically 27000), used as substrate for the determination of D-galacturonanase activity, was prepared from apple pectin by repeated alkaline deesterification in 0·1M-NaOH and precipitation at pH 2·5.

Sodium pectinate (esterified to 65·1%), used as substrate for the determination of pectinesterase activity, was prepared by washing citrus pectin with 60% ethyl alcohol containing 5% of HCl, and then with 60 and 90% ethyl alcohol.

Pectic acid cross-linked by epichlorohydrine was prepared according to Tibenský and Kuniak[12] from citrus pectin (Genu Pectin, Type B, Rapid Set, Københavns Pektinfabrik, Denmark) as described elsewhere[9]. The preparation contained one cross link per 3·4 units of D-galactopyranuronic acid.

Determination of Enzymic Activity

The activity of *endo*-D-galacturonanase was determined in terms of initial rate of liberation of reducing groups by spectrophotometry using the Somogyi reagent[13]. A 0·5% solution of sodium pectate in 0·1M acetate buffer, pH 4·4, containing 0·2M sodium chloride was used as substrate. The specific activity was expressed in μmol of reducing saccharides liberated by 1 mg of protein in 1 min. D-Galactopyranuronic acid was used as a standard. The protein content was determined by the method of Lowry and coworkers[14].

Pectinesterase activity was determined by continuous titration in an automatic titrator (TTT 11, Radiometer, Copenhagen, Denmark), in a thermostatized vessel under a nitrogen barrier. Pectinesterase activity is expressed in μmol of ester groups hydrolyzed in 1 min at pH 7·5 and 30°C.

Starch Gel Electrophoresis

Starch gel electrophoresis was effected in the horizontal arrangement[15]; hydrolyzed cross-linked starch[16] was used. The electrode vessels were filled with 0·3M Tris-HCl buffer, pH 7·5, and the

same buffer (molarity 0·03) was used for the preparation of the gel. The electrophoresis was run at 4°C and at a potential gradient of 3 V/cm. After 18 h of the run the gel was sliced horizontally, one layer was used for the detection of pectinesterase activity and proteins. Pectinesterase was detected by the paper replica technique using Whatman No 1 paper impregnated with 1% pectin solution in 0·1M sodium chloride adjusted to pH 7·5 and by 0·2% alkaline solution of bromothymol blue. Yellow spots on a blue background appeared at sites where pectinesterase was present. The gel was subsequently used for protein staining with amido black 10 B. The other part of the gel was dipped in 0·05M acetic acid for 15 min and D-galacturonanase was detected by the paper replica technique: Whatman No 1 paper impregnated with 1% sodium pectate solution in 0·1M acetate buffer at pH 4·5, containing 0·2M sodium chloride, was placed on the gel. The replica was dried and subsequently dipped in a ruthenium red solution (50 mg/1 000 ml) overnight and then washed several times with water. White spots on a red background indicated the location of D-galacturonanase activity.

Affinity Chromatography on Cross-Linked Pectic Acid

The crude product (1 g) of pectic enzymes from tomatoes was applied to a column (3·4 × 10 cm) of cross-linked pectic acid equilibrated with 0·05M sodium chloride solution in 0·1M acetate buffer, pH 4·2. The column was eluted first by the equilibrating buffer until the fraction which had not been adsorbed was eluted. The pH of the eluting buffers was increased linearly up to 6·9. The separation was monitored by absorbance measurement at 280 nm (Fig. 1). Aliquots taken from 14 ml fractions collected at 30 min intervals served for the determination of D-galacturonanase and pectinesterase activity. Fractions showing identical activity were pooled, lyophilized, desalted by gel filtration on Sephadex G-25 and again lyophilized. Identical experimental conditions were used for partly purified endo-D-galacturonanase (Fig. 2).

RESULTS

The fractionation of the crude enzyme preparation on cross-linked pectic acid and the distribution of enzymic activities are shown in Fig. 1 and 3*a* and in Table I. The enzyme preparation designated M-2, which was applied to the column, contained 46000 units of pectinesterase, corresponding to its five molecular forms, and 700 units of D-galacturonanase, present as three molecular forms. Colored components and enzymically inactive proteins emerged from the column in the hold-up volume of the first buffer. All basic proteins including D-galacturonanase remained adsorbed to the column and were eluted by buffers of increasing pH-value. All five molecular forms of pectinesterase were eluted as one peak (*I*) in the pH-range 4·5 – 5·2. D-Galacturonanase remained firmly attached at this pH. Starting from pH 5·2, three fractions of D-galacturonanase were eluted in the order of increasing basicity; these fractions corresponded to the three molecular forms present in the starting material. The first fraction was contaminated with traces of the most basic form of pectin esterase. The last, major fraction of D-galacturonanase emerged at pH 5·5. As obvious from Table I, the individual molecular forms corresponded to 4·1, 2·4, and 80% of the original 86·5% recovery of D-galacturonanase. The recovery of pectinesterase activity was 90%.

The fractionation of partly purified *endo*-D-galacturonanase (M-3/II) prepared by gel filtration of the crude extract on Sephadex G-75, is shown in Fig. 2 and 3*b*. The preparation whose D-galacturonanase activity was seven times higher con-

TABLE I

Distribution of Enzyme Activities after Separation of Crude Preparation of Pectic Enzymes from Tomatoes by Affinity Chromatography on Cross-linked Pectic Acid

Product	Pectinesterase		D-Galacturonanase	
	units	total activity %	units	total activity %
M-2	46 000	100	700	100
I	39 400	86·3	0	0
II	1 620	3·5	28·8	4·1
III	0	0	17·0	2·4
IV	0	0	560·0	80·0
Yield	41 020	89·9	605·8	86·6

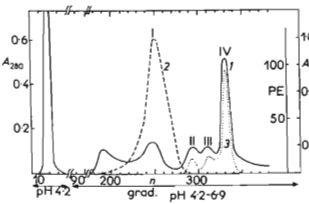

FIG. 1

Fractionation of Crude Preparation of Pectic Enzymes from Tomatoes on Column of Cross-linked Pectic Acid

1 A_{280}; 2 activity of pectinesterase (PE) expressed in μmol of ester groups hydrolyzed in 1 min at pH 7·5; *3* activity of D-galacturonanase expressed in A_{530}. *n* number of fractions. I—IV enzyme fractions. The conditions of the separation are given in the text.

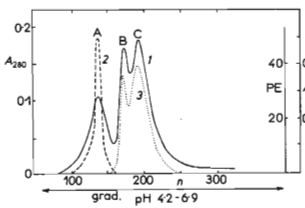

FIG. 2

Fractionation of Partly Purified *endo*-D-Galacturonanase on Column of Cross-linked Pectic Acid

1 A_{280}; 2 activity of pectinesterase (PE) expressed in μmol of ester groups hydrolyzed in 1 min at pH 7·5; *3* activity of D-galacturonanase expressed in A_{530}. *n* number of fractions. A—C enzyme fractions. The conditions of the separation are given in the text.

tained basic proteins only, including two forms of D-galacturonanase and one form of pectinesterase. The two other forms of pectinesterase and the third form of D-galacturonanase were present as traces only. The chromatography was carried out under conditions described for the preceding case. All proteins were adsorbed to the column. The separation of pectinesterase from the two main forms of D-galacturonanase was again effected by a linear elution gradient.

DISCUSSION

The course of the fractionation of the crude enzyme preparation from tomatoes on cross-linked pectic acid essentially differs from the elution profile obtained with the product of *A. niger*[9]. The chief cause of the differences in the behaviour of these products is their different net charge. Cross-linked pectic acid contains ionizable groups and therefore an ion-exchange effect may also play a role in the separation process, in addition to the mechanism controlled by the active center which is responsible for selective binding of the enzyme.

The pectic enzymes and the remaining components present in the product of *A. niger* are acidic proteins (p*I* of *endo*-D-galacturonanase ~5·1, ref.[17]) which are bound by anion exchangers[18]; when they come in contact with cross-linked pectic acid, *endo*-D-galacturonanase only is bound to the cross-linked substrate exclusively

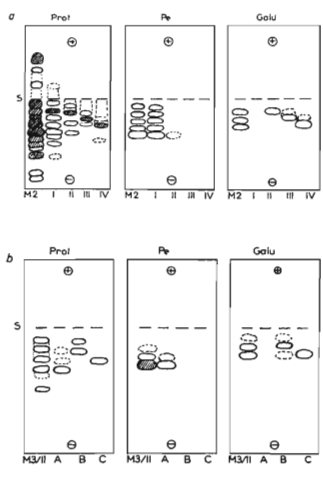

FIG. 3

Starch Gel Electrophoresis

a Crude enzyme preparation (M2) and fractions I-IV obtained by fractionation on cross-linked pectic acid (Fig. 1) *b* partly purified *endo*-D-galacturonanase (M-3/II) and fractions A–C obtained by its fractionation on cross-linked pectic acid (Fig. 2). Detection of proteins (Prot), pectinesterase (Pe), and D-galacturonanase (Galu). The electrophoresis was carried out in Tris-HCl buffer, pH 7·5 at 4°C and at a potential gradient of 3 V/cm.

according to the functional affinity of its binding site[19]. The remaining pectic enzymes as well as contaminants do not interact with the support under the conditions used.

The acidic components of the enzyme preparation from tomatoes are likewise not adsorbed to the column. Since the pH of activity of tomato D-galacturonanase as well as the pH of formation and decomposition of its complex with cross-linked pectic acid are the same, it is probable that similarly to *endo*-D-galacturonanase from *A. niger*[19] identical groups of the enzyme are responsible for the interaction with the native and the cross-linked substrate. The liberation of the individual molecular forms from the complex in the order of their increasing basicity indicates that an ion--exchange effect simultaneously participates on this process. By contrast pectinesterase is bound to the column and liberated (even its forms which are more basic than D-galacturonanase) at a pH which is considerably lower than its pH-optimum[2] and lower than the pH at which D-galacturonanase is displaced; this shows that it is only the ion-exchange effect and not the biospecific interaction which is responsible for the adsorption of pectinesterase to the column of cross-linked pectic acid.

The combination of these two binding principles permits us to separate not only D-galacturonanase from pectinesterase but also the three molecular forms of D-galacturonanase from each other; this is the main advantage of the method described.

REFERENCES

1. Lee M., Macmillan J. D.: Biochemistry *7*, 4005 (1968).
2. Markovič O., Slezárik A.: This Journal *34*, 3820 (1969).
3. Delincée H., Radola B. J.: Biochim. Biophys. Acta *214*, 178 (1970).
4. Nakagawa H., Yanagawa Y., Takehana H.: Agr. Biol. Chem. *34*, 991 (1970).
5. Pressey R., Avants J. K.: Biochem. Biophys. Acta *309*, 363 (1973).
6. Rexová-Benková Ľ., Markovič O.: Advan. Carbohyd. Chem. Biochem. *33*, 323 (1976).
7. Horton D.: Private communication.
8. Markovič O., Slezárik A.: This Journal, *42*, 173 (1977).
9. Rexová-Benková Ľ., Tibenský V.: Biochem. Biophys. Acta *268*, 187 (1972).
10. Foglietti M. J., Girerd H., Percheron F.: Biochimie *57*, 667 (1975).
11. Markovič O.: This Journal *39*, 908 (1974).
12. Tibenský V., Kuniak Ľ.: Czech. 140713/70.
13. Somogyi M.: J. Biol. Chem. *195*, 19 (1952).
14. Lowry O. H., Rosebrough M. J., Farr N. J., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
15. Smithies O.: Biochem. J. *61*, 629 (1955).
16. Markovič O., Kuniak Ľ.: J. Chromatogr. *91*, 873 (1974).
17. Rexová-Benková Ľ.: This Journal *33*, 1965 (1967).
18. Rexová-Benková Ľ., Slezárik A.: This Journal *31*, 122 (1966).
19. Rexová-Benková Ľ.: Biochim. Biophys. Acta *276*, 215 (1972).

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