TOMATO PECTIN ESTERASE — CHARACTERIZATION OF ONE OF ITS MULTIPLE FORMS

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Pectin esterase isolated in an earlier study from tomatoes was found to represent one of the multiple forms of the enzyme, whose quantity is dominant. It was subjected to additional characterization in this study. The lipid components were separated from the multiple forms of this enzyme and the form isolated was subjected to an analysis of fatty acid content. From these experiments the possibility could be excluded that this form of tomato pectin esterase has a lipoprotein character. Attempts to confirm the assumed glycoprotein character of this form of pectin esterase were unsuccessful because the glycidic, arabinose-containing component could be separated by desalting of the final product. The results of sedimentation analysis permitted the earlier data on the sedimentation coefficient of this enzyme to be corrected.

Endo[1] has described the multiple forms of pectin esterase (pectin pectyl hydrolase, EC 3. 1. 1. 11) isolated from an extract of the culture medium of *Coniothyrium diplodiella*, and Hultin[2–4] has obtained three fractions of this enzyme from banana.

In our preceding paper[5], we have pointed at the possible existence of isozymes of tomato pectin esterase; later, we reported[6] on the resolution of five zones showing pectin esterase activity by starch-gel electrophoresis of tomato extracts. Pressey and Avants[7] were recently able to identify four forms of pectin esterase in tomato extracts by chromatography on DEAE-Sephadex A 50. Lee and Macmillan[8] have made a preliminary conclusion that tomato pectin esterase may have a lipoprotein character whereas Delincée and Radola[9] have considered the possibility of the glycoprotein nature of this enzyme.

This paper describes the use of starch-gel electrophoresis with simultaneous detection of enzymatic activity and the staining of the gel for proteins and lipids. Tomato pectin esterase, which had been isolated as described in our preceding paper[5], was found to be identical with one of the multiple forms of this enzyme. This form was used by us to prove the assumed lipoprotein or glycoprotein character of the enzyme and to verify the data of sedimentation analysis.

EXPERIMENTAL

Enzyme preparation and assay. Pectin esterase was prepared from ripe tomatoes (*Lycopersicum esculentum*, var. *Immuna*) as described earlier[5]. Purified citrus pectin (Type B, Rapid Set, Københayns Pektifabrik, Denmark), degree of esterification 65·1%, was used as substrate. Pectin esterase activity was determined by the modified titration method[5] in an automatic titrator (Type SRB 2c/ABU 1c/PHM 28/TTT 11, Radiometer, Copenhagen). One enzyme unit was defined as 1 μequivalent of ester hydrolyzed in 1 min at pH 7·0 and 30°C.

Starch-gel electrophoresis was carried out in the horizontal arrangement at 4°C for 18 h at a potential gradient of 3 V/cm, in 0·3M-Tris-HCl buffer at pH 7·5. The gel was prepared from 11·5 g of hydrolyzed starch[10] and 100 ml of 0·03M-Tris-HCl buffer at pH 7·5. One gel layer cut off horizontally was used for the detection of pectin esterase by the printing technique[9]. The inner part of the gel was covered by Whatman No 1 paper, first impregnated with 1% pectin solution in 0·1M-NaCl adjusted to pH 7, then dried and dipped in an alkaline solution of bromothymol blue. Yellow spots on a blue background appeared at sites where pectin esterase was present. The spots were either photographed or marked when the color intensity was maximal and before the spots became diffuse. After the print had been taken, proteins were stained in the same part of the gel with nigrosine or amido black 10 B. The second part of the gel was used for the staining of lipids (0·3 g of Sudan black B was dissolved in 150 ml of ethanol, 100 ml of water, and 0·5 ml of 1M-NaOH; the gel was allowed to stay in this solution for 12 h and was then washed several times with 60% ethanol).

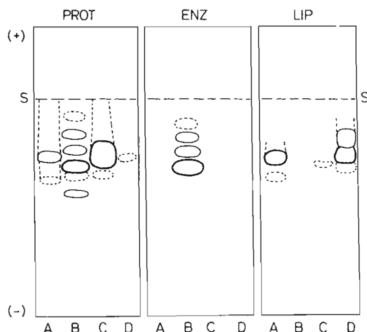

FIG. 1

Starch-Gel Electrophoresis of Fractions M3/I—M3/III (1 st through 3rd peak) from Sephadex G 75 Column

Prot: gel stained with nigrosine for proteins. Enz: detection of pectin esterase activity by the printing technique using a paper impregnated with pectin and bromothymol blue. Lip: gel stained with Sudan black B for lipids. S: origin. A: product M3/I. B: product M3/III. C and D: samples of fractions obtained by separation of product M3/II on CM-Sephadex C 50.

Discontinuous electrophoresis in polyacrylamide gel was carried out as usual for basic proteins[11]. The proteins were stained with amido black 10 B, glycoproteins with periodic acid and the Schiff reagent (PAS-staining)[12].

Analytical methods. The protein concentration was determined by the method of Lowry and coworkers[13]. The nitrogen content of the desalted samples was determined according to Dumas.

The saccharides were identified after the hydrolysis of the samples in 1N-H_2SO_4 for 16 h at 105°C in sealed tubes, neutralization of the acid by $BaCO_3$, and passage through a column of Dowex 50 W (in H^+-form). The samples were concentrated *in vacuo*, dissolved in water, and chromatographed on Whatman No 1 paper in the following systems: n-butanol–pyridine–benzene–water (7 : 3 : 1 : 2) (ref.[14]) and ethyl acetate–pyridine–water (8 : 2 : 1) (ref.[15]). Aniline hydrogen phthalate[16] was used for the detection. Fatty acids were analyzed after methanolysis[17] by gas chromatography on Perkin-Elmer F 11 gas chromatograph.

Hydroxyproline was identified after hydrolysis of the samples in 6M-HCl at 105°C for 16 h by paper chromatography in the solvent system pyridine–n-butanol–acetic acid–water (60 : 90 : : 18 : 72) on Whatman No 3MM paper. *p*-Dimethylaminobenzaldehyde was used for the detection after preceding oxidation of the samples by spraying of the chromatogram with 1·35% solution of chloramine T. The hydroxyproline content was determined by the method of Stegemann[18].

Ultracentrifugation analysis. The sedimentation analysis was carried out in Spinco Model E Ultracentrifuge at 56100 rev./min. The samples were dissolved in 0·1M phosphate buffer at pH 7·8, containing 0·1M-NaCl, to give 0·8, 0·6 and 0·4% solutions. The sedimentation equilibrium was studied with 0·03% solution of the enzyme in the same buffer at 39 460 rev./min.

RESULTS AND DISCUSSION

In the course of the purification of tomato pectin esterase, we were able to detect five zones of the multiple forms of this enzyme by starch-gel electrophoresis. All these zones migrated toward the cathode.

In all studies on the purification of tomato pectin esterase, which have been reported[8,9,19] until present, an active fraction, obtained by gel chromatography on Sephadex G 75 or G 100 and homogeneous with respect to its molecular weight and sedimentation characteristics, was regarded as the final product. This product, which we have designated M3/III (third peak obtained by chromatography of product M3 on Sephadex G 75) in our preceding paper[5], gave after rechromatography four multiple forms of pectin esterase on starch-gel electrophoresis (Fig. 1, sample B). An additional purification of this product was achieved by chromatography on CM-Sephadex C 50. The sample of pectin esterase isolated, designated M4, was identical with one of the multiple forms of this enzyme which is present in dominant quantities in tomatoes. When subjected to starch-gel electrophoresis, this form (M 4) behaved as one zone, showing both the presence of protein and enzymatic activity (Fig. 2, samples A and D). The specific activity of this form determined in the fractions emerging from the CM-Sephadex C 50 column was 1075 μequiv/min/mg of protein. After this product had been desalted and lyophilized, a white substance was obtained

which was not completely soluble either in water or in 0·2M-NaCl and remained partly flocculated. The specific activity dropped to 704 μequiv/min/mg of protein.

The presence of lipid components during the purification was examined to advantage by starch-gel electrophoresis with simultaneous detection of pectin esterase activity, proteins, and lipids (Fig. 1). Five zones giving the positive color test for the presence of lipids can be observed in tomato extracts; three of them are on the cathodic side and two on the anodic side. The anodic components were removed by chromatography on DEAE-Sephadex A 50 and the cathodic components on Sephadex G-75. We did not find any zones giving a positive color test for lipids in the third peak containing product M3/III with maximum pectin esterase activity (Fig. 1, sample B). The presence of lipids was revealed in products contained in the first and second peak from the Sephadex G 75 column, designated M3/I and M3/II (Fig. 1, samples A and D).

The form of pectin esterase isolated (both the soluble and the insoluble part) was analyzed by gas chromatography after methanolysis. Not even traces of fatty acids were found. In view of these results, the assumed lipoprotein character of this form of tomato pectin esterase could be eliminated.

The possibility that tomato pectin esterase was a glycoprotein seemed to be suggested by the results of the PAS-staining of the enzyme fractions and by their behavior on electrophoresis[9] in the borax buffer. When product M3/III was examined by discontinuous electrophoresis in polyacrylamide gel, zones stained with amido black which were identical with the zones stained with the Schiff reagent (PAS-staining) were found. This product therefore was hydrolyzed in 1N-H_2SO_4 and analyzed

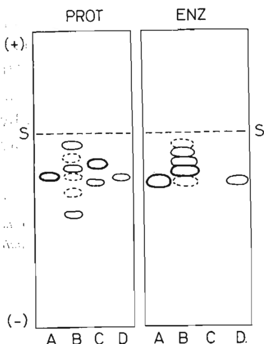

FIG. 2

Starch-Gel Electrophoresis of Fractions after Chromatography of Product M3/III on CM-Sephadex C 50.

Prot: *cf.* Fig. 1. Enz: *cf.* Fig. 1. S-origin. A: 500 μg of isolated form of pectin esterase-product M4. B: fraction at 0·15 M concentration of buffer. C: fraction at 0·20M concentration of buffer. D: 250 μg of product M4.

by paper chromatography for the presence of sugars. The presence of arabinose as the main component of neutral sugars was identified both in product M3/III as well as in the isolated form of pectin esterase (product M4) before their desalting on a column of Sephadex G 25 Fine. The presence of hydroxyproline was revealed in both these products after their hydrolysis in 6M-HCl. These findings seemed to indicate the presence of glycoproteins of a type similar to that isolated from tomato cell walls by Lamport[20] who found a glycosidic binding of arabinose through hydroxyproline in glycopeptides. Since pectin esterase is one of the enzymes localized in the cell walls of higher plants[19], it could be assumed that – similarly to horseradish peroxidase whose isozymes are glycoproteins containing hydroxyproline, arabinose, and galactose[20,21] – the character of pectin esterase could also be that of a glycoprotein. When we desalted the isolated form of pectin esterase (product M4) on a column of Sephadex G 25 Fine, we obtained two peaks. In the first peak, which contained the isolated form of pectin esterase only (Fig. 2; samples A and D), the presence of neither arabinose nor hydroxyproline was determined. Both these components were identified in the second peak emerging before the salts. It appears therefore that the glycoprotein component, which accompanied the enzyme through the entire purification process, can be removed by desalting of the final product. In view of this finding, the originally assumed glycoprotein character of this form of tomato pectin esterase cannot be confirmed.

The isolated form of pectin esterase moved on ultracentrifugation as a symmetrical peak with a sedimentation coefficient $s^0_{20,w} = 3{\cdot}25$ S. This value is very close to the value of 3·3 S found by Delincée and Radola[9] and similar to the values obtained by other authors: 3·08 S (ref.[8]) and 3·17 S (ref.[19]), for purified tomato pectin esterase. The value of 2·34 S, given in our preceding paper[5], should be corrected in view of the results (3·25 S) of our recent experiments carried out under more suitable conditions.

The value of molecular weight of the isolated form of tomato pectin esterase derived from the sedimentation equilibrium experiments is 27800, on condition that the partial specific volume is 0·73 ml/g. This value is also close to the value 26300 obtained by this method under different experimental conditions[9]. The values of molecular weights of the purified preparations of tomato pectin esterase, obtained by the method of gel filtration on Sephadex[5,7], can be affected by contaminating polysaccharides[9] and should therefore be considered with reservation.

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