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THIN-LAYER ISOELECTRIC FOCUSING OF MULTIPLE FORMS OF TOMATO PECTINESTERASE

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; pectinesterase; isoenzymes; thin-layer isoelectric focusing.

Abstract—Commercial tomato pectinesterase has been separated into at least eight multiple forms by thin-layer isoelectric focusing. The enzyme components were basic proteins in the range pH 7–9.3, the predominant form having an isoelectric point of 8.6. The enzyme was detected with a staining procedure, employing the reaction of hydroxylamine with the ester groups of pectin. The MW's of the multiple forms of pectinesterase were in the range of 27000 ± 5000.

INTRODUCTION

Gel filtration [1], ion-exchange chromatography [2] and starch gel electrophoresis [3] have indicated that tomato pectinesterase (EC 3.1.1.11) occurs in multiple forms. However, in other work with tomato pectinesterase (using commercial preparations as well as freshly prepared extracts) only a single enzyme has been observed by gel filtration, sedimentation analysis in the ultracentrifuge, paper and disc electrophoresis and isoelectric focusing [4–6]. The present paper describes the separation of multiple forms of commercial pectinesterase from tomatoes by thin-layer isoelectric focusing. The enzyme was detected after focusing by a novel staining procedure, which depends on the reaction of hydroxylamine with pectin, and subsequent coloring with ferric chloride [7]. By this procedure relatively low levels of enzyme activity were detected in the paper print taken after focusing [8,9].

RESULTS

Detection of pectinesterase

In previous studies, pectinesterase in gel or paper electrophoresis was detected after elution by conventional enzyme assay [4,10]. The print technique in thin-layer gel filtration which employs a paper impregnated with pectin and a suitable pH-indicator, enabled the direct detection of pectinesterase [4]. Markovic used this technique successfully in starch gel electrophoresis to detect 5–6 zones of pectinesterase activity [3]. This principle cannot be used in isoelectric focusing, and with the elution procedure only a single enzyme zone could be found [4]. A new procedure for detecting pectinesterase in thin-layer isoelectric focusing was therefore developed (Table 1). A print was taken with a chromatographic paper buffered at pH 7.5, impregnated with pectin. The pectinesterase partly de-esterified the pectin, and the remaining ester groups were reacted with alkaline hydroxylamine and stained with ferric chloride [7]. Zones with pectinesterase activity appeared as yellow–white bands on a red–brown background. The same procedure was used in thin-layer gel filtration.

The technique used for detection of pectin depolymerizing enzymes, e.g. polygalacturonase and pectin lyase [11,12], cannot be used for the detection of pectinesterase, since the de-esterifying action of pectin-esterase on the pectin does not destroy its ability to be stained with ruthenium red.

Thin-layer isoelectric focusing

Figure 1 shows the focusing of commercial tomato pectinesterase in a mixture of pH 3–10 and pH 8–9.5

Table 1. Detection of pectinesterase activity with the print technique in thin-layer isoelectric focusing

Step	Paper treatment	Time interval (min)
1	Impregnate a SS 2043 b Mgl paper (Schleicher and Schüll, Dassel, W. Germany) with a 0.35% pectin soln in 0.3 M Pi buffer, pH 7.5 (*ca* the pH optimum of tomato pectinesterase).	5
2	Dry the paper at room temp.	180
3	Repeat Step 1 and 2. The paper can now be stored for several weeks until use.	185
4	Take the print after focusing and dry at 110°.	10
5	Immerse the print in *ca* 20 ml of the alkaline hydroxylamine reagent, prepared by mixing equal vol of soln A and B. A = 14 g of hydroxylamine chloride dissolved in 100 ml of 60% EtOH. B = 14 g of NaOH in 100 ml of 60% EtOH.	7
6	Acidify with 20 ml of soln C. C = HCl (25%) and EtOH (95%) 1:2.	10
7	Discard the above soln and stain the print in 20 ml of soln D. D = 2.5 g of $FeCl_3$ dissolved in 100 ml of 0.1 N HCl in 60% EtOH	2
8	Dry the paper at room temp.	30

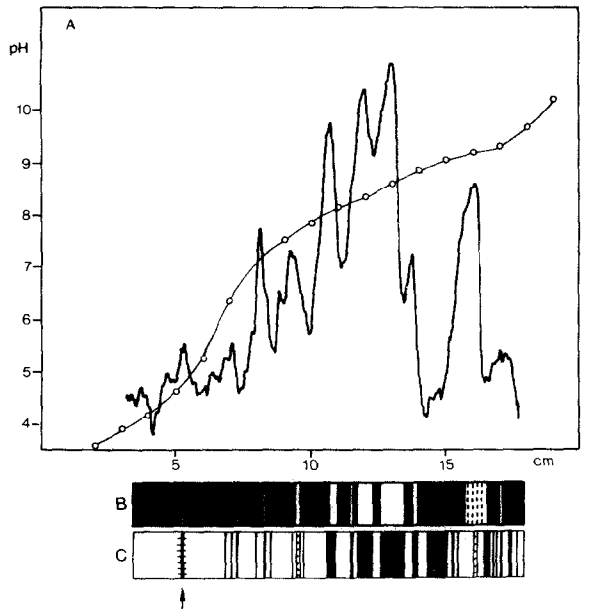

Fig. 1. Thin-layer isoelectric focusing of tomato pectinesterase. Separation distance, 20 cm; carrier ampholytes, 1% Ampholine pH 3–10 and pH 8–9.5 at a 1:1 ratio; carrier gel, Sephadex Superfine G-75. Focusing at 400 V for 3 hr followed by 1000 V for 4 hr. The commercial pectinesterase was applied 5 cm from the anode. (a) Densitometric scan (remission) at 480 nm of the print stained for enzyme detection. The pH gradient —○—○—○— was measured in the gel layer at 25°. (b) Focusing print stained for enzyme detection according to Table 1. (c) Focusing print stained for protein with Coomassie Brilliant Blue G-250. The arrow indicates the application site.

carrier ampholytes at a 1:1 ratio. With this ampholyte mixture an efficient resolution of the multiple components in the alkaline range was achieved. The tracing (A) was obtained by densitometry of the paper print after enzyme staining (B). At least eight distinct components of pectinesterase activity were demonstrated, a heterogeneity which had not been found previously by any other method. The enzyme components had isoelectric points in the range pH 7–9.3, the main band having an isoelectric point of 8.6, in agreement with earlier results [4]. The protein staining (c) was basically identical with the pattern previously described [4], although the components were resolved to a better degree. Not all protein bands (see Fig. 1c) correspond to an enzyme band (Fig. 1b), indicating the presence of several contaminating proteins.

The sensitivity of pectinesterase activity staining by the print technique in focusing is demonstrated in an experiment using decreasing amounts of pectinesterase (Fig. 2). The three main bands in the pH 8.0–8.6 range could still be detected when 85 nkat of the preparation were applied. At the highest level of 2000 nkat, overloading was evident, but some minor zones appear only at this level of loading. Different preparations of tomato pectinesterase showed similar enzyme patterns.

Size properties

Thin-layer gel filtration of the commercial tomato pectinesterase revealed about 6 protein fractions, F I–F VI (ref. [4]). When the gel filtration print was stained for enzyme detection by the pectin hydroxylamine reaction, a relatively broad zone of enzyme activity appeared.

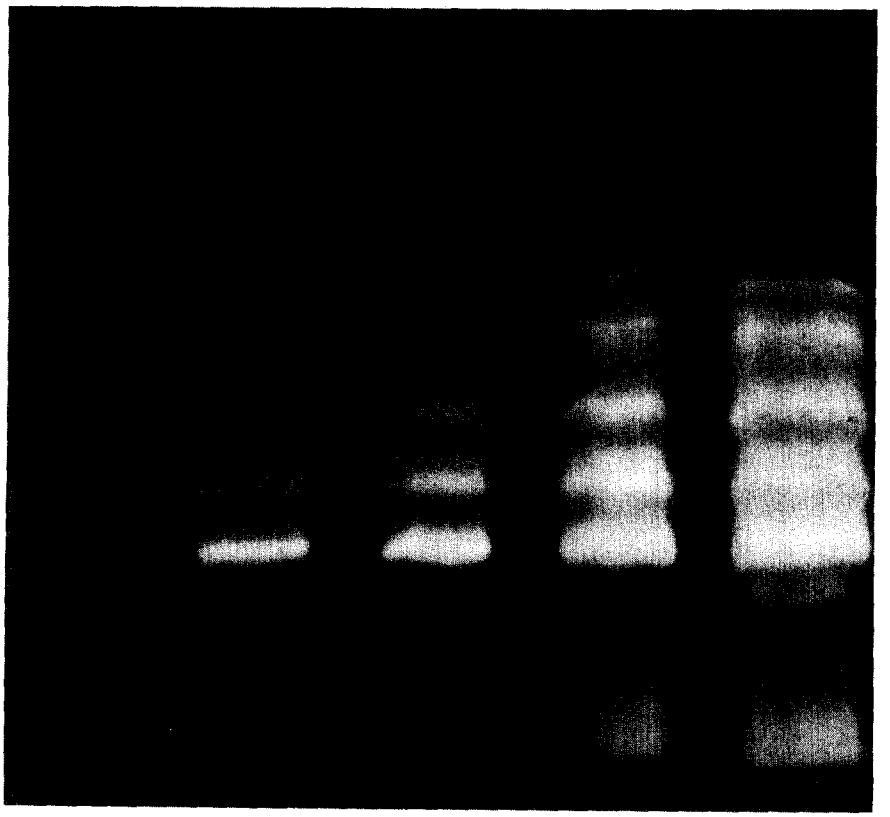

Fig. 2. Detection of pectinesterase activity in thin-layer isoelectric focusing. From left to right, the amounts of pectinesterase ranged from 85, 170, 500, 1000 to 2000 nkat (pH 7.5, 30.0°). Focusing as in Fig. 1. Anode at the top. Enzyme detection.

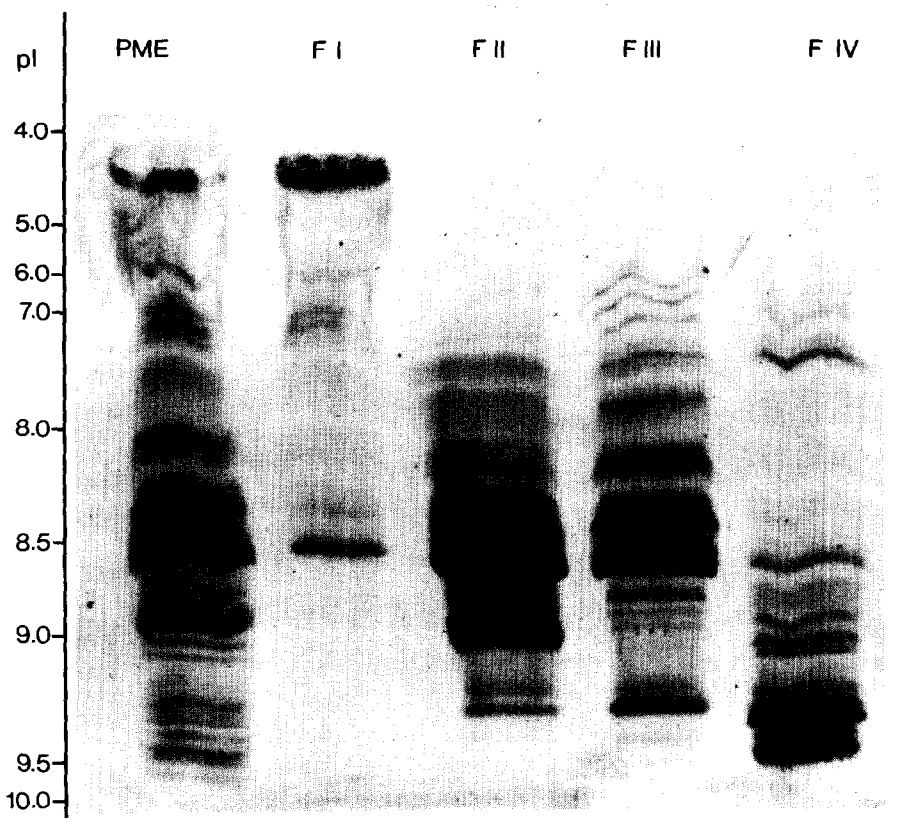

Fig. 3. Thin-layer isoelectric focusing of protein fractions isolated by preparative gel filtration. Protein staining with Coomassie Brilliant Blue G-250. To the left the unfractionated pectinesterase.

This zone corresponded to a MW of *ca* 27000. When larger amounts of the sample were applied, a faint zone with enzyme activity was observed in the high MW fraction (FI), which was excluded from the Sephadex G-75 gel. To obtain further evidence for size heterogeneity the enzyme preparation was fractionated by preparative column gel filtration, by which the four fractions F I–F IV were isolated as described previously [4]. The separation of the fractions with different size was confirmed by thin-layer gel filtration of these isolated fractions followed by protein staining. When the print was stained for enzyme activity, rather than for proteins, all of the isolated fractions showed pectinesterase activity. Most activity was located in the broad zone of F III. In F IV, enzyme activity did not coincide exactly with protein staining; activity was only noted in the leading part of the zone, which in turn corresponded to the trailing part of the enzyme zone of F III.

Thin-layer isoelectric focusing of the isolated gel chromatographic fractions revealed that all pectinesterase bands observed in the unfractionated preparation were present in F III. F II also comprised most of these bands, whereas in F IV only the three main components in the pH 8.0–8.6 range and the larger cathodic component with an isoelectric point of 9.2 were recognized (compare with Fig. 1). In F I traces of the three main components and the components with lower isoelectric points were observed. By protein staining of the focusing print the difference in the patterns of the four isolated fractions was evident (Fig. 3). In F III basically all protein bands corresponded to bands with pectinesterase activity, whereas in the other fractions inactive bands, such as the pI 8.95-band in F II were enriched. Possibly, an association of the active enzyme with the inert accompanying proteins could be responsible for the observed size heterogeneity.

For further elucidation of the size heterogeneity of pectinesterase the reverse procedure was chosen, namely gel filtration of a number of fractions isolated by preparative isoelectric focusing. Eight fractions with different isoelectric points corresponding to the enzyme zones (compare with Fig. 1) were isolated. Analytical refocusing of the isolated isoelectric fractions demonstrated that no interconversion between these fractions occur, and that essentially homogeneous fractions were obtained. When the print was stained for pectinesterase detection, thin-layer gel filtration revealed almost identical migration distances for all fractions. This indicates that all enzyme components have similar MW's, contradicting the abovementioned gel filtration results. To confirm these previous findings, the gel filtration study was repeated using the stored fractions F I–F IV. In this instance it was somewhat surprising to find that the enzyme activity in all fractions was confined to the area occupied by the broad band of F III. F IV still showed activity in the trailing part of the band of F III, and now F II exhibited activity in the leading part. The activity of F I was also found to have the same migration distance as F III. By overstaining the print for enzyme detection with Amidoblack, it was shown that the protein zones were still well separated but did not coincide with the enzyme zones.

DISCUSSION

Until recently, studies on the purification of tomato pectinesterase were solely concerned with a single form of this enzyme [4–6]. Pressey and Avants first demonstrated the heterogeneity of tomato pectinesterase by ion-exchange chromatography [2] and attributed the lack of earlier detection of some of the enzyme forms to the fact that they were present at low levels only. The lack of a sensitive detection method thus seems to present a major obstacle for the recognition of multiple forms of pectinesterase. The introduction of the print technique which employs a pectin-impregnated paper with pH-indicator [4], enabled Markovic to detect 5-6 components with pectinesterase activity in starch gel electrophoresis. A still greater heterogeneity has now been revealed using thin-layer isoelectric focusing followed by a novel staining procedure (Table 1), by which at least 8 distinct enzyme components were separated (Figs. 1 and 2). Instead of the about 10 protein zones in the commercial preparation described previously [4], approximately 20 protein zones were recognized under the refined conditions of focusing (Figs. 1 and 3). The enzyme component with the isoelectric point of 8.6 was the major form in all our commercial preparations. However, the amount of the individual components and the actual number of components may vary with the variety and ripeness of the tomato [2].

The heterogeneity of pectinesterase may be partly based on size differences. The preliminary results of gel filtration indicated the presence of isoenzymes differing in size, in agreement with results reported in ref. [1]. However, this may be due to some unspecific adsorption of the enzyme to, or association with the inert accompanying proteins. Aggregation of pectinesterase seems improbable since, for example, the enzyme activity in the earlier gel filtration experiments coincided exactly with the protein staining in F II, the MW of which is not a multiple of the MW of F III [4]. Possibly small differences in the MW of the different isoenzymes exist [2], as indicated by the slightly different migration behaviour of the gel chromatographic fractions. The MW of these fractions was calculated to be in the range of 27000 ± 5000.

The pectinesterase isoenzymes thus differ mainly with respect to their charge properties. The fact that all these isoelectric fractions showed similar migration characteristics in thin-layer gel filtration, indicates that no binding to inert proteins occurred in focusing. Furthermore, it seems improbable that multiple forms are caused by recombination of subunits, since the enzyme exhibited a relatively low MW. Possibly, heterogeneity is caused by variations in a carbohydrate or lipid moiety of the enzyme, however, discussions concerning the content of carbohydrates or lipids in tomato pectinesterase are controversial [3–5]. Experiments in which the commercial pectinesterase was stained for lipoprotein after focusing with Sudanblack or Sudan III showed only faint zones coinciding with the protein zones. When the pectinesterase was stained for glycoproteins by the PAS-reaction, again only faint zones coinciding with the major protein zones were observed. In this case the polyacrylamide gel Bio-Gel P-60 was used as carrier in focusing instead of the carbohydrate gel Sephadex.

The possibility of artifact formation as discussed by Hultin [13] after the separation of pectinesterases in banana, is still not resolved. The reason for heterogeneity must be studied after isolation of the various enzyme components, e.g. by preparative isoelectric focusing, and a more exact characterization of each isoenzyme.

EXPERIMENTAL

Two preparations of tomato pectinesterase were purchased from Worthington Biochem. Co., N.J., USA: PE 7 HA with 1500 nkat/mg and PE 7 JC with 500 nkat/mg. The third came from Sigma Chem. Co., St. Louis, USA: 97B-1870 with 210 nkat/mg. For the enzyme assay [4] citrus pectin "Exchange" No. 3442 N.F. from Sunkist Growers, California, USA was used. Thin-layer or column gel filtration and the MW determinations were carried out as described previously [4,14]. Thin-layer or preparative layer isoelectric focusing was performed on glass plates coated with Sephadex or Bio-Gel as carrier gel and with LKB Ampholine as carrier ampholytes [9,15]. In the preparative focusing experiment 0.2 g of commercial pectinesterase were separated on a 20×40 cm plate, coated with a 1-mm layer of Sephadex Superfine G-200 containing 1% Ampholine (pH 3–10 and pH 8–9.5 at a ratio of 1:1). After focusing, 2 cm-wide guide strips were applied, and stained for pectinesterase (Table 1). The fractions were removed from the gel plate with a spatula, transferred to small glass columns and eluted with buffer.

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