Phytochemistry, 1972, Vol. 11, pp. 3139 to 3142. Pergamon Press. Printed in England.

MULTIPLE FORMS OF PECTINESTERASE IN TOMATOES

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(*Revised Received* 8 *May* 1972. *Accepted* 11 *July* 1972)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; pectinesterase isozymes.

Abstract—Four forms of pectinesterase have been identified in extracts of tomatoes by chromatography on DEAE-Sephadex A50. Green fruit of the variety Marion contained two pectinesterases. On ripening, the level of one of the enzymes decreased while the second increased several fold and a third component developed. Fruit of the variety Pixie also contained three pectinesterases, but one of the Pixie enzymes was not present in the other varieties. The pectinesterases were different in terms of MW, stability to heat, and their responses to pH and cation concentration.

INTRODUCTION

DURING studies on the purification of commercial tomato pectinesterase, we observed that the activity separated into two peaks on columns of DEAE-Sephadex A50. Subsequent examination of extracts of three varieties of tomatoes revealed multiple forms of pectinesterase in each. We found that the number of components and their relative concentrations vary with the variety and the ripeness of the fruit. This paper describes the separation of the tomato pectinesterases and some of their properties.

RESULTS

Separation of Tomato Pectinesterases

Our preliminary studies were conducted with tomato pectinesterase purchased from Sigma Chemical Co. The activity in this sample appeared to be homogeneous when chromatographed on Sephadex G100. However, passage of the enzyme solutions through columns of DEAE-Sephadex A50 yielded two peaks of activity. Maximal resolution and the sharpest peaks were obtained with 0·15 M NaCl at pH 6·0, and these conditions were adopted for the analysis of our extracts of tomatoes.

The pectinesterase in an extract of green Marion tomatoes was separated into two peaks of activity (Fig. 1). The first peak possessed a shoulder of activity extending toward the second peak. The second peak was nearly twice as large as the first. An extract of ripe Marion tomatoes contained the same two enzymes but there was also a distinct third peak where only the shoulder of activity was found in green tomatoes. Other changes that occurred on ripening were that the first peak decreased while the second peak increased several fold.

The elution pattern for the pectinesterase in an extract of ripe Homestead tomatoes was similar to that for ripe Marion fruit (Fig. 2). The total activity was somewhat higher in the variety Homestead and the middle peak was less distinct, but the ratios of the different enzymes were about the same for the two varieties. An extract prepared from ripe fruit of the dwarf variety Pixie contained two pectinesterases corresponding to the peaks of activity

in green Marion tomatoes. But in contrast to the samples described earlier, Pixie tomatoes contained a component which was eluted at about fraction 19. This peak was eluted clearly to the right of the middle peak in Marion tomatoes and it was the major component in Pixie fruit. Because the first two peaks are so close together, it is not possible to tell whether there is an additional pectinesterase at about fraction 17 as in the other ripe samples.

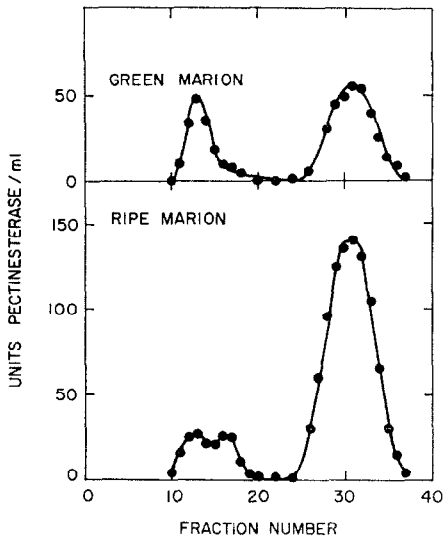

FIG. 1. ELUTION OF PECTINESTERASES IN EXTRACTS OF GREEN AND RIPE MARION TOMATOES ON A DEAE-SEPHADEX A50 COLUMN.

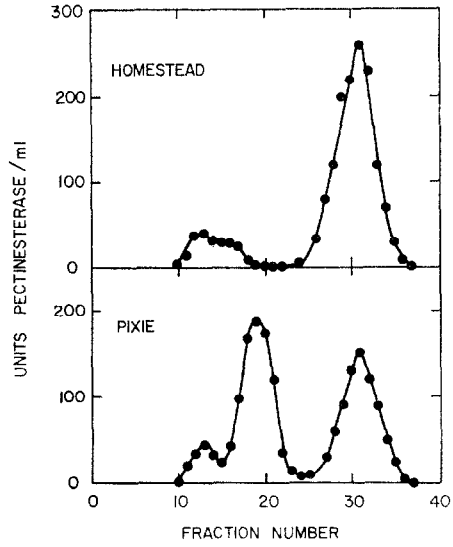

FIG. 2. ELUTION OF PECTINESTERASES IN EXTRACTS OF HOMESTEAD AND PIXIE TOMATOES ON A DEAE-SEPHADEX A50 COLUMN.

The enzymes were designated pectinesterase I (PE I), PE II, PE III and PE IV, in the order of their elution off the DEAE-Sephadex A50 column. For characterization studies, PE III was separated from Pixie tomatoes, PE I from green Marion fruit and PE II and PE IV from ripe Marion tomatoes.

Effects of pH and NaCl *concentration*

It is well known that cations activate plant pectinesterases and shift their pH optima to lower pH values.[1,2] The effects of pH and NaCl concentration on the four tomato pectinesterase were therefore studied in detail to determine whether the enzymes differ in their responses to these factors. Because the enzymes were in 0·15 M NaCl, the contributions of NaCl from the enzyme solutions must be considered. This residual amount of NaCl was reduced by using concentrated enzyme solutions and the final concentration of NaCl in the reaction mixture was 6×10^{-4} M, in the absence of added salt. All of the enzymes, except PE I, deesterified pectin at pH 9 in the presence of 6×10^{-4} M NaCl, although the rate of hydrolysis was by far the highest for PE IV. At pH 8, PE II was only slightly active at this level of NaCl and PE I and PE III were completely inactive, while PE IV was almost as active as at pH 9. PE IV deesterified the substrate in the presence of only 6×10^{-4} M NaCl as low as pH 6·0, but the rate decreased sharply as the pH was lowered.

[1] H. LINEWEAVER and G. A. BALLOU, *Arch. Biochem.* **6**, 373 (1945).
[2] L. R. MACDONNELL, E. F. JANSEN and H. LINEWEAVER, *Arch. Biochem.* **6**, 389 (1945).

The four enzymes further differ in their responses to NaCl added to the reaction mixture. At the lower pH values, deesterification was not obtained below NaCl concentrations characteristic for each enzyme. In general, the threshold level of NaCl for activity increased as the pH was lowered. The minimal levels of NaCl for deesterification at pH 5·0 were 0·2, 0·1, 0·05 and 0·01 M for PE I, PE II, PE III and PE IV, respectively. After the threshold NaCl concentration was reached at each pH, increasing the salt concentration increased the rate of deesterification for all four enzymes, At the higher pH values, the activity increased to a maximum with increasing NaCl concentration and then decreased. The NaCl concentration for maximum activity was lowest at pH 9 for all of the enzymes but the optimum NaCl concentration varied for the different enzymes. The values were about 0·15, 0·2, 0·1 and 0·03 M NaCl for PE I, PE II, PE III and PE IV, respectively.

The salt concentration required for maximal activity increased as the pH of the reaction solution was lowered. For example, highest activities at pH 5 were obtained at about 0·15 M NaCl for PE IV and 0·26 M NaCl for PE III, while the rates of deesterification for PE I and PE II were still increasing at 0·5 M NaCl. PE II consistently required the highest NaCl levels for maximal activation at all pH values.

Effect of Sodium Dodecyl Sulfate

Sodium dodecyl sulfate (SDS), an effective inactivator of tomato pectinesterase,[3] provided additional information that the four pectinesterases are different. The tests were conducted by adding the enzymes to 25 ml of 0·2 M NaCl containing SDS. After stirring at pH 7·0 for 5 min, 25 ml of 1% pectin was added and the reaction was titrated at pH 7·0. SDS at $3{\cdot}5 \times 10^{-4}$ M did not affect any of the pectinesterases. At 6×10^{-4} M, the activity of PE I was unchanged, PE II was reduced to 83% of the control, PE III was reduced to 18% and PE IV was reduced to 42%. SDS at $7{\cdot}5 \times 10^{-4}$ M completely inactivated all 4 enzymes.

Stability to Heat

Solutions of the enzymes in 0·15 M NaCl at pH 6·0 were heated to various temperatures and held for 5 min. After cooling, the residual activities were determined at the optimum pH and NaCl concentration for each enzyme. The temperature for 50% inactivation was 56° for PE I, 59° for PE II and 62° for both PE III and PE IV.

Molecular Weights

The MWs of the four pectinesterases were estimated by analytical column gel filtration using a $2{\cdot}5 \times 95$ cm column of Sephadex G100 in 0·15 M NaCl. The elution volumes for the standard proteins cytochrome c, ovalbumin, bovine serum albumin monomer, and bovine serum albumin dimer were 330, 233, 202 and 160 ml, respectively. The elution volumes for the pectinesterases and their calculated MWs were: PE I, 250 ml, 35 500; PE II, 268 ml, 27 000; PE III, 284 ml, 23 700; PE IV, 281 ml, 24 300.

DISCUSSION

Pectinesterase is widely distributed in higher plants. It has been isolated and partly characterized from several sources including alfalfa,[1] orange,[2] banana[4] and tomato.[5] Hultin and Levine[4] have presented evidence that the pectinesterase in banana pulp consists

[3] S. I. KERTESZ, *The Pectic Substances*, p. 371, Interscience, New York (1951).
[4] H. O. HULTIN and A. S. LEVINE, *Arch. Biochem. Biophys.* **101**, 396 (1963).
[5] R. J. MCCOLLOCH, H. C. MOYER and Z. I. KERTESZ, *Arch. Biochem.* **10**, 479 (1946).

of at least three molecular forms. Extensive studies have been conducted on tomato pectinesterase, reflecting the availability of this enzyme and also its importance in tomato products. In two recent studies[6,7] it was concluded that the pectinesterase activity in tomatoes is due to a single enzyme.

We have now demonstrated that there are multiple forms of prectinesterase in tomatoes. One reason why this was not recognized previously is that some of the enzymes are present at low levels. Furthermore, the closeness in their MWs explains the inability to separate them by gel filtration which has been used as a criterion of homogeneity. The major component in most of our extracts and the one commercial sample of pectinesterase we examined was PE IV. Its properties appear to be very similar to that for the tomato pectinesterase described by Lee and Macmillan[6] and it may be the most important enzyme in most samples of tomatoes. But, as we have found, the actual number of components and their activities may vary considerably with ripeness and tomato variety.

The physiological significance of the occurrence of more than one pectinesterase in tomatoes may be related to the complexity of pectin and the variety of changes it undergoes during fruit development and ripening. The individual enzymes may exhibit specificity for substrates of particular MW and degree of esterification. Lee and Macmillan[8] found that their preparation of tomato pectinesterase acted at both the reducing end and another locus on the substrate molecule. They concluded that a free carboxyl group in the molecule may be necessary to serve as an initiation point for enzymatic attack. The function of one of the pectinesterases in tomatoes may be to create initiation points for the other enzymes.

The differences in elution patterns of the pectinesterase activities in green and ripe tomatoes suggest specialized roles for the isoenzymes. In the growing fruit, biosynthesis and hydrolysis of the ester groups in pectin must be an important process in the formation and elongation of the cell walls. In ripe tomatoes, the pectinesterases probably participate in tissue softening, and the large increases in two enzymes in Marion tomatoes indicate that they may be involved in this process.

EXPERIMENTAL

Field-grown tomatoes were picked when full-sized at both the green and ripe stages and stored at −20°. Extracts were prepared by blending 100 g of tissue with 100 ml of cold H_2O and 2 ml of 0·8 M sodium bisulfite in a VirTis homogenizer for 2 min. 5 homogenates of each tomato sample were combined and the particles were further broken down with a Polytron (Brinkmann Instruments, Inc.) for 2 min. After centrifugation at 8000 *g* for 30 min, the insoluble material was suspended in 500 ml of 1·0 M NaCl, adjusted to pH 6·0 and stirred overnight at 3°. The solubilized proteins were then fractionated with $(NH_4)_2SO_4$; the precipitate obtained between 30 and 75% saturation was dissolved in 10 ml of 0·5 M NaCl and dialyzed overnight against 0·15 M NaCl.

The dialyzed solution was clarified by centrifugation at 15 000 *g* for 30 min and applied to a 2·5 × 80 cm column of DEAE-Sephadex A50 in 0·15 M NaCl. The gel was adjusted to pH 6·0 before packing the column. Elution was conducted with 0·15 NaCl at a rate of 12 ml/hr. The individual fractions were assayed for pectinesterase by adding 1 ml to 50 ml of 0·5% pectin in 0·1 M NaCl, pH 8·0. The pH was maintained at 8·0 with an automatic titrator by addition of 0·02 N NaOH. A unit of pectinesterase is defined as that amount which catalyzes the hydrolysis of 1 μeq ester/min at 25°C. The fractions containing each enzyme were combined and concentrated to small volumes by ultrafiltration (Amicon Corporation) using a PM10 membrane.

The substrate was pectin from citrus fruits obtained from Sigma Chemical Company. The substrate was dissolved in H_2O, adjusted to the desired pH and diluted to 1%. The pectin solution was diluted with an equal volume of H_2O or NaCl solution of appropriate concentration, warmed to 25° and the pH was readjusted, if necessary, before the addition of the enzyme.

[6] M. LEE and J. D. MACMILLAN, *Biochemistry* **7**, 4005 (1968).
[7] H. DELINCEE and B. J. RADOLA, *Biochim. Biophys. Acta* **214**, 178 (1970).
[8] M. LEE and J. D. MACMILLAN, *Biochemistry* **9**, 1930 (1970).