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PECTIC ENZYMES ASSOCIATED WITH THE SOFTENING OF TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit ripening; pectic enzymes; pectin; pectic acid; transeliminase; enzymes.

Abstract—Some degree of softening of maturing tomato fruit takes place before the usually accepted mechanism for the loss of firmness, involving the sequential action of pectin esterase and polygalacturonase, is operative to any extent. The possibility that pectin and pectic acid transeliminases were implicated, providing an alternative pathway for the breakdown of pectin in the tomato, was investigated. Direct spectrophotometric determination of these enzymes proved unreliable and the thiobarbituric acid test, which should give a specific colour reaction with the products, was negative. It is concluded that transeliminases do not play a significant part in the softening of tomato fruit either prior to or during ripening.

INTRODUCTION

MEASUREMENTS carried out on tomato fruit as they approach full ripeness[1] indicate that loss in firmness accelerates progressively with time so that a maximum rate coincides with the orange–green to orange colour stage.[2] During ripening, a series of pectic enzymes is thought to attack the intercellular binding material. Increasing solubilization of the pectic substances is closely followed by demethoxylation by pectin esterase,[3] while chain shortening and degradation of the products of this reaction are attributed to hydrolytic polygalacturonases.[4] These latter enzymes are not active, however, in tomatoes prior to the green–orange stage,[4] and therefore the softening of fruit up to this point cannot be due to the above mechanism. A possible alternative pathway lies in degradation by the enzymes pectin transeliminase (PTE) and pectic acid transeliminase (PATE); these split pectin or pectic acid by a transeliminative cleavage of the α-1,4-glycosidic bonds to produce C_4–C_5 unsaturated galacturonic acid derivatives. These enzymes, if present in tomato fruit, would interfere with previously adopted methods for the determination of hydrolytic polgalacturonases and might account for the initial softening which accompanies incipient ripening.

RESULTS AND DISCUSSION

The products of transeliminase action contain unsaturated groups which absorb strongly in the region of 235–240 nm, and this forms a basis for the assay of the enzymes.[5] Two fungal sources of PTE were assayed by this method and the activities are recorded in Table 1. As a confirmatory test on the products of the reaction, Edstrom and Phaff[6] used a method originally described by Weissbach and Hurwitz[7] in which the unsaturated bonds are cleaved

[1] G. E. HOBSON, *J. Hort. Sci.* **40**, 66 (1965).
[2] G. E. HOBSON, *Rep. Glasshouse Crops Res. Inst. 1958*, p. 66 (1959).
[3] G. E. HOBSON, *Biochem. J.* **86**, 358 (1963).
[4] G. E. HOBSON, *Biochem. J.* **92**, 324 (1965).
[5] P. ALBERSHEIM, H. NEUKOM and H. DEUEL, *Helv. Chim. Acta* **43**, 1422 (1960).
[6] R. D. EDSTROM and H. J. PHAFF, *J. Biol. Chem.* **239**, 2403 (1964).
[7] A. WEISSBACH and J. HURWITZ, *J. Biol. Chem.* **234**, 705 (1959).

TABLE 1. PECTIN TRANSELIMINASE ACTIVITY IN TWO PECTINASE SAMPLES

Source of the enzyme	Specific activity* pH 5·2	pH 7·5
Crude Pectinase (Koch-Light Ltd.)	0	0·16
Crude Pectinol R-10 (Rohm & Haas)	2·71	2·90
Purified Pectinol R-10	72·10	—

* The specific activity is measured as units per mg protein, where a unit is regarded as that amount of enzyme that will cause the absorption of light at 235 nm to be increased by one optical density unit per min at 25° and pH 5·2 or 7·5 as appropriate, when acting on 2 ml of 0·5% citrus pectin solution in 0·1 M citrate-phosphate buffer contained in a 1-cm cuvette.

with periodate to form β-formyl pyruvate, which reacts with thiobarbituric acid (TBA) to give a coloured complex having a strong absorption peak at 550 nm. However, this procedure is not entirely specific for the products of transeliminase action since pure galacturonic acid, which shows no absorption peak at 235 nm implying a freedom from double bonds, also gives a peak in the TBA test at the same wavelength (550 nm) (Fig. 1). Free galacturonic acid is produced by some polygalacturonases. A modified method employed by Ayres *et al.*[8] avoids the use of periodate, and possible interference due to galacturonic acid is thus

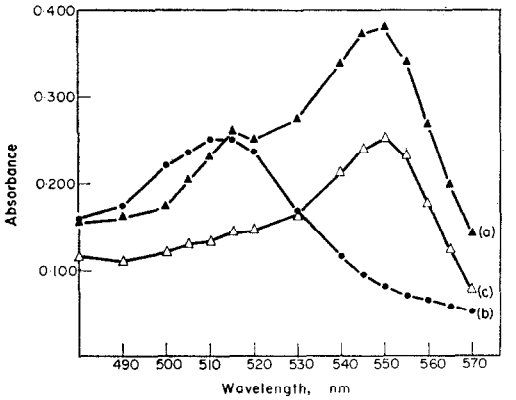

FIG. 1.

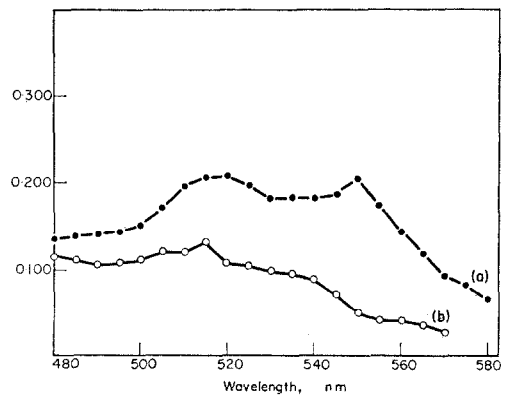

FIG. 2.

FIG. 1. INTERFERENCE BY GALACTURONIC ACID IN THE THIOBARBITURIC ACID TEST FOR 4,5-UNSATURATED TERMINAL GALACTURONIDE UNITS FOLLOWING PERIODATE OXIDATION.
(a) Spectrum of the product when α-D-galacturonic acid is subjected to periodate oxidation followed by reaction with thiobarbituric acid (method according to Weissbach and Hurwitz[7]). (b) Spectrum of the product when α-D-galacturonic acid is directly reacted with thiobarbituric acid (method of Ayres *et al.*[8]). (c) Spectrum of the product when pectin transeliminase, purified by DEAE-cellulose from Pectinol R-10, is incubated with 0·5% pectin in 0·1 M citrate-phosphate buffer pH 5·2, purified with $Zn(OH)_2$ (Ayres *et al.*[8]), and then reacted with thiobarbituric acid.

FIG. 2. TESTS FOR PTE ACTIVITY IN TOMATO FRUIT EXTRACTS.
(a) Absorption spectrum of the products from the TBA test involving periodate oxidation following incubation of 1 ml enzyme and 10 ml 0·5% citrus pectin in 0·1 M citrate-phosphate buffer, pH 5·2, at 25°. (b) Absorption spectrum of the products from the TBA test without periodate oxidation following incubation of enzyme and substrate under the above conditions.

obviated (Fig. 1). The spectrum of the TBA complex with the products of PTE action using the method of Ayres *et al.*[8] (Fig. 1) indicates that the compound has a single absorption peak near 550 nm. This test was adopted in subsequent studies, although it is somewhat less sensitive than the direct spectrophotometric method.

Tomato fruit at selected stages of ripeness[2] were extracted with a number of salt and buffer solutions detailed in the Experimental in order to bring the transeliminases into solution. The enzymes were assayed by following the change in absorption at 235 nm with time. Small increases in absorptivity were found using pectin (at pH 5·2 and 7·5) and sodium polypectate (at pH 7·5) as substrates, suggesting a limited amount of PTE and PATE activity corroborated by a positive TBA test if periodate oxidation is used (Fig. 2a). However, the modified TBA test was negative even after several hours incubation (Fig. 2b). Acetone powders from the fruit were also prepared and extracted with the same selection of buffers. Similar results to those with fresh fruit were found.

In order to test the possibility that inhibitory substances were affecting transeliminase activity, the crude extract from an acetone powder preparation was subjected to DEAE-cellulose column chromatography. In no case could the minor increases in absorbancy at 235 nm given by some fractions be substantiated by the TBA test.

Since these increases in absorption at 235 nm might be the shoulder of a peak having a maximum at another wavelength, the entire spectrum of the reaction medium from 220–300 nm was recorded at the beginning of the experiment and again 1 hr later. Examples of the traces when the reaction were carried out at pH 5·2 and 7·5 are given in Figs. 3a and 3b. In neither case was a distinct peak shown at 235 nm in contrast to the behaviour of a similar medium exposed to an authentic sample of PTE (Fig. 3c). Thus the small absorptivity rises given by tomato fruit extracts at 235 nm are very probably not due to the action of PTE.

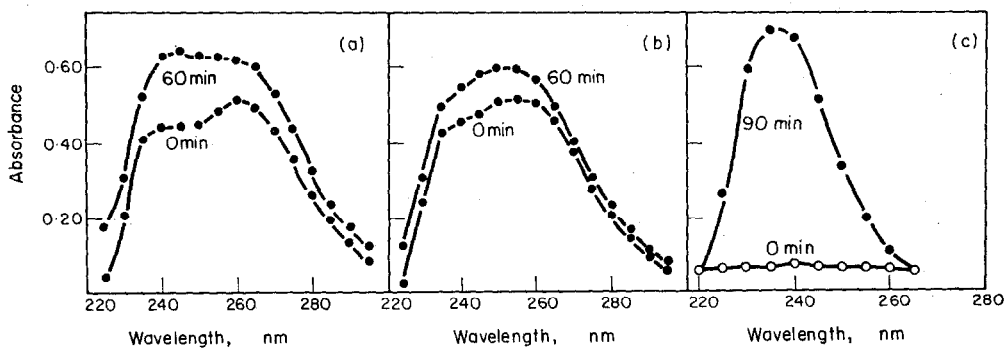

FIG. 3. ABSORPTION SPECTRA OF TOMATO FRUIT EXTRACTS AND OF AUTHENTIC PTE INCUBATED WITH PECTIN SOLUTION

(a) Spectrum of a reaction mixture of 1 ml tomato fruit enzyme (containing 3·2 mg protein) and 10 ml 0·5% citrus pectin in 0·1 M citrate-phosphate buffer, pH 5·2, immediately after the reactants had been mixed and again after incubation at 25° for 1 hr. (b) Spectrum of a similar reaction mixture at pH 7·5 both before and after incubation at 25° for 1 hr. (c) Spectrum of a reaction mixture of 0·1 ml PTE preparation from Pectinol R-10 (containing 14 μg protein) and 10 ml 0·5% citrus pectin in 0·1 M citrate-phosphate buffer, pH 7·5, immediately after mixing and again after incubation at 25° for 90 min.

[8] W. A. AYRES, G. C. PAPAVIZAS and A. F. DIEM, *Phytopath.* **56**, 1006 (1966).

Although the transeliminases are principally associated with microorganisms, pectin transeliminase has been reported in pea seedlings.[9] In attempts to confirm this work, pea seedling extracts incubated with pectin showed a slow increase in absorption at 235 nm, but the TBA test with the reaction products was negative. Even in the presence of infective strains of the symbiotic bacterium *Rhizobium leguminosarum* there was no increase in absorption at 235 nm or a positive TBA test.

The validity of the chromatographic methods was assessed by the partial purification of enzymes from Pectinol R-10 since, according to Albersheim and Killias,[9] this is a good source of PTE as well as containing a number of polygalacturonases. PTE from this source was found to be about as active at pH 7·5 as at pH 5·2 (Table 1). The specific activity was increased 25-fold by DEAE-cellulose column chromatography, and it appeared that complete separation from polygalacturonases had been achieved since there was no peak near 515 nm following the TBA test which would have been given by any free galacturonic acid produced (Fig. 1b).

From these results it seems unlikely that pea seedlings contain an active transeliminase even in the presence of their host-specific nodule bacteria. Nor is there any sign of transeliminase activity in tomato fruit at any stage of maturation or development, and these enzymes do not therefore appear to contribute to the softening mechanism.

Previous methods for the assay of polygalacturonases in tomato fruit based on the increase of reducing groups or on the reduction in viscosity of pectin or sodium polypectate solutions are still valid, as interference by transeliminases does not occur.

EXPERIMENTAL

Extraction procedures with tomato fruit. Fresh or previously frozen (−20°) whole tomato fruit tissue (variety 'Kingley Cross' or 'Potella') was macerated in the cold with 10 × (w/v) of one of the following extractants: (i) 0·5, 1·0 or 2·0 M NaCl-0·1 M Na_2EDTA, (ii) 0·05, 0·5 or 1·0 M NaOAc, (iii) 0·2 M citrate-phosphate buffer, pH 5·2 or pH 7·0, (iv) 0·1 M Tris-phosphate buffer, pH 8·0. Acetone powders were also prepared from previously frozen tissue and extracted with the above buffer systems in the ratio of 1 g powder to 50 ml liquid for 15 min at 2°. For column chromatography, 2 g of acetone powder from tomato fruit were extracted with 100 ml of 0·2 M citrate-phosphate buffer, pH 5·2, for 15 min at 2° and the solids filtered off. The filtrate was dialysed against 2 l. of 0·05 M citrate-phosphate buffer, pH 5·2, for 10 hr at 2°, the buffer and dialysis sack being changed every 2 hr. The contents were then subjected to DEAE-cellulose (Whatman DE 32) chromatography according to the method of Albersheim and Killias.[9]

Extraction procedures with pea seedlings. Pea seeds (variety 'Alaska') were soaked for 24 hr in a 0·2% suspension of thiram,[10] washed in sterilized H_2O and grown in vermiculite for 10 days under weak red light. Lyophilized cultures of *Rhizobium leguminosarum* (Cat. Nos. 1021–6, kindly donated by Dr. F. A. Skinner, Rothamsted Experimental Station, Harpenden, Herts.) were transferred aseptically to mannitol-yeast-agar plates,[11] incubated for 2 weeks at 25°, and then transferred to a mannitol-yeast nutrient solution for a further week at the same temperature. The bacteria were centrifuged at 5000 *g* for 15 min at 2°, suspended in sterilized H_2O and centrifuged again. The roots of 4-day-old pea seedlings were soaked in a suspension of the washed *R. leguminosarum* for 2 hr, replanted in vermiculite and allowed to grow for a further 6 days. Acetone powders from both infected and non-infected plants were prepared, extracted, dialysed and subjected to column chromatography according to the method of Albersheim and Killias.[9]

Assay of PTE and PATE. The pectin substrate for PTE assay was prepared by centrifuging a 2% (w/v) suspension of pectin at 34 000 *g* for 1 hr[12] and the supernatant solution diluted with 3 vol. of the appropriate buffer. 10 ml of this substrate were incubated with 1 ml of enzyme preparation. Optical density changes were measured at 25°.

For a TBA test, the method of Ayres *et al.*[8] was followed. 1 ml of enzyme purified by DEAE-cellulose from Pectinol R-10 was incubated with 3 ml of diluted pectin solution for 3 hr at 25° and the mixture diluted to 10 ml. The solution was clarified by the addition of 0·6 ml of 9% $ZnSO_4 \cdot 9H_2O$ and 0·6 ml of 0·5 N

[9] P. ALBERSHEIM and U. KILLIAS, *Arch. Biochem. Biophys.* **97**, 107 (1962).
[10] R. B. MAUDE, *Ann. Appl. Biol.* **57**, 193 (1966).
[11] P. S. NUTMAN, *J. Bact.* **51**, 411 (1946).
[12] R. J. W. BYRDE and A. H. FIELDING, *J. Gen. Microbiol.* **52**, 287 (1968).

NaOH, shaken and then centrifuged at 16 000 *g* for 15 min at 2°. To 5 ml of the supernatant solution, 3 ml of 0·04 M TBA and 1·5 ml of 1 N HCl were added. After heating at 100° for 30 min, the tubes were cooled and the spectrum between 480 and 570 nm plotted against a blank prepared from an identical mixture but containing enzyme that had been boiled for 3 min.

For the PATE assay, 10 ml of 0·5% Na polypectate solution in 0·1 M citrate–phosphate buffer at pH 7·5 were mixed with 1 ml of enzyme preparation. Optical density changes at 235 nm were measured while the mixture was incubated at 25°, and the TBA procedure was used as for PTE assay. The presence of 1 mM $CaCl_2$ had no effect on the activity of the enzyme extract.[8]

Protein determination. The protein content of the various enzyme solutions was measured by the method of Thompson and Morrison[13] as modified by Biale *et al.*[14]

Chemicals. Citrus pectin (150 grade) was supplied by Hopkin & Williams, Romford, Essex, and sodium polypectate for PATE assay by Sunkist Growers, Ontario, California, U.S.A. Pectinase was supplied by Koch-Light Ltd., Pectinol R-10 by Rohm & Haas, Philadelphia, U.S.A., and α-D-galacturonic acid by Sigma, London.

[13] J. F. THOMSON and G. R. MORRISON, *Analyt. Chem.* **23**, 1153 (1951).
[14] J. B. BIALE, R. E. YOUNG, C. S. POPPER and W. E. APPLEMAN, *Physiologia Pl.* **10**, 48 (1957).