Butler, L. G. *J. Agric. Food Chem.* **1982**, *30*, 1090.
Diagayete, M. *Landwirtsch. Forsch.* **1981**, *37*, 416.
Goldstein, J. L.; Swain, T. *Phytochemistry* **1965**, *4*, 185.
Hagerman, A. E.; Butler, L. G. *J. Agric. Food Chem.* **1978**, *26*, 809.
Jain, P. M.; Beniwal, R. K., Proceedings of the All India Seminar on Top Feed Resources, their Production, Utilization and the Constraints, Central Sheep & Wool Research Institute, Avikanagar, India, Dec 27–28, 1982, p 11.
Lohan, O. P.; Lall, D.; Pal, R. N.; Negi, S. S. *Indian J. Anim. Sci.* **1980**, *50*, 881.
Malik, N. S.; Nath, K. *Indian J. Anim. Sci.* **1970**, *40*, 41.
McLeod, M. N. *Nutr. Abstr. Rev.* **1974**, *44*, 804.
McManus, J. P.; Davis, K. G.; Lilley, T. H.; Haslam, E. *J. Chem. Soc., Chem. Commun.* **1981**, 309.
Naser, J. A.; Coetzer, J. A. W.; Boomker, J.; Cable, H. *J. S. Afr. Vet. Assoc.* **1982**, *53*, 151.
Nath, K.; Malik, M. S.; Singh, O. N. *Aust. J. Agric. Res.* **1969**, *20*, 1137.
Price, M. L.; Butler, L. G. *J. Agric. Food Chem.* **1977**, *25*, 1268.
Price, M. L.; Scoyoc, S. V.; Butler, L. G. *J. Agric. Food Chem.* **1978**, *26*, 1214.
Sandusky, G. E.; Fosnaugh, C. J.; Smith, J. B.; Mohan, R. *J. Am. Vet. Med. Assoc.* **1977**, *171*, 627.
Shirlaw, D. W. G. In "A practical course in Agricultural Chemistry"; Pergamon Press: Oxford, 1967; p 87.
Wolter, R. *Rev. Med. Vet. (Toulouse)* **1974**, *125*, 1481.

Ravindra Kumar
Division of Grass, Fodder, Agronomy & Nutrition
Central Sheep & Wool Research Institute
Avikanagar, Rajasthan, India 304 501

Received for review May 18, 1983. Accepted August 1, 1983.

Electrophoretic Analyses of Selected Enzymes from Tomato Cultivars with Different Fruit Maturation

Acetone powders from Chico III and Homestead-24, two tomato (*Lycopersicon esculentum* M.) cultivars with different maturing characteristics, were prepared at the small green, mature green, turning, and ripe stages of fruit development, and their proteins were extracted. The electrophoretic patterns of six enzyme systems were investigated by disc gel electrophoresis. Malate dehydrogenase (EC 1.1.1.37), alcohol dehydrogenase (EC 1.1.1.1), acid phosphatase (EC 3.1.3.2), and leucine aminopeptidase (EC 3.4.11.2) patterns changed, and the number of their isozymes varied during maturation. The number of peroxidase (EC 1.11.1.7) isozymes increased from the mature green to the ripe stage in both cultivars. The number of esterase (EC 3.1.1.1) isozymes varied but was higher at the ripe stage in Chico III extracts than for Homestead-24. Total protein, on a fresh weight basis, decreased in both cultivars during maturation.

The softening that occurs during the ripening of the tomato fruit is the result of enzymatic breakdown of the cell wall polysaccharides. Hobson (1965) found a relation between firmness and polygalacturonase activity of tomatoes. Babbitt et al. (1973) associated the solubilization of the cell wall protopectin material by pectinase with fruit softening. The possibility that β-glycosidases may contribute to cell wall modification leading to softening has also been reported (Wallner and Walker, 1975). Recently, the loss of fruit firmness in the nonripening rin tomato mutant related to a decrease in cell wall components, notably galactose (Gross and Wallner, 1979). This decrease occurred in the absence of polygalacturonase activity and polyuronide solubilization, suggesting that other components could be involved in cell wall modification.

The enzymes involved during tomato ripening are not known. However, the presence of several enzyme systems in developing tomato fruit has been revealed by disc gel electrophoresis (Hobson, 1974). Several of these enzyme systems were reported to be present in dormant seeds and young tomato seedlings (Stein and Lime, 1978). Since many enzymes are in multiple molecular forms within a single cell, certain enzyme systems have been investigated in depth with respect to their physiological roles in fruit ripening (Hobson, 1974; Hulme, 1972). Pressey and Avants (1972) identified four forms of pectinesterase in extracts of green tomatoes and separated two forms of polygalacturonase forms from ripe tomato extracts using chromatographic techniques (Pressey and Avants, 1973a). Multiple polygalacturonase activity has also been found in ripening peaches (Pressey and Avants, 1973b) and cucumbers (Pressey and Avants, 1975).

The objectives of this study were to (1) confirm the presence of several selected enzymes at four stages of the tomato fruit ripening process and (2) determine the number of molecular forms of each enzyme at each development stage and ascertain if differences existed in enzymic forms between Homestead-24 (soft maturing) and Chico III (firm maturing) tomato cultivars.

MATERIALS AND METHODS

Plant Material. Field-grown tomato fruit from Chico III (C-III) and Homestead-24 (H-24) cultivars were used at the small green, mature green, turning, and ripe fruit stages (Besford and Hobson, 1973). Acetone powders were prepared by immersing 250 g of tomato slices in an acetone–dry ice mixture of −70 °C (Clements, 1965). This technique minimizes the action of proteinases on the enzymes present. The frozen powders were stored at −5 °C to delay denaturation.

Extraction of Proteins. Protein extracts were prepared from 2 g of acetone powder for each fruit stage, according to Hobson's method (1974). The protein content of the crude extract was measured by using the method of Lowry et al. (1951).

Polyacrylamide Disc Gel Electrophoresis. Polyacrylamide gels (7%) were prepared after Davis (1964). Four microliters of the crude protein extract was layered on the gel, and electrophoresis was carried out for 1 h using a current of 3 mA/tube. Electrophoretic separations were

Table I. Protein Content of Chico III and Homestead-24 Tomato Fruit at Different Stages of Fruit Development

developmental stage	protein extracted[a] (acetone powder), mg/g		protein extracted[a] (fresh wt), mg/100 g		% moisture in acetone powder	
	C-III	H-24	C-III	H-24	C-III	H-24
small green	25.19 (20.48-34.20)	23.00 (20.84-25.16)	92.01 (82.08-101.94)	84.23 (75.86-92.59)	7.5	6.7
mature green	31.77 (23.47-41.31)	28.09 (26.11-29.10)	91.62 (73.26-106.34)	70.67 (51.23-81.40)	7.5	6.4
turning	30.12 (16.10-46.89)	33.52 (26.66-43.15)	65.02 (35.40-90.41)	67.85 (53.90-86.32)	6.8	9.6
ripe	16.30 (13.07-18.53)	19.01 (11.70-29.15)	28.74 (22.58-32.52)	28.49 (19.66-36.15)	8.3	6.6

[a] Mean of three extractions. Range in parentheses.

Table II. Number of Enzyme Forms in Chico III and Homestead-24 Extracts at Different Stages of Fruit Development

	small green		mature green		turning		ripe	
	C-III	H-24	C-III	H-24	C-III	H-24	C-III	H-24
malic dehydrogenase (MDH)	5	4	4	4	2	2	4	3
peroxidase (Per)	6	6	5	6	9	10	9	9
alcohol dehydrogenase (ADH)	1	1	4	4	0	0	2	2
esterase (Est)	3	2	4	4	3	3	6	4
acid phosphatase (H^+Ptase)	1	1	3	3	2	2	1	1
leucine aminopeptidase (LAP)	0	0	2	2	0	0	4	2
total	16	14	22	23	16	17	26	21

conducted at 5 °C. Immediately after electrophoresis, the gels were removed from the tubes and stained by incubating in enzyme-specific substrate mixtures. The distance that each band migrated toward the anode from the point of origin was measured.

Enzyme Assays. Peroxidase (Per.) and nonspecific esterases (Est.) were assayed by the method of Brewbaker et al. (1968). The method of Thomas and Neucere (1973) was used to detect malate dehydrogenase (MDH). Alcohol dehydrogenase (ADH) was detected by Kadam's method (1973). The method of Desborough and Peloquin (1971) was used to detect acid phosphatase (H^+Ptase). Aminopeptidase (LAP) was detected by using the staining procedure of Rudolph and Stahman (1966).

RESULTS AND DISCUSSION

The levels of protein in extracted acetone powders from ripening tomatoes were dependent on fruit maturation (Table I). Total protein, on a fresh weight basis, decreased as maturity increased.

The isozymes of MDH, ADH, LAP, and H^+Ptase varied in number among stages, and no definite trend was established (Table II). This observation agrees with the premise (Hobson, 1974) that during fruit maturation enzyme systems are formed to facilitate a specific change associated with the developmental process and are then degraded and represent enzymes systems in varying stages of degradation. Isozymes of peroxidase, however, increased progressively from the mature to the ripe stage in both cultivars. The number of esterase isozymes changed among stages and increased to six in the ripe stage of the C-III cultivar, an observation previously reported (Hobson, 1963).

The electrophoretic patterns of peroxidase indicated shifts in isozymes for C-III and H-24 tomatoes at different stages of ripening (Figure 1). The pattern for both cultivars was similar at the small green stage, except for a second band that was present in the H-24 but was absent in C-III gel. The staining of two fastest moving bands in H-24 was more pronounced than corresponding bands in C-III, indicating greater peroxidase activity and thus greater amounts of protein. At the mature green stage two peroxidase isozymes migrated the same distance in both cultivars. The activities of these forms in C-III were the same as the corresponding bands in H-24. All other forms at this stage had different migrating distances. A greater number of peroxidase isozymes were observed for the

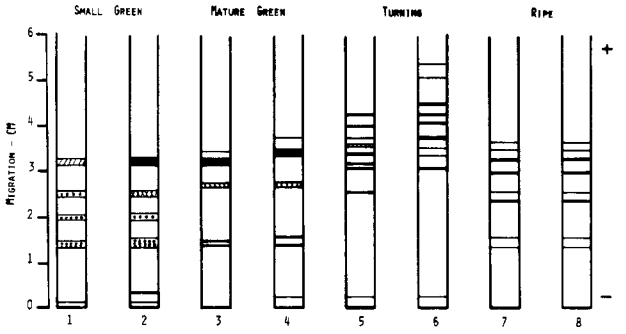

Figure 1. Diagramatic representation of the disc gel electrophoretic separation of Chico III (1, 3, 5, 7) and Homestead-24 (2, 4, 6, 8) peroxidase isozymes in small green, mature green, turning, and ripe tomato fruit. Very light (···), light (///), medium (XXX), and dark bands (■).

turning stage tomato extracts than for the previous stages. However, six of these isozymes had identical migrating distances in both cultivars. Ripe tomato extracts had identical peroxidase isozymes patterns in both cultivars. A wide, prominent band that did not migrate was present in the gels of both ripe fruit extracts. It was not observed in any of the extracts of the other stages. In general, peroxidase isozymes underwent pattern shifts in both cultivars as the tomato fruit matured. This observation is consistent with documented evidence from tissue of other plant species (Scandalios, 1974). Recently, an anionic peroxidase from green and ripe tomato fruit was isolated, identified, and characterized (Kokkinakis and Broods, 1979). More than 80% of the enzymic activity of this peroxidase was due to one peroxidase isozyme; however, its physiological role has not been established. This fact illustrates that isozymes can differ as to enzymic activity and that observed electrophoretic differences between isozymes cannot be ignored. However, the interpretation of an electrophoretic enzyme pattern must be made cautiously, since the isozymes of an enzyme are not always distinctly different molecules produced by different genetic sites but can be the result of proteinase action on the enzyme or other alteration in the structure of the polypeptide and thus in vitro artifacts.

The presence of several previously identified enzymes has been confirmed, and the number of isozymes of selected enzymes at each stage of the fruit developmental process of two tomato cultivars has been established.

Changes in protein, on a fresh weight basis, were noted with increased fruit maturity. The number of isozymes of each enzyme system varied among stages and no trend was observed, except for peroxidase. Disc gel electrophoresis revealed minor differences between peroxidase enzyme patterns of Chico III (firm) and Homestead-24 (soft) tomato cultivars. During fruit development, wide peroxidase bands with low electrophoretic mobilities were replaced by thinner higher mobility bands in both cultivars. At the color-turning stage of fruit development, three peroxidase forms from Homestead-24 extracts exhibited higher mobilities than those of Chico III.

ACKNOWLEDGMENT

The technical assistance of Amelia T. Murray is acknowledged.

Registry No. Peroxidase, 9003-99-0; esterase, 9013-79-0; MDH, 9001-64-3; ADH, 9031-72-5; acid phosphatase, 9001-77-8; LAP, 9054-63-1.

LITERATURE CITED

Babbitt, J. K.; Powers, M. J.; Patterson, M. E. *J. Am. Soc. Hortic. Sci.* **1973**, *98*, 77.
Besford, R. T.; Hobson, G. E. *Photochemistry* **1973**, *12*, 1255.
Brewbaker, J. L.; Upadlya, M. D.; Makinen, Y.; MacDonald, T. *Physiol. Plant.* **1968**, *21*, 930.
Clements, R. L. *Anal. Biochem.* **1965**, *13*, 390.
Davis, B. J. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.
Desborough, S.; Peloquin, S. J. *Phytochemistry* **1971**, *10*, 571.
Gross, K. C.; Wallner, S. J. *Plant Physiol.* **1979**, *36*, 117.
Hobson, G. E. *Biochem J.* **1963**, *68*, 358.
Hobson, G. E. *J. Hortic. Sci.* **1965**, *40*, 66.
Hobson, G. E. *Phytochemistry* **1974**, *13*, 1383.
Hulme, A. C. *J. Food Technol.* **1972**, *7*, 343.
Kadam, S. S.; Singh, J.; Mehta, S. L. *Phytochemistry* **1973**, *12*, 1221.
Kokkinakis, D. M.; Broods, J. L. *Plant Physiol.* **1979**, *63*, 93.
Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randal, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
Pressey, R.; Avants, J. K. *Phytochemistry* **1972**, *11*, 3139.
Pressey, R.; Avants, J. K. *Biochim. Biophys. Acta* **1973a**, *309*, 363.
Pressey, R.; Avants, J. K. *Plant Physiol.* **1973b**, *52*, 252.
Pressey, R.; Avants, J. K. *J. Food Sci.* **1975**, *40*, 937.
Rudolph, K.; Stahman, M. A. *Plant Physiol.* **1966**, *41*, 389.
Scandalios, J. G. *Annu. Rev. Plant Physiol.* **1974**, *25*, 225.
Stein, E. R.; Lime, B. J. *J. Agric. Food Chem.* **1978**, *26*, 239.
Thomas, D. L.; Neucere, J. J. *J. Agric. Food Chem.* **1973**, *21*, 479.
Wallner, S. J.; Walker, J. E. *Plant Physiol.* **1975**, *55*, 94.

Eduardo R. Stein
Agricultural Products Quality Research Unit
Subtropical Agricultural Research Laboratory
Oklahoma-Texas Area, Southern Region
Agricultural Research Service
U.S. Department of Agriculture
Weslaco, Texas 78596

Received for review March 3, 1983. Revised manuscript received June 21, 1983. Accepted August 1, 1983.

Toluene as an Alternative to Benzene in the Woessner Determination of Hydroxyproline

Toluene extraction is a suitable alternative to benzene extraction in the Woessner procedure for the chemical quantitation of 4-hydroxyproline.

Intramuscular collagen content is usually estimated through the chemical determination of 4-hydroxyproline, an amino acid confined essentially to connective tissue proteins. The Woessner (1961) modification of the Stegemann (1958) procedure is the most frequently used method for the determination of hydroxyproline in meat (Etherington and Sims, 1981). This approach involves extractions with benzene, a solvent known to be chronically toxic and carcinogenic (Sax, 1975), even at concentrations much too low to detect through the sense of smell. Its use is either prohibited, discouraged, or permitted with extreme caution. Although the much less commonly used Prockop–Udenfriend (1960) method makes use of extractions with toluene instead of with benzene, the purpose is to extract the reaction intermediates, pyrrole-2-carboxylic acid and pyrrole, from the impurities. On the other hand, the Woessner (1961) procedure makes use of benzene to extract impurities from the chromaphore that is to be analyzed spectrophotometrically. The present study tests the use of toluene as an alternative to benzene in the Woessner (1961) procedure.

MATERIALS AND METHODS

Ten 75% or 100% Simmental or Limousin steers were slaughtered at approximately 15 months of age and 450 kg live weight. One steak was removed from the longissimus dorsi muscle of the left side of the carcass 6 days postslaughter. A thin slice (2 mm thick) was removed from each steak, freeze-dried under vacuum for approximately 72 h, and then broken up by blending with a Virtis homogenizer. A 0.5-g portion was subjected to the salt extraction procedure of Hill (1966) to yield salt-soluble and salt-insoluble fractions. These fractions were hydrolyzed in 6 N HCl at 113 °C for 18 h, neutralized, and made to a volume of 150 mL. The hydrolysate of the salt-insoluble fraction was diluted 5-fold with distilled water before analysis. All hydrolysates were then analyzed for hydroxyproline content using "method II" of Woessner (1961). This analysis includes (1) oxidation by Chloramine T, (2) perchloric acid treatment, (3) incubation with *p*-(dimethylamino)benzaldehyde at 60 °C followed by cooling, (4) extraction with benzene, and (5) measurement of the absorbance at 557 nm before and after peroxide treatment. Each muscle sample was analyzed in triplicate and each standard was analyzed in quadruplicate. The volumes of all sample and reagent solutions added prior to benzene extraction were increased 3-fold over those used by Woessner (1961). Following step 3, two 5-mL aliquots were removed from each reaction mixture. One was extracted with benzene in the normal fashion and the other

0021-8561/83/1431-1368$01.50/0 Published 1983 by the American Chemical Society