cesses and eluent volume should be an important factor influencing its displacement.

Increasing eluent volume from 25 to 50 ml significantly increased thiamine recovery (mean recovery increased from 90 to 97.5%) and decreased the standard deviation from 2.15 to 1.53 (Table V). Hence, eluent volume is an important factor affecting both the quantity and reproducibility of thiamine recovery from Decalso.

Elution to 50 ml requires more reagent and time than for 25 ml. However, the acid-KCl eluent is not expensive so time is the more important consideration. It averaged 15.9 min longer (about 29% longer) per column to elute to 50 ml than to 25 ml (Table VI). However, the additional time is not cumulative when 6–12 columns are run at once.

Hence, for maximum precision and accuracy in the thiochrome method the important effects of eluent temperature and volume on thiamine recovery from Decalso must be recognized and controlled. Both factors could cause inaccuracy when sample extracts are purified on Decalso by hot elutions on unthermostated columns to 25-ml eluent volume. The nearly quantitative recovery of thiamine possible on unthermostated columns by hot elution to 50 ml suggests a standard curve may now be used in the thiochrome method instead of standards run for every set of determinations as is now generally recommended.

LITERATURE CITED

Association of Official Agricultural Chemists, "Official Methods of Analysis," 11th ed, 1970, pp 772, 39.027.
Bechtel, W. G., Hollenbeck, C. M., *Cereal Chem.* **35**, 1 (1958).
Freed, M., "Methods of Vitamin Assay," 3rd ed, Interscience, New York, N.Y., 1966, p 129.
Harris, L. J., Wang, Y. L., *Biochem. J.* **35**, 1050 (1941).
Hennessy, D. J., Cerecedo, L. R., *J. Am. Chem. Soc.* **61**, 179 (1939).
Jowett, M., *Biochem. J.* **34**, 1348 (1940).
Strohecker, R., Henning, H. M., "Vitamin Assay: Tested Methods," translated by D. D. Libman, Chemical Rubber Publishing Co., Cleveland, Ohio, 2nd Printing, revised, 1966, pp 65–97.

Received for review August 5, 1974. Accepted December 29, 1974. Reference to a company or product name does not imply recommendation by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Gel Filtration and Disc Gel Electrophoresis of Tomato Pectic Substances

Eduardo R. Stein and Harold E. Brown*

Pectic substances were extracted from tomato alcohol insoluble solids (AIS) according to their solubility in water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide. Gel filtration with Sephadex G-200 indicated that all fractions were heterogeneous in molecular size, the majority having molecular weights of 2×10^5 or more. Small differences were noted in the gel filtration patterns of all the solubility fractions indicating differences in molecular weights of pectic substances from the Chico III and Homestead-24 cultivars. Disc gel electrophoresis also revealed a heterogeneous set of molecules with respect to molecular charge. At least three distinct bands or zones were easily distinguishable in each solubility fraction, and discernible differences in electrophoretic patterns were noted in pectic substances of the two cultivars.

Pectic substances of fruits and vegetables have been associated with the texture and firmness of fresh and processed products (Kertesz, 1951) as well as with the viscosity of juices (Kertesz and Loconti, 1944) and purees (McColloch and Kertesz, 1949). The softening of certain fruits upon ripening has been attributed to a decrease in the molecular size of the pectic substances (McCready and McComb, 1954). Both quality and quantity of pectic substances influence the viscosity of tomato pastes (McColloch et al., 1950) and the consistency of pureed tomato products (Luh et al., 1954).

The characterization of tomato pectic substances has dealt mainly with the composition of those found primarily in the water soluble fraction. Methods for extracting pectic substances have not been standardized, but a common procedure involves the sequential extraction of tissue with hot water, dilute mineral acid, and dilute alkali or ammonium oxalate (Kertesz, 1951; King and Bayley, 1963; Owens et al., 1952). Molecular weights of pectic substances have been shown to exhibit a high degree of variability and are dependent on both source and isolation technique (Worth, 1967). Furthermore, the shape of tomato has been implicated in the quantity of pectic substances (McColloch et al., 1950) and could possibly affect the molecular distribution of pectic substances. Therefore, it was the purpose of this study to determine and compare the molecular species of pectic substances occurring in the alcohol-insoluble solids following hot water, dilute mineral acid, dilute ammonium oxalate, and dilute sodium hydroxide extraction of two tomato cultivars, Chico III (pear-shaped) and Homestead-24 (round-type) tomatoes.

Food Crops Utilization Research Laboratory, Subtropical Texas Area, Southern Region, Agricultural Research Service, U.S. Department of Agriculture, Weslaco, Texas 78596.

MATERIALS AND METHODS

Preparation of Tomato Sample. Chico III and Homestead-24 tomato cultivars were planted in the spring of 1970 and 1971 by the Texas Agricultural Experiment Station, Weslaco, Tex. Fully red ripe samples were handpicked to avoid damaged and nonuniform fruit, and were carefully washed. Samples of approximately 600 g were selected, frozen solid with liquid nitrogen, finely crushed, sealed in cans, and stored at −20° until the alcohol-insoluble solids (AIS) were prepared. Quick freezing of the tomato tissue prevented the degradation of pectic substances observed during the crushing process.

The crushed, frozen samples were weighed and slowly added to boiling 95% ethanol (2 parts ethanol to 1 part tomatoes) in a Waring Blendor equipped with a stainless steel blendor vessel and heavy duty heating straps to keep the alcohol near boiling during constant blending. The

Table I. Alcohol-Insoluble Solids (AIS) and Pectic Substances from Chico III and Homestead-24 Tomatoes

	Chico III, %	Homestead-24, %
Alcohol insoluble solid[a]	1.73	1.40
Total pectic substances[b]	22.6	26.1
H_2O soluble	10.5	12.0
Oxalate soluble	4.2	5.6
HCl soluble	2.6	3.2
NaOH soluble	5.3	5.3

[a] Alcohol insoluble solids, percent of fresh weight, are an average of duplicate determinations on 40 samples for Chico III and 30 samples for Homestead-24 tomatoes over 2-year period. [b] Percent of alcohol insoluble solids.

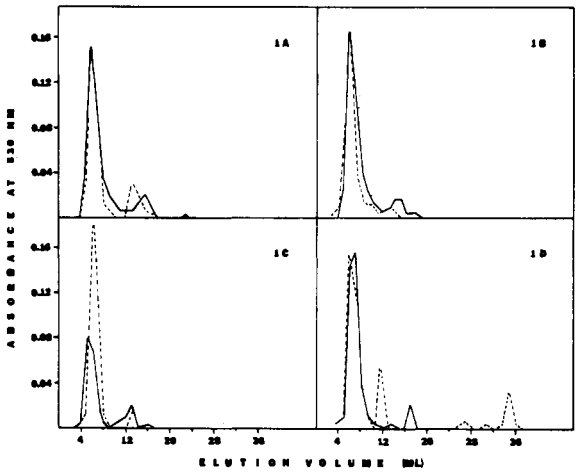

Figure 1. Elution pattern for Homestead-24 (—) and Chico III (- - -) pectic fractions, pH 5.5, at an elution rate of 5.0 ml/hr: column size, 0.9 cm × 30 cm; bed volume, 17.3 ml; bed material, Sephadex G-200; eluent, water; (A) water-soluble pectin; (B) 0.2% ammonium oxalate soluble pectin; (C) 0.05 *N* hydrochloric acid soluble pectins; and (D) 0.05 *N* sodium hydroxide soluble pectins.

macerate was blended for 2 min, placed in a 2-l. beaker, heated to boiling, and allowed to stand overnight. The sample weight was adjusted to the original weight by carefully removing the excess supernatant liquid. Samples were stored at −20° for subsequent analysis.

Preparation of Alcohol-Insoluble Solids (AIS). The AIS were determined according to the methods of McColloch et al. (1950) except that a soxhlet extractor was used. The tomato macerate was weighed into an extraction thimble, extracted six times with 95% ethanol, and then extracted with diethyl ether until the extractant was colorless. The tomato AIS was then dried in a vacuum oven at 70°, cooled, and weighed. Larger batches of AIS were prepared in a similar manner by using a large soxhlet extractor. These larger batches of AIS were used for the extraction of the pectic substance.

Extraction of Pectic Substances. Pectic substances were extracted from the AIS by using exhaustive extraction with water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide. All extractions were performed at 70°. Each set of extractions was combined, concentrated to 300 ml with a rotary evaporator, and adjusted to a pH of 5.5. Pectic substances in the concentrated extract were determined according to the carbazole–hexuronic acid–sulfuric acid method of Dische (1946).

Gel Filtration. Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was used for the gel filtration fractionation of the pectic substances. The gels were prepared according to the recommendations of the supplier using a 0.9 cm × 30 cm column. Approximately 80 μg of pectin sample was placed on the column and eluted with distilled water at a flow rate of about 5 ml/hr. A Buchler Fractomat automatic fraction collector equipped with a 1.0-ml sample collection tube was used for fraction collection. The pectic substances collected in each 1.0-ml fraction were measured colorimetrically by the carbazole–hexuronic acid–sulfuric acid method of Dische (1946) by using galacturonic acid as the standard.

Disc Gel Electrophoresis. The pectic samples used for disc gel electrophoresis studies were precipitated and washed prior to electrophoretic analysis. Pectic substances in each solubility fraction were precipitated by the addition of 2 vol of 95% ethanol containing 0.05 *N* hydrochloric acid. The gelatinous precipitate was washed three times with 70% ethanol and was collected and dried under vacuum in a Stokes Food Freeze Drier. The dried material was ground and used in the analyses.

Disc gel electrophoresis was performed by using 7.5% polyacrylamide gels according to the method of Do et al. (1971). Gel columns were prepared according to the method of Davis (1964). A 1% solution of each of the dried pectic samples was prepared for addition to the columns. Deesterified samples were prepared by adding 25 μl of 5 *N* sodium hydroxide to a 2.0-ml aliquot of the 1% pectic solution; they were allowed to stand at room temperature for 30 min and were neutralized with 25 μl of 5 *N* hydrochloric acid. Thirty-microliter samples of pectin and deesterified pectin were added to the gel column. Electrophoresis was performed on a Canalco Model 1200 disc gel electrophoresis apparatus by using 0.2 *M* borax–boric acid buffer, pH 9.3. A current of 1 mA per tube was applied for 15 min followed by 2 mA per tube for 4 hr. The gels were immediately removed from the tubes and stained with a periodic acid–Schiff's reagent (Hotchkiss, 1948) for 30 min for visualization of the bands.

RESULTS AND DISCUSSION

The use of water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide provided a degree of fractionation of the pectic substances in the tomato AIS. The sequence in which these extractants were used had an influence on the amount of pectic materials extracted. Therefore, the sequence of extractants as listed in Table I was used in order to obtain comparable pectic fractions between the two cultivars using the different solvents. Morre and Olson (1965) had noted several different fractions of *Avena* coleoptile pectins based on the solubility in various extractants. The results obtained by extracting the pectic substances from Chico III and Homestead-24 AIS appear in Table I.

Gel fractionation separated the pectic substances in each pectic fraction into several molecular components. Typical fractionation patterns for each pectic fraction from two varieties of tomatoes are shown in Figure 1. Sephadex G-200 separated each solubility fraction of pectins from both varieties of tomatoes into two or more component peaks, each representing at least one molecular size or shape. The major peak from each fraction represents one or more molecular components having an average molecular weight greater than 2×10^5. The molecular weight is based entirely on the molecular exclusion limits of the Sephadex G-200 gel. Barrett and Northcote (1965) were able to separate apple pectic substances into a pure pectinic acid and a neutral arabinan–galactan complex which was further separated into two components having a molecular weight, as estimated by gel filtration, of 2×10^5 or more. The values for molecular weight of tomato pectin are in agreement with those estimated by Barrett and Northcote (1965), but greater than those reported by Jos-

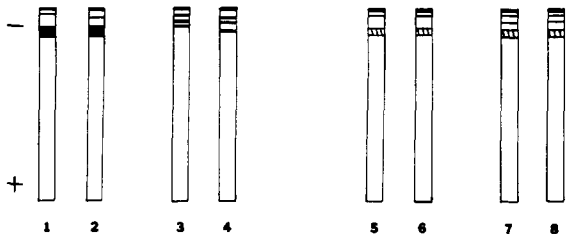

Figure 2. Diagrammatic representation of electrophoretic patterns of water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide soluble pectin fractions from Homestead-24 and Chico III tomatoes. (Diagram numbers 1, 3, 5, and 7 represent Homestead-24 pectin fractions and numbers 2, 4, 6, and 8 represent Chico III pectin fractions.) Dark bands represent heavy staining and moderate staining is represented by single hatching. The pectins were separated anodally.

lyn (1962) and Worth (1967) except for apple and lemon pectin leached out without boiling. The apparent high molecular weight of tomato pectin may have been caused by an aggregation of smaller molecular species as suggested by other investigators (Kertesz, 1939; Pallman et al., 1944; Joslyn, 1962; Barrett and Northcote, 1965).

All solubility fractions of tomato pectic substances exhibited slight differences in these fractionation patterns when subjected to Sephadex G-200 gel filtration. Each of the fractions from the two cultivars differed in the number of component peaks or in the elution volumes of the peaks (Figure 1).

The water-soluble pectic fractions from the two cultivars differed in number of peaks and elution volumes. Homestead-24 water-soluble pectins yielded four fractions while Chico III pectins had two component peaks. Homestead-24 minor peaks were eluted from the column at 10, 15, and 22 ml while the comparable fraction from Chico III had a single minor peak at 13 ml on the chromatogram. The major peaks of both water-soluble fractions were comparable (Figure 1A).

The elution patterns of the ammonium oxalate soluble fractions showed a difference in elution volumes for the minor peaks. The Homestead-24 oxalate soluble fraction had minor peaks being eluted at 14 to 15 and 17 ml compared to minor peaks appearing at 10 and 13 ml for the Chico III pectic fraction (Figure 1B).

The hydrochloric acid soluble pectic fraction from the two cultivars differed in the number of minor peaks as well as the relative size of the major peak. The Homestead-24 fraction had minor peaks being eluted at 10, 13, and 16 ml compared to a single minor peak for the Chico III fraction being eluted at 14 ml (Figure 1C).

The slight variations in elution patterns of the pectic fractions from the two cultivars may indicate slight differences in the molecular components of the pectins or may be due to degradation products formed during preparation and extraction. However, different loading effects due to different amounts of pectic substances in the fractions could cause a similar molecular behavior.

The elution patterns of the sodium hydroxide extracted pectins were not consistent. Inconsistencies were observed in the number of component peaks and in their elution volumes. There were always three or more components appearing on the chromatograms. Size of the peaks and elution volumes were inconsistent and no trend was established (Figure 1D). However, the major component peak always eluted at the exclusion volume of the gel. It has been reported that sodium hydroxide extraction of pectic substances will degrade pectins into smaller units (Barrett and Northcote, 1965). This may possibly explain the inconsistencies observed and the numerous component peaks obtained on gel filtration of the sodium hydroxide pectic fraction. Electrophoretic patterns of pectins extracted from both cultivars with water and dilute hydrochloric acid showed three distinct and separate component bands representing three molecular species with different charges (Figure 2). One major band, of intense color, did not migrate and was always present at the origin; a second minor band migrated 2 or 3 mm from the origin. Another major band, the last to appear, migrated a distance of 6 mm from the origin. Electrophoresis of pectins from both varieties of tomatoes extracted with dilute ammonium oxalate and dilute sodium hydroxide showed a pattern similar to the patterns of other fractions; in addition a fourth component band was always present 4 mm from the origin (Figure 2). The component bands of the water- and oxalate-soluble pectins were of a more intense color than were those of the hydrochloric acid and sodium hydroxide soluble pectins. The electrophoretic patterns of Homestead-24 and Chico III deesterified water-soluble pectin possessed one more band than did the water-soluble pectin (Figure 3). This same pattern was observed in the Chico III acid-soluble and deesterified acid-soluble pectins (Figure 3). A difference was observed between the mobility pattern of one minor component band from the Homestead-24 acid soluble pectins and deesterified acid-soluble pectins (Figure 3). No differences in the number of bands were observed between the electrophoretic patterns of the ammonium oxalate soluble pectins and deesterified ammonium oxalate soluble pectins of both varieties (Figure 3). Slight mobility differences between minor component bands were observed for deesterified ammonium oxalate soluble pectins from both varieties.

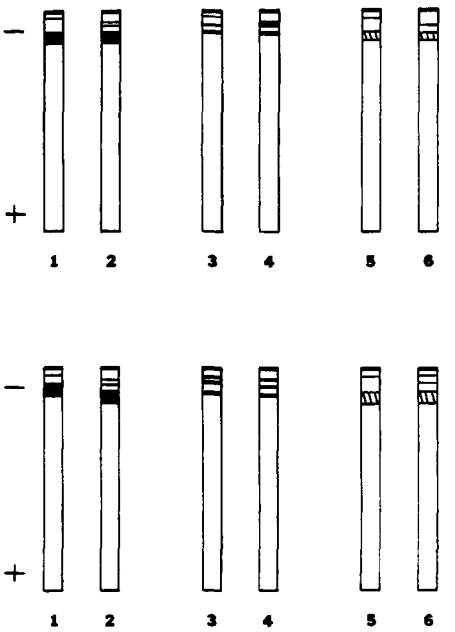

Figure 3. Diagrammatic representations comparing the electrophoretic patterns of Homestead-24 (top diagram) and Chico III (bottom diagram) pectic fractions (diagram numbers 1, 3, and 5) and deesterified pectic fractions (diagram numbers 2, 4, and 6). Diagram numbers 1 and 2 represent water-soluble fractions, 3 and 4 0.2% ammonium oxalate fractions, and 5 and 6 0.05 *N* hydrochloric acid fractions. Dark bands represent heavy staining and moderate staining is represented by single hatching. The pectins were separated anodally.

Do et al. (1971) reported that the electrophoretic pattern of deesterified citrus pectin differs from the pattern of citrus pectin by one component band. These researchers observed a greater mobility of the deesterified pectin major bands than for the pectin major bands. The observations on deesterified tomato pectins differ in that the minor component bands exhibited the greater mobility. Electrophoretic patterns of tomato pectins also showed fewer component bands than did the pattern reported for citrus.

The fourth band and the greater mobility observed after deesterification of the water- and acid-soluble pectins could be explained via the hypothesis that the basic structure of pectins might involve linkages of different types and of different degrees of vulnerability and molecular configuration (Kertesz, 1951). Treatment with alkali may liberate sterically hindered esterified carboxyl groups which can only be deesterified after they are exposed thus increasing the net molecular charge and giving rise to changes in molecular size and shape.

CONCLUSIONS

The study showed that tomato pectic substances extracted from alcohol-insoluble solids according to their solubilities in water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide can be further fractionated by using the techniques of gel filtration and disc gel electrophoresis.

Gel filtration, based on the properties of Sephadex G-200, demonstrated that the water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide soluble pectins are heterogenous in molecular size with a majority of the pectic substances having molecular weight of 2×10^5 or more. Small differences in the pectic substances of the two cultivars were detected by using gel filtration. These small differences were observed in each of the solubility fractions.

Disc gel electrophoresis indicated at least three distinct groups of pectic compounds possessing slightly different molecular charges, further confirming the heterogeneity of each pectic fraction. Electrophoretic patterns of the pectic fractions from the two varieties showed quantitative differences when the intensities of their colored bands were compared. Deesterification of the same pectic fractions showed differences between the number of component bands of the water-soluble pectins from both cultivars and of the hydrochloric acid soluble pectin from the Chico III variety.

LITERATURE CITED

Barrett, A. J., Northcote, D. H., *Biochem. J.* **94,** 617 (1965).
Davis, J., *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).
Dische, J., *J. Biol. Chem.* **167,** 189 (1946).
Do, J. Y., Ioannou, J., Haard, N. F., *J. Food Sci.* **36,** 1137 (1971).
Hotchkiss, R. D., *Arch. Biochem.* **16,** 131 (1948).
Joslyn, M. A., *Adv. Food Res.* **11,** 63 (1962).
Kertesz, Z. I., "The Pectic Substances", Interscience, New York, N.Y., 1951.
Kertesz, Z. I., Loconti, J. D., *N. Y. State Agric. Exp. Stn. Bull.*, 272 (1944).
King, N. J., Bayley, S. T., *Can. J. Bot.* **41,** 1141 (1963).
Luh, B. S., Dempsey, W. D., Leonard, S., *Food Technol.* **8,** 576 (1954).
McColloch, R. J., Kertesz, Z. I., *Food Technol.* **3,** 94 (1949).
McColloch, R. J., Nielson, B. W., Beavens, E. A., *Food Technol.* **4,** 339 (1950).
McCready, R. M., McComb, E. A., *Food Res.* **19,** 530 (1954).
Morre, D. J., Olson, A. C., *Can. J. Bot.* **43,** 1083 (1965).
Owens, H. S., McCready, R. M., Shepherd, A. D., Schults, T. H., Pippen, E. L., Swenson, H. A., Mier, J. C., Erlandsen, R. F., McClay, W. D., U.S. Department of Agriculture, Bureau of Agriculture and Industrial Chemical Agriculture Research Administration Mimeo Publication A.I.C. 340, 1952.
Pallman, H., Weber, F., Deuel, H., *Schweiz. Landwirtsch. Monatsh.* **22,** 306 (1944).
Worth, H. D., *J. Chem. Rev.* **67,** 465 (1967).

Received for review July 10, 1974. Accepted December 24, 1974. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Fractionation and Characterization of Major Reserve Proteins from Seeds of *Phaseolus vulgaris*

Keizo Ishino*[1] and María Luisa Ortega D.

The globulin fraction (75% of the total protein) of "negro mecentral" bean (*Phaseolus vulgaris*) consisted of four major components, α, β, γ, and δ, designated in order of decreasing electrophoretic mobility, which accounted for 50, 19, 10, and 12% of the globulin, respectively. The α component (14.55% of nitrogen) had a $s_{20,w}{}^{0.53\,\%} = 7.42$ S and an approximate molecular weight of 170,000. Also this component was a glycoprotein which contained 4.95% of carbohydrate (as mannose) and 1.19% of hexosamine (as glucosamine), and was deficient in the sulfur-containing amino acids. Although neither 8 *M* urea nor 0.2 *M* 2-mercaptoethanol treatments induced dissociation of the α component, alkali treatment (pH 12.5) caused dissociation into the subunits, although not completely even after 24 hr. The β and γ components dissociated immediately with alkali. At pH 4.1, 62% of the globulin remained soluble in acetate buffer with $\mu = 0.5$, and 57% with $\mu = 0.1$. The resolubility of acid precipitated protein was 65% in the phosphate buffer (pH 7.5), $\mu = 0.5$. The α and β components seemed to form insoluble complexes during acidification.

In Latin American countries, many kinds of bean have been used for food from prehistoric times and, together with corn, still hold an important position in the diet of the people. At present, processing of beans is limited to boiling, but it is possible that new, more highly processed foodstuffs could be developed based on the reserve proteins in the seeds. Moreover, studies of bean protein comparable to those of soybean have not been done. Therefore, we think that it is important to investigate the nature of the reserve proteins of bean.

As constituents of seed protein (*Phaseolus vulgaris*), phaseolin, phaselin, and conphaseolin have been known for a long time (Osborne, 1894; Waterman *et al.*, 1923; Jones *et al.*, 1937–1938). It has been reported by Osborne (1894) that phaseolin accounts for about 20% of the seed dry weight, that is, 85% of crude total protein. The two other fractions, phaselin and conphaseolin, make up 2%

Laboratorio de Bioquímica, Rama de Botánica, Colegio de Postgraduados, Escuela Nacional de Agricultura, Chapingo, México.
[1] Present address: Asahimatsu-Koritofu Co., Ltd., Matsuo Iida-City, Nagano-Prefecture, Japan.