

Analysis of Pectate Lyases Produced by Soft Rot Bacteria Associated with Spoilage of Vegetables

CHING-HSING LIAO

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

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Isoelectric focusing (IEF) profiles of pectate lyases (PLs) produced by five different groups of soft rot bacteria were analyzed by using the combined techniques of thin-layer polyacrylamide gel IEF and agarose-pectate overlay activity staining. Four strains of soft rot *Erwinia* spp. produced three or more PL isozymes. All of eight *Pseudomonas viridiflava* strains examined produced one single PL with a pI of 9.7. All 10 of *Pseudomonas fluorescens* strains produced two PLs; the major one had a pI of 10.0 and the minor one had a pI of 6.7. A single PL with a pI of ≥ 10.0 was detected in one strain each of *Xanthomonas campestris* and *Cytophaga johnsonae*. PLs of six representative strains were purified from culture supernatants by ammonium sulfate precipitation and anion-exchange chromatography. All purified PL samples macerated potato slices, but to different degrees. The M_r s of alkaline PLs produced by *P. viridiflava*, *P. fluorescens*, *X. campestris*, and *C. johnsonae* were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 42,000, 41,000, 41,500, and 35,000, respectively. IEF profiles of PLs were distinct among the bacterial species. Profiles of non-*Erwinia* spoilage bacteria were considerably simpler than those of *Erwinia* spp. The PL with an alkaline pI appeared to be the principal or the sole enzymatic factor involved in tissue maceration caused by most strains of soft rot bacteria.

The ability of soft rot bacteria to cause vegetable spoilage results mainly from their ability to produce massive amounts of pectic enzymes. Pectate lyase (PL; EC 4.2.2.2), which cleaves polygalacturonates by β -trans-elimination, is believed to be the principal enzyme responsible for tissue maceration, electrolyte loss, and cell death (22). Activities of PL have been detected previously in culture supernatants of diverse groups of soft rot bacteria (20), including *Erwinia* spp. (27, 34, 36), *Pseudomonas fluorescens* (or *Pseudomonas marginalis*) (7, 9, 10, 23, 37), *Pseudomonas viridiflava* (10, 15), *Xanthomonas campestris* (17, 24), and *Cytophaga johnsonae* (13, 16, 35). The PL produced by *Erwinia* spp. has been studied extensively and is unique for its occurrence as multiple (≥ 3) isozymic forms (12). In *Erwinia chrysanthemi* for example, five PL isozymes with pIs ranging from 4.0 to 10.0 have been detected in most strains examined so far (2, 29, 34). Several studies indicate that alkaline PL (pI, ≥ 9.5) is more efficient than neutral or acidic PLs in inducing tissue disintegration or tissue maceration (1, 25, 30, 31, 33) and that alkaline PL by itself is sufficient to macerate potato tuber and other plant tissues (11, 25, 33). The physiological basis for the production of more PLs than are required to macerate plant tissue by *Erwinia* spp. is not understood. It has been suggested that some PL activities (or isozymes) may be required for other pathological functions such as recognition of the host (1, 33) and induction of disease resistance (5).

At present, little is known about the enzymatic mechanism which controls the soft rot pathogenicity of non-*Erwinia* spoilage bacteria. Two recent studies (15, 32) suggest that pectic enzyme systems of non-*Erwinia* spoilage bacteria are not as complex as those demonstrated in *Erwinia* spp. Schlemmer et al. (32) have shown that *P. fluorescens* W51 produces a pectin lyase (pI, 9.4) that is required for maceration of plant tissue. Liao et al. (15) have isolated and purified a single PL (pI 9.7) from culture supernatants of *P. viridiflava* SF312 and have shown that the bacterium became

nonpathogenic when the gene encoding for PL synthesis or secretion was inactivated by Tn5 transposition. It remains to be determined whether the simple pectic enzyme system, as demonstrated in the two pseudomonads described above, represents a common feature of non-*Erwinia* soft rot bacteria.

The objectives of this study were (i) to examine the isoelectric focusing (IEF) profiles of PLs produced by five different groups of soft rot bacteria, including 4 strains of *Erwinia* spp., 8 strains of *P. viridiflava*, 10 strains of *P. fluorescens*, and 1 strain each of *X. campestris* and *C. johnsonae*; and (ii) to determine the pIs, M_r s, and macerating abilities of PLs purified from six representative strains.

(A preliminary account of the results of this study have been presented previously [C.-H. Liao, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P-28, p. 278].)

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the origins and sources of the 25 strains of soft rot bacteria used in this study. These strains were isolated from rotten specimens of various vegetables. With the exception of *P. fluorescens* PJ-08-14, all strains have been shown to macerate potato slices and other plant tissues by producing large amounts of PLs (18). *P. fluorescens* PJ-08-14 produced PL intracellularly and exhibited little or no tissue-macerating ability. All of the bacterial strains were maintained on *Pseudomonas* Agar F (Difco Laboratories, Detroit, Mich.) and were grown to the late-stationary phase in MMY broth medium at 20°C for enzyme preparation. The MMY medium (pH 7.1) contained K_2HPO_4 , 0.7%; KH_2PO_4 , 0.2%; $MgSO_4 \cdot 7H_2O$, 0.02%; $(NH_4)_2SO_4$, 0.1%; $CaCl_2$, 1 mM; yeast extract, 0.1%; and pectin (grade 1, 0.3%; Sigma Chemical Co., St. Louis, Mo.).

Assay of PL activity. PL activities were determined by a spectrophotometric method (37). One unit of activity was defined as the amount of enzyme which produced an in-

TABLE 1. Bacterial strains used in this study

Strain ^a	Host origin	Source or reference
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
SR319	Potato	A. Kelman
CU-06-1	Cucumber	18
<i>Erwinia carotovora</i> subsp. <i>atro-septica</i> SR8		
	Potato	A. Kelman
<i>Erwinia chrysanthemi</i> EC16		
	Chrysanthemum	A. Chatterjee
<i>Pseudomonas fluorescens</i>		
CY091	Celery	18
17816	Dahlia	ATCC ^b
SJ-08-2	Squash	18
SJ-08-3	Squash	18
PJ-08-30	Pepper	18
PJ-08-14	Pepper	18
BC-05-1B	Broccoli	18
BC-05-2B	Broccoli	18
LC-04-2B	Lettuce	18
AJ-06-2A	Asparagus	18
HU-08-11B	Spinach	18
<i>Pseudomonas viridiflava</i>		
SF312	Squash	18
13223	Bean	ATCC
SF-05-3	Squash	18
PJ-09-6A	Pepper	18
PJ-08-6B	Pepper	18
PJ-08-9	Pepper	18
PJ-08-16B	Pepper	18
TU-04-2A	Tomato	18
<i>Xanthomonas campestris</i> CJ091		
	Cucumber	17
<i>Cytophaga johnsonae</i> PF062		
	Pepper	16

^a The ability to macerate potato slices was demonstrated in all strains except *Pseudomonas fluorescens* PJ-08-14, which produced PL intracellularly but did not excrete the enzyme out of the cell.

^b ATCC, American Type Culture Collection, Rockville, Md.

crease of 1.73 units in the A_{232} per min at 30°C. An increase in the A_{232} of 1.73 was considered to represent the formation of 1 μ mol of unsaturated uronides. The reaction mixture (2 to 3 ml) contained 100 mM Tris hydrochloride (Tris-HCl) (pH 8.5), 0.25% sodium polygalacturonate (Sigma), 1 mM CaCl_2 , and enzyme sample.

Preparation of enzyme samples. Culture supernatants obtained after centrifugation (10,000 $\times g$, 10 min) were concentrated and equilibrated with 50 mM Tris-HCl (pH 7.5) by ultrafiltration (Centricon 10 microconcentrator; Amicon Corp., Danvers, Mass.). The concentrated samples containing 0.1 to 0.3 U of PL activity per μ l were used to determine extracellular PL profiles. For the preparation of intracellular PL samples, the following procedures were used. Cell pellets obtained after centrifugation were washed twice and suspended in 50 mM Tris-HCl (pH 7.5) buffer in a 1/100 volume of the culture medium. Cells were disrupted by ultrasonication, and cell debris was removed by centrifugation (350,000 $\times g$, 30 min). The clear supernatant was used directly to determine intracellular PL profiles.

Purification of PLs. The purification scheme previously described for the extracellular PL of *P. viridiflava* was followed (15). Culture supernatants obtained from six representative strains (*P. fluorescens* CY091 and BC-05-1B, *P.*

viridiflava SF312, *X. campestris* CJ091, *C. johnsonae* PF062, and *E. chrysanthemi* EC16) were treated with ammonium sulfate. The precipitate that formed at 50 to 85% saturation was dialyzed against 50 mM Tris-HCl (pH 7.5) buffer and subsequently applied onto a DEAE-cellulose column equilibrated with 50 mM Tris-HCl (pH 8.0). The PL was eluted stepwise with 50 mM Tris-HCl (pH 8.0) buffer containing 0.00, 0.05, 0.10, or 0.20 M NaCl.

Thin-layer polyacrylamide gel IEF. IEF techniques were performed by the procedures described by Ried and Collmer (28, 29). Thin-layer (1 mm) polyacrylamide gel plates (pH 3.5 to 9.5) containing a gel concentration of 5% and an Ampholine concentration of 2.4% were purchased from LKB/Pharmacia Instruments (Piscataway, N.J.). The method of Guo and Bishop (8) was used to prepare gels, with the alkaline end of the pH gradient extending above pH 9.5. Gels were run on a Multiphor II 2117 apparatus (LKB), and enzyme samples (3 to 8 μ l) were applied directly onto the gel. Prefocusing was carried out at 9 W for 30 min, and focusing was carried out at 12 W for 40 min after application of samples. Phosphoric acid (0.1 M) and sodium hydroxide (0.1 M) were used, respectively, as the anolyte and the catholyte throughout the study. The wide range of pI markers (LKB/Pharmacia Instruments) were included in each run. After electrofocusing, gels were either stained with Coomassie blue or subjected to enzyme activity staining as described below.

Enzyme activity staining. Agarose-pectate overlay stain techniques for detecting PL activities have been described previously (2, 4, 28, 34). Thin-layer (0.75 mm) agarose substrate gels were prepared by casting a melted mixture of agarose (0.8%), sodium polygalacturonate (0.2%), Tris-HCl (100 mM, pH 8.5), and CaCl_2 (1 mM) onto an Agarose Gelbond Film (FMC Corp., Marine Colloids Div., Rockland, Maine). The agarose-pectate gel bond on the film support was overlaid onto the polyacrylamide gel immediately after electrofocusing, and incubation was carried out at room temperature for 30 min. PL activity was visualized 1 h after submerging the agarose-pectate gel in 1% mixed alkyltrimethyl ammonium bromide (Sigma). Alkyltrimethyl ammonium bromide precipitates pectate and leaves a clear zone where the pectate was enzymatically degraded (34).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by previously described procedures (14). Resolving gels contained 10% acrylamide, 0.33% bisacrylamide, and 0.1% sodium dodecyl sulfate. Enzyme samples containing 4 to 6 μ g of protein or 6 to 12 U of PL activity were added to each well. The protein concentration was determined based on the method of Lowry et al. (19), as described in the Protein Assay Kit (Sigma). Protein molecular weight markers purchased from Bethesda Research Laboratories (Gaithersburg, Md.) were included in each run to estimate the M_r s of PL samples.

Assay of tissue maceration. Purified or partially purified PL samples from six representative strains of soft rot bacteria were compared for their abilities to macerate potato tuber. Potato slices were prepared as described previously (16, 17), except that the slices were treated with 30 μ g of kanamycin per ml before they were placed onto 0.6% agar plates. Each slice was inoculated with 5 μ l of an enzyme sample containing 0.1 to 5.0 U of PL activity. The maceration zone was examined after 2 days of incubation at 20°C.

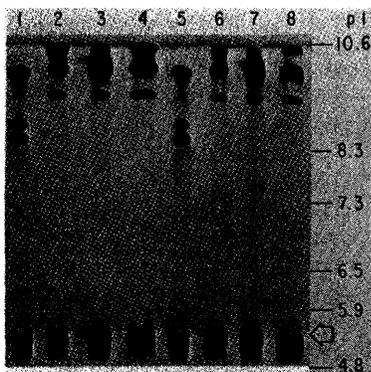


FIG. 1. IEF profiles of extracellular PLs produced by *E. chrysanthemi* EC16 (lanes 1 and 5), *E. carotovora* subsp. *carotovora* SR319 (lanes 2 and 6), *E. carotovora* subsp. *atroseptica* SR8 (lanes 3 and 7), and *E. carotovora* subsp. *carotovora* CU-06-1 (lanes 4 and 8). The arrow indicates the sites where the samples were applied.

RESULTS

IEF profiles of extracellular PLs. Concentrated culture supernatants from 25 strains of soft rot bacteria were subjected to thin-layer polyacrylamide gel IEF and subsequently assayed for PL activity by agarose-pectate overlay staining techniques. IEF profiles of PLs produced by *Erwinia* spp. *P. viridiflava*, *P. fluorescens*, *X. campestris*, and *C. johnsonae* were distinct. An alkaline PL (pI, ≥ 9.7) was identified as the sole or the principal PL produced by all groups of non-*Erwinia* spoilage bacteria. The specific PL profiles of each group of organisms are described below.

(i) *Erwinia* spp. Two strains of *E. carotovora* subsp. *carotovora* and one strain each of *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* were examined. All four strains produced at least three PL isozymes (Fig. 1). However, the PL profile of *E. chrysanthemi* differed greatly from that of *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora*. *E. chrysanthemi* EC16 produced one basic (pI, 10.0), two neutral (pIs, 8.8 to 9.0), and one acidic (pI, 4.2) PLs. *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* strains produced three PL isozymes, all of which had alkaline pIs (pI, ≥ 9.5). No differences in PL profiles were observed between *E. caroto-*

vora subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* strains. It should be noted that the acidic PL of *E. chrysanthemi* EC16 is not visible in Fig. 1. This isozyme is produced in extremely small quantities (29), and its migration in the IEF gel was interfered with by the enzyme sample that was retained at the application site (Fig. 1).

(ii) *P. viridiflava*. All of the eight *P. viridiflava* strains included in this study produced a single PL at nearly identical pIs (9.7) (Fig. 2A). Neutral or acidic PLs were not detected even when a large quantity of enzyme sample (containing up to 1.6 U of PL activity) was applied (Fig. 2B). The formation of bands with minor activity, similar to those shown in lanes 4 and 7 of Fig. 2A, likely resulted from the sample-trailing effect, since these bands did not appear consistently in every experiment. Bands with irregular activities were often observed in gel sites where enzyme samples were initially applied (Fig. 2), indicating that a portion of enzyme sample was bound to the gel and remained immobilized during electrofocusing.

(iii) *P. fluorescens*. Ten strains of *P. fluorescens* isolated from eight different plants were examined (Table 1). These strains differed somewhat in their nutritional and physiological properties and have been classified and grouped into biovar II (five strains) or biovar V (five strains) of *P. fluorescens* (18). All 10 strains produced, in addition to the alkaline PL (pI, 9.7), a neutral PL with a pI of 6.7. The production of neutral PLs by strains PJ-08-30, HU-08-11B, CY091, and BC-05-1B is shown in Fig. 3A (lanes 5 and 10) and Fig. 3B (lanes 1 and 6). The neutral PL produced by strains 17816, SJ-08-2, SJ-08-3, BC-05-2B, LC-04-2A, AJ-06-2A is hardly identified in Fig. 3A (lanes 2 to 4 and lanes 7 to 9, respectively), but it has been detected in other gels (data not shown). When decreasing amounts of enzyme samples were analyzed by IEF, neutral PL was hardly detected in samples containing <0.25 U of PL activity (Fig. 3B, lanes 4 and 8). Alkaline PL thus constituted the major proportion whereas neutral PL constituted the minor proportion of the total PL produced by *P. fluorescens* strains.

(iv) *C. johnsonae* and *X. campestris*. One strain each of *C. johnsonae* and *X. campestris* was examined. Both organisms produced a single PL with a pI of ≥ 10.0 . Neutral or acidic isozymes were not detected, even when samples containing 2.4 U of PL activity were applied (Fig. 4, lanes 1 and 5).

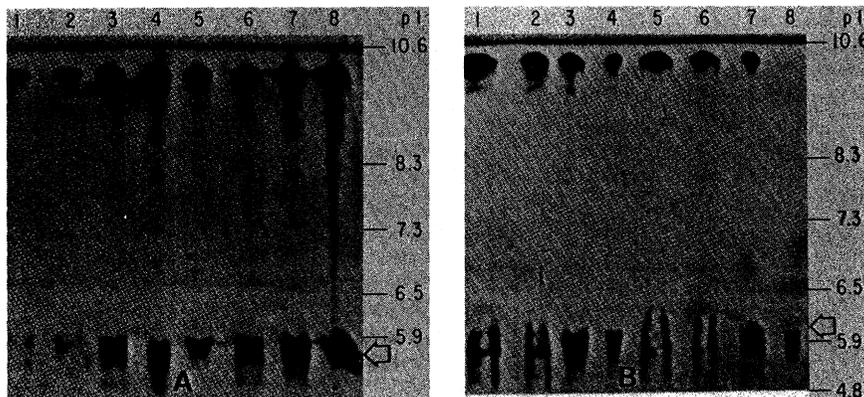


FIG. 2. (A) Extracellular PL profiles of *P. viridiflava* SF312 (lane 1), 13223 (lane 2), SF-05-3 (lane 3), PJ-08-6A (lane 4), PJ-08-6B (lane 5), PJ-08-9 (lane 6), PJ-08-16B (lane 7), and TU-04-2A (lane 8). (B) Comparison of PL profiles of strains PJ-08-16B (lanes 1 to 4) and SF312 (lanes 5 to 8) by using decreasing quantities of PL samples: 1.6 U (lanes 1 and 5), 0.8 U (lanes 2 and 6), 0.4 U (lanes 3 and 7), and 0.2 U (lanes 4 and 8). The arrows indicate the sites where the samples were applied.

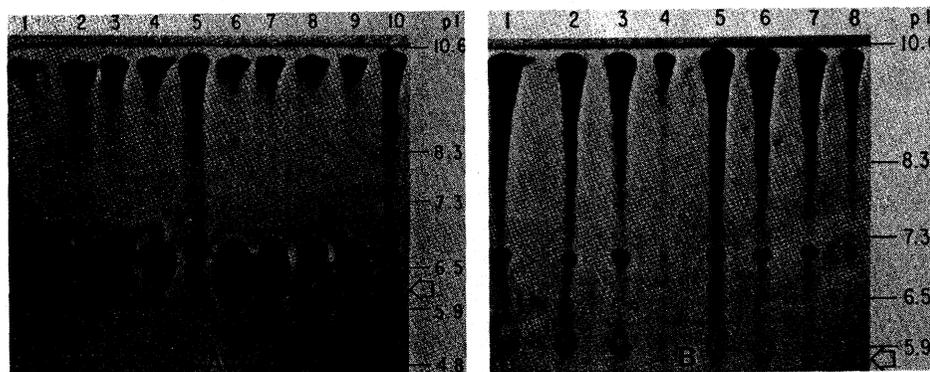


FIG. 3. (A) IEF profiles of extracellular PLs produced by *P. fluorescens* CY091 (lane 1), 17816 (lane 2), SJ-08-2 (lane 3), SJ-08-3 (lane 4), PJ-08-30 (lane 5), BC-05-1B (lane 6), BC-05-2B (lane 7), LC-04-2A (lane 8), AJ-06-2A (lane 9), and HU-08-11B (lane 10). (B) Comparison of extracellular PL profiles of strains BC-05-1B (lanes 1 to 4) and CY091 (lanes 5 to 8) by using decreasing quantities of PL samples: 2.0 U (lanes 1 and 5), 1.0 U (lanes 2 and 6), 0.5 U (lanes 3 and 7), and 0.25 U (lanes 4 and 8). The arrows indicate the sites where the samples were applied.

IEF profiles of intracellular PLs. To determine whether the bacteria produced PL species which were not readily excreted into the medium, IEF profiles of PLs prepared from culture supernatants and from intracellular fluids of seven representative strains were compared. Results show that the extracellular and intracellular PL profiles of *P. viridiflava*, *P. fluorescens*, *X. campestris*, and *C. johnsonae* were identical. There was no indication that these organisms produced PL species in addition to the PLs that were readily detected in the culture supernatant. *P. fluorescens* PJ-08-14 caused very mild or no maceration of potato slices. The PL produced by this strain could be detected in intracellular fluid but not in culture supernatant. The intracellular PL of strain PJ-08-14 was similar, at least in terms of the pI, to the alkaline PL of soft rot strains (CY091 and BC-05-1B) of *P. fluorescens*.

Purification of PLs. Extracellular PLs were purified from culture supernatants of *E. chrysanthemi* EC16, *P. fluorescens* CY091 and BC-05-1B, *P. viridiflava* SF312, *X. campestris* CJ091, and *C. johnsonae* PF062 by ammonium sulfate precipitation and anion-exchange chromatography. About 40 to 60% of total PL activity was recovered in the precipitate obtained between 50 and 85% saturation with ammonium sulfate. The dialyzed enzyme samples were subjected to DEAE-cellulose (Cl^-) chromatography and

were eluted with the Tris-HCl buffer (50 mM, pH 8.0) containing 0.00, 0.05, 0.10, or 0.20 M NaCl. For PLs of *E. chrysanthemi* EC16, two peaks of enzyme activity were observed. The major peak was eluted with the buffer alone, whereas the minor peak was eluted with buffer containing 0.10 M NaCl. For PLs of non-*Erwinia* strains, the enzyme was eluted as a single peak with buffer containing 0.05 M NaCl.

Purified PL samples were subsequently examined for purity, M_r , and pI by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5) and thin-layer polyacrylamide gel IEF (Fig. 6). PLs produced by two strains of *P. fluorescens* and one strain of *P. viridiflava* were purified almost to homogeneity by the two-step procedures described above (Fig. 5, lanes 5 to 7; Fig. 6, lanes 2 to 4). However, PL samples prepared from *E. chrysanthemi* EC16 (Fig. 5, lanes 4, Fig. 6, lane 5), *X. campestris* CJ091 (Fig. 5, lane 3; Fig. 6, lane 6), and *C. johnsonae* PF062 (Fig. 5, lane 2; Fig. 6, lane

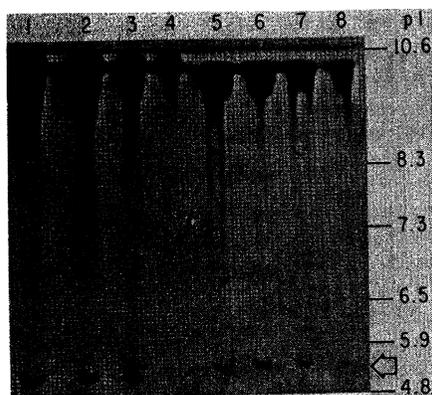


FIG. 4. Extracellular PL profiles of *C. johnsonae* PF062 (lanes 1 to 4) and *X. campestris* CJ091 (lanes 5 to 8). The amounts of PL sample used were as follows: 2.4 U (lanes 1 and 5), 1.2 U (lanes 2 and 6), 0.6 U (lanes 3 and 7), and 0.3 U (lanes 4 and 8). The arrow indicates the site where the samples were applied.

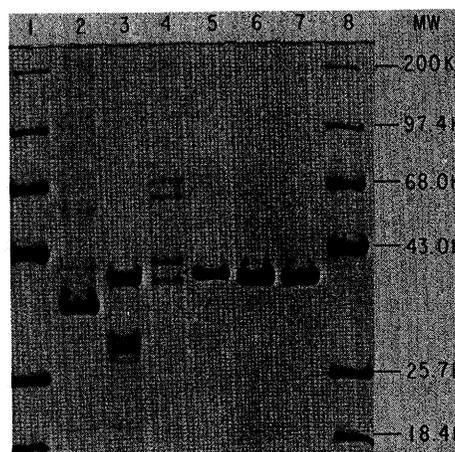


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracellular PLs purified from culture supernatants of *C. johnsonae* PF062 (lane 2), *X. campestris* CJ091 (lane 3), *E. chrysanthemi* EC16 (lane 4), *P. viridiflava* SF312 (lane 5), and *P. fluorescens* BC-05-1B (lane 6) and CY091 (lane 7). The M_r markers (MW [in thousands in the figure]; lanes 1 and 8) used were myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α -chymotrypsinogen (25,700), and β -lactoglobulin (18,400).

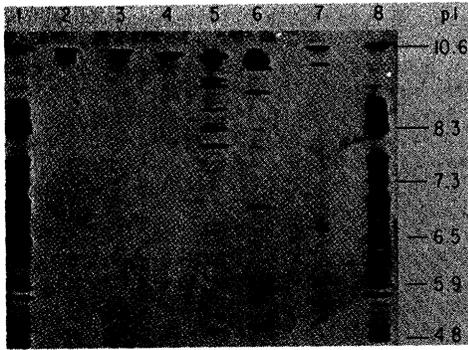


FIG. 6. Thin-layer polyacrylamide gel IEF of extracellular PL purified from culture supernatants of *P. fluorescens* CY091 (lane 2) and BC-05-1B (lane 3), *P. viridiflava* SF312 (lane 4), *E. chrysanthemi* EC16 (lane 5), *X. campestris* CJ091 (lane 6), and *C. johnsonae* PF062 (lane 7). The gel was stained with Coomassie blue. The pI markers (lanes 1 and 8) were cytochrome *c* (horse heart) (10.6), myoglobin (sperm whale) (8.3), myoglobin (equine) (7.3), myoglobin (porcine) (6.5), trifluoroacetylated myoglobin (porcine) (5.9), and C-phycocyanin (4.8).

7) were contaminated with other proteins, since several protein bands were detected by Coomassie blue staining and only one of them was shown to contain PL enzyme activity by the agarose-pectate overlay staining technique (28). PLs produced by the various groups of soft rot bacteria were different not only in pI but also in molecular mass. The M_r s of PLs produced by *P. fluorescens*, *P. viridiflava*, *X. campestris*, and *C. johnsonae* were estimated to be 41,000, 42,000, 41,500, and 35,000, respectively.

Induction of tissue maceration by purified PLs. PLs purified from culture supernatants of six representative strains were capable of inducing soft rot of potato slices, but to different degrees. The enzyme preparation of *E. chrysanthemi* EC16 containing 0.5 U or less of PL activity was sufficient to cause visible maceration of potato slices. On the contrary, the PL preparation of *C. johnsonae* was the least effective in inducing tissue maceration. An enzyme preparation containing 5.0 U or more of PL activity was required to induce visible maceration. The macerating ability of PL produced by *P. fluorescens*, *P. viridiflava*, and *X. campestris* was moderate, falling between the PL of *E. chrysanthemi* and that of *C. johnsonae*.

DISCUSSION

The data presented in this study demonstrate that PL profiles of non-*Erwinia* soft rot bacteria are much simpler than those of *Erwinia* spp. All of the *P. viridiflava*, *P. fluorescens*, *X. campestris*, and *C. johnsonae* strains that were examined produced one or two PLs, whereas all of the *Erwinia* strains that were studied here and elsewhere (2, 27, 29, 34, 36) produced three or more. *Erwinia* spp. as a whole differ in their pathological capacity from non-*Erwinia* soft rot bacteria, in that the former attack plants both in the field and in storage (26) and the latter usually cause spoilage of detached plant products after harvest (16–18, 20). The simple pectic enzyme system detected in non-*Erwinia* soft rot bacteria may account, at least in part, for the low efficiency of these microorganisms to attack actively growing plants. Davis et al. (5) have reported that PLs purified from cultures of *E. carotovora* are capable of eliciting phytoalexin accumulation in plants. They proposed that soft rot pathogens

must produce large quantities or multiple forms of PL to overcome the host defense mechanism triggered by pectic enzymes or other elicitors. The correlation between the low PL activity and the inability to infect field plants, as observed in non-*Erwinia* soft rot bacteria, seems to provide indirect evidence supporting the above hypothesis. Moreover, it is worth noting that the postharvest pathogen *X. campestris* CJ091 examined in this study produced a single PL. This organism is indistinguishable from the black rot pathogen *X. campestris* pv. *campestris* in terms of its physiological and nutritional properties (17). In contrast to the single PL detected in strain CJ091, the field pathogen *X. campestris* pv. *campestris* has been shown to produce at least three PL isozymes (6).

Two lines of evidence suggest that PL isozymes of *E. chrysanthemi* may play a role in the recognition of host plants. Ried and Collmer (29) and Thurn et al. (33) have reported that PL profiles of *E. chrysanthemi* strains isolated from different hosts are distinct, but the profiles are similar among strains isolated from the same host. Barras et al. (1) have shown that the reaction patterns catalyzed by four PLs of *E. chrysanthemi* EC16 are different. The 8 strains of *P. viridiflava* and the 10 strains of *P. fluorescens* examined in this study were isolated from 10 different hosts (Table 1). Despite the difference in host origins and in nutritional and physiological properties (18), strains of *P. viridiflava* and *P. fluorescens* appear to possess a common PL pattern specific for either species. The PL genes of *P. viridiflava* or *P. fluorescens* strains are possibly derived from the same origin and are well conserved during evolution. Because of the homogeneous pattern, it is unlikely that PLs of *P. fluorescens* or *P. viridiflava* serve a function in differentiating one host from another.

PLs with alkaline pIs appear to be the principal or the sole enzymatic factor involved in tissue maceration caused by most strains of soft rot bacteria. Results obtained from a number of recent studies, including the one reported here, strongly support this conclusion. Kotoujansky (12) and Collmer and colleagues (25, 30) have shown that deletion of alkaline PL genes from the genome of *E. chrysanthemi* greatly diminishes the virulence or macerating ability of the organism. Chatterjee and colleagues (1, 33) have reported that alkaline PL constitutes 40 to 60% of total PLs produced by *E. chrysanthemi* EC16 and that alkaline PL is more efficient in inducing tissue maceration, electrolyte loss, and cell death than are neutral or acidic PLs. Acidic PLs produced by certain strains of *E. chrysanthemi* (1, 11, 12, 30, 33) and species of *Yersinia* (3, 21) and *Klebsiella* (3) usually exhibit very little or no macerating ability. The biochemical basis for the fact that alkaline PLs are more effective in inducing tissue maceration than are neutral or acidic PLs is not known but warrants further investigation.

Because of their simple pectic enzyme systems, non-*Erwinia* soft rot bacteria could provide useful sources for the preparation of pure PLs. In this study, PLs produced by representative strains of *P. viridiflava*, *P. fluorescens*, *X. campestris*, and *C. johnsonae* were purified or partially purified following two simple steps: ammonium sulfate precipitation and anion-exchange chromatography. Purified or partially purified PL preparations differed in pIs, M_r s, and tissue-macerating abilities. The enzymological basis on which one PL is more efficient in inducing maceration of potato slices than another is not understood. Preliminary results of a study of enzyme kinetics have indicated that PLs produced by the aforementioned organisms degrade polygalacturonates by the *endo* mode of action but exhibit some-

what different cleavage patterns (A. L. Hotchkiss, K. B. Hicks, and C. H. Liao, unpublished data). It remains to be determined whether the difference in enzymological properties detected *in vitro* can be correlated with the difference in their ability to digest the pectic components of various plant cell walls.

Previous studies of PLs from a limited number of strains of *P. fluorescens* (7, 9, 10, 23, 37), *P. viridiflava* (10, 15), *X. campestris* (17, 24), and *C. johnsonae* (13, 16, 35) have focused on the detection and inducibility of the enzymes. Results presented in this study provide further information about the isoelectric properties, M_r s, and tissue-macerating abilities of PLs produced by the organisms listed above. The relatively simple pectic enzyme system demonstrated in non-*Erwinia* soft rot bacteria indicates that these microorganisms may be used as a model to study how procaryotes synthesize and excrete extracellular proteins at the molecular level.

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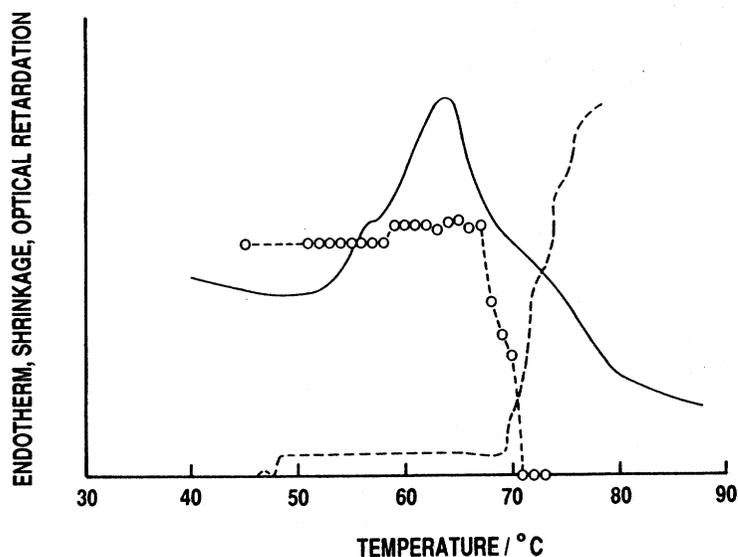


FIGURE 2 Endotherm (—), thermal shrinkage (---), and change in birefringence (o...o...o) of collagen in calf dermis during thermal denaturation. Whole ordinate spans 0.2 mW, 30% shrinkage, or 0.5 wavelengths.

Samples were taken from the calorimeter at selected temperatures and quenched for observation with the electron microscope. The rationale for choosing these particular temperatures can best be inferred from Fig. 1a, where 59°C, 66°C, 69°C, and 80°C mark points where the denaturation processes seemed to change. At 65°C the second process was being completed but the sample had not yet shrunk, whereas at 68°C the sample had begun to shrink but the third process was still fully underway.

Embedded in melamine resin and stained with tannic acid-uranyl acetate as described in METHODS, the unheated fibrils in transverse section appeared against a light background and had lightly stained interiors (Fig. 3a); the longitudinal sections (Fig. 3b) had positively stained Schmitt-Gross bands.¹⁴ As can be seen in Figs. 3a and 3b, the edges and bands of the fibrils were well defined. Any changes that occur when the dermis was heated through the first peak to 59°C were not evident by direct examination in the electron microscope, as shown in Fig. 4.

After heating through the second peak, however, to 65°C, dark central cores appeared in the transverse sections (Fig. 5a). These cores coincided in their locations with dark streaks on the axes of the fibrils in the longitudinal sections of Fig. 5b. These streaks and their correspondence with the cores are shown more clearly in Fig. 5c, which was obtained from a sample heated separately at a higher rate, which did not alter the thermogram qualitatively. The dark cores of Fig. 5a would have been expected to show as central dark streaks against the cross-bands in Fig. 5b because the sections were 60 nm thick, more than half the diameter of the fibrils. Thus, dark cores were observed to be superposed on the banding pattern of the fibril sheath. Fig. 5 shows then that the fibrils in the sample that

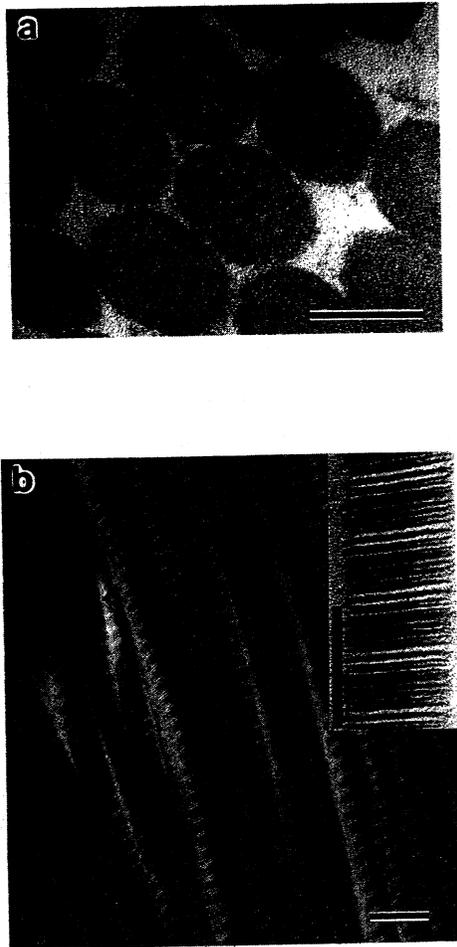


FIGURE 3 Collagen fibrils in unheated calf-skin dermis. (a) transverse section. Bar, 100 nm; (b) longitudinal section. Bar, 200 nm. Inset: bar, 100 nm.

had not yet undergone thermal shrinkage, but had been 55.6% denatured (from data in Fig. 1a), had sheaths with obvious intermolecular order still preserved.

A striking feature of the heated samples in Fig. 5 was the dark background. Although samples heated to 65°C appeared to be either negatively stained or photographed in reverse contrast in their micrographs, the normal, positively stained Gross-Schmitt bands showed them to be neither. This did not seem to be an observational photographic artifact: neither unusual microscope settings nor photographic processes were used to develop these bands at their usual intensity and contrast. There seems to have been a stain-binding material in these spaces, at least after the heating. Even before being heated this material might also have been present in a latent, unstainable form, perhaps undergoing a physical change under heating which caused it to bind uranyl ions. Studies under way to identify this interfibrillar material and also the material in the cores show it to vanish after

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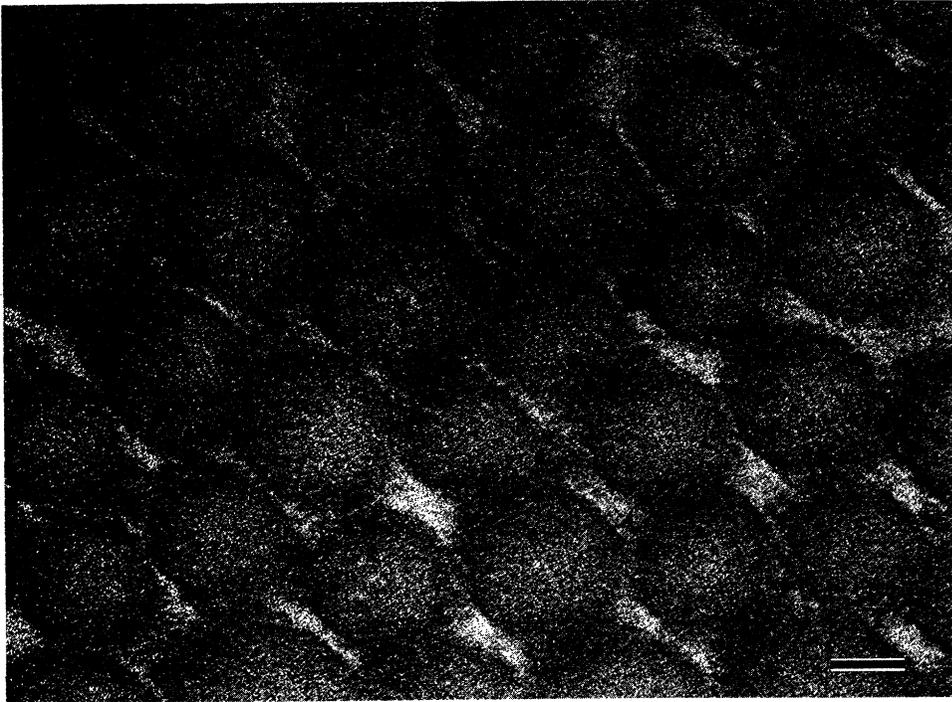


FIGURE 4 Collagen fibrils in calf-skin reticular dermis heated at 1.25°C/min to 59°C. Bar, 100 nm.

treatment with trypsin. Unlike the stands¹³ observed to be associated with the d-bands¹⁴ of the fibrils when the tissue is embedded in epoxy resin, this material was dispersed. We noticed that it was stained with either uranyl acetate or ruthenium red even without tannic acid (micrograph not shown). Tannic acid has been found to enhance the binding of metal cations to polysaccharides.¹⁵ The behavior then was consistent with the properties of a small proteoglycan, perhaps proteodermatan sulfate.¹⁶ It appears (Figs. 4 and 5) to have been stained more deeply and to have been more dispersed after it had been heated, perhaps because of its own denaturation. Its presence in the cores was unexpected and unexplained, but ruthenium-red stained cores have been shown in collagen fibrils of bovine heart valves in epoxy resin,¹⁷ and uranyl acetate-lead citrate staining cores, in fibrils of quick-frozen and freeze-substituted dermis in epoxy resin.¹⁸

The sheaths were first observed to have become denatured along the lengths of the fibrils at 68°C (Fig. 6), at which the fibers had just become isotropic (Fig. 2) and the sample had begun to shrink. The sheaths, like the cores, appeared to be transformed into a dark-staining amorphous material. This denaturation of the sheaths appeared to be progressive, since at 80°C, at which denaturation was virtually complete and shrinkage was severe, fibril definition and banding were not observed (Fig. 7a). In the dermis that has been reduced with borohydride, however, the fibrils were preserved even when heated to 120°C, although the

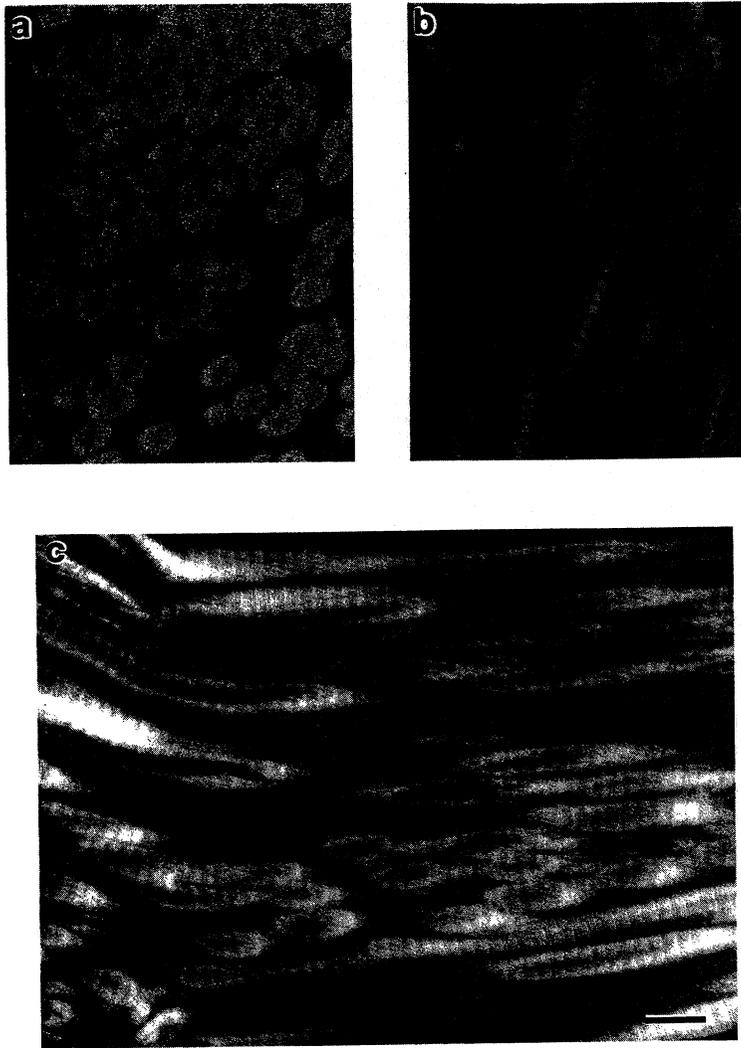


FIGURE 5 Collagen fibrils in calf-skin reticular dermis heated to 65°C. (a) transverse sections heated at 1.25°C/min; (b) longitudinal sections heated at 1.25°C/min; (c) longitudinal sections heated at 10°C/min. Bar, 200 nm.

lateral register of the molecules, indicated by the bands, was lost (Fig. 7b). Fibrils have expanded in diameter and appear to have coiled into helices.

DISCUSSION

The endotherm of calf skin in Fig. 1, with three components, was very similar to those reported for the skin of young rats.¹⁹ Peak 1 has been shown to be related to a class of collagen, which comprised 13% of the total in the calf reticular

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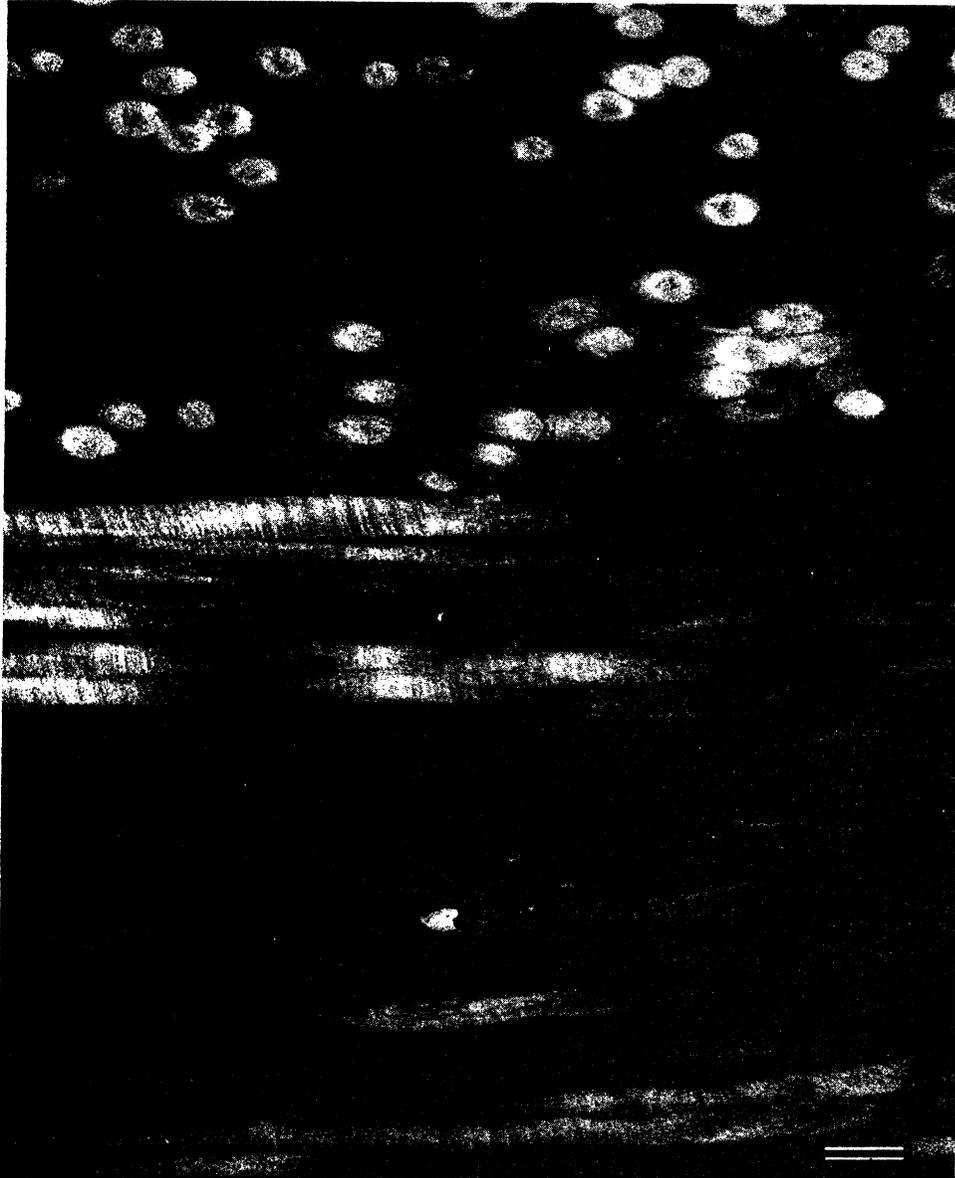


FIGURE 6 Collagen fibrils in calf-skin reticular dermis heated at 1.25°C/min to 68°C. Bar, 200 nm.

dermis and was not crosslinked, since it occurs only in dermis of young or lathyritic rats.¹⁹ On quenching after this denaturation step, however, we observed no change in the morphology of the fibrils (Fig. 4). Clearly, this most labile fraction of collagen was not segregated in any one part of the fibrils; it might not have been distributed uniformly, but could not occur by itself.

The core regions of the fibrils were comprised of material that was unstable at

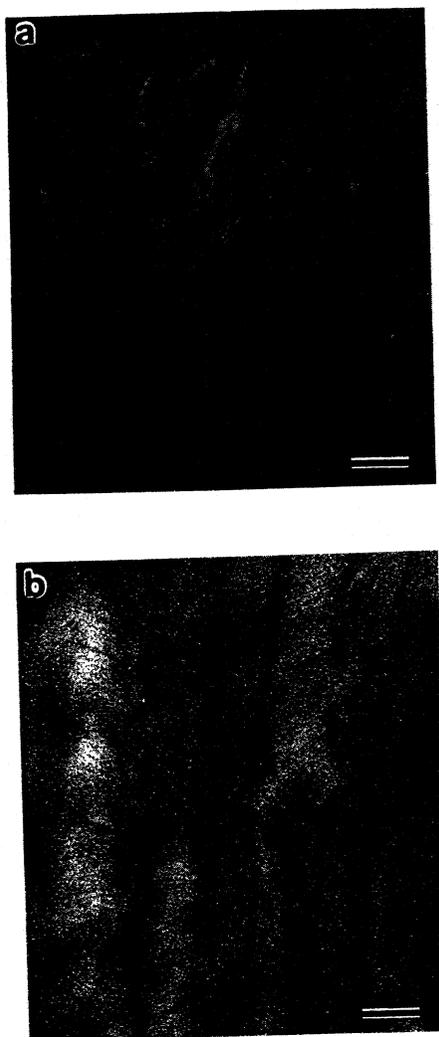


FIGURE 7 Collagen fibrils in heated reticular dermis. (a) Native calf dermis heated at 1.25°C/min to 80°C; (b) mature dermis, reduced with borohydride and heated at 10°C/min to 120°C. Bar, 200 nm.

temperatures between 52°C and 72°C, the region of the second denaturation peak (Fig. 1). Unlike the first peak, this one appeared in the skin of both young and mature animals^{11,19} and thus was not due to recently synthesized collagen. During this denaturation step, the shape of the fibril was preserved by the stable sheath, in which lateral order among the molecules was not disturbed. That is, there was no macroscopic shrinkage, no change in form birefringence, no significant change in fibril diameter, nor loss of cross-banding.

Figs. 5 and 6 show that the most stable collagen fraction in the dermis comprised a peripheral sheath about each fibril. The presence of such stable material was also revealed in collagen denaturation endotherms of rat skin,¹⁹ where it was

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identified as collagen with thermally labile crosslinks.¹⁹⁻²¹ It may be noteworthy that a peak or shoulder on the same temperature interval (75°C) was completely absent in the endotherm of reconstituted collagen fibrils,⁵ which would have contained fewer crosslinks. Labile crosslinks were shown to decompose on the same temperature interval as the third endotherm, allowing collagen helices to be denatured.²⁰ We found that during this process the fibrils swelled, the edges were lost, and, as also shown before,^{20,21} the sample shrank. After stabilization of the molecules by chemical reduction of the crosslinks, the third endotherm was spread out and was greatly diminished (Fig. 1b), and the fibrils persisted in Fig. 7, although they swelled about 50% in diameter, and their edges became diffuse. Reduction, however, did not eliminate thermal shrinkage (Fig. 1b) nor loss of ordered molecular packing.

The helicoid filamentous substructure, developed by thermal denaturation of the reduced collagen (Fig. 7b), resembled that seen previously in tissue collagen denatured by 4M guanidinium chloride.²²

The crosslinked collagen molecules were evidently segregated into circumfibrillar sheaths as we show here because they must have reacted extracellularly with lysyl oxidase, which, however, could not easily diffuse into the fibril itself. Its reaction product was therefore localized at the periphery of the fibril, where it subsequently formed the crosslinks involving allysine and lysine. This reaction must have occurred after the fibrils organized laterally and thickened. We also found DSC evidence that this sheath-core substructure persists even as the animal matures, more stable crosslinks evolving in the sheath but not in the core, with preservation of the constituent collagen in the native state at temperatures up to 110°C in reticular dermis of 18 mo old steers.¹¹

Since the crosslinks are segregated into half the collagen of the dermis, their local density is at least twice the average concentration. This concentration should be considered in trying to relate crosslink concentrations to mechanical properties, remodelling, swelling, and industrial tanning of this tissue.

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