Plant Physiol. (1988) 87, 616–621
0032-0889/88/87/0616/06/$01.00/0

Enzymic Cross-Linkage of Monomeric Extensin Precursors *in Vitro*[1]

Received for publication March 1, 1988 and in revised form February 29, 1988

DANIEL S. EVERDEEN, STEFANIE KIEFER, JAMES J. WILLARD, E. PATRICK MULDOON, PRAKASH M. DEY, XIONG-BIAO LI, AND DEREK T. A. LAMPORT*
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312

ABSTRACT

Rapidly growing tomato (*Lycopersicon esculentum*) cell suspension cultures contain transiently high levels of cell surface, salt-elutable, monomeric precursors to the covalently cross-linked extensin network of the primary cell wall. Thus, we purified a highly soluble monomeric extensin substrate from rapidly growing cells, and devised a soluble *in vitro* cross-linking assay based on Superose-6 fast protein liquid chromatography separation, which resolved extensin monomers from the newly formed oligomers within 25 minutes. Salt elution of slowly growing (early stationary phase) cells yielded little or no extensin monomers but did give a highly active enzymic preparation that specifically cross-linked extensin monomers in the presence of hydrogen peroxide, judging from: (a) a decrease in the extensin monomer peak on fast protein liquid chromatography gel filtration, (b) appearance of oligomeric peaks, and (c) direct electron microscopical observation of the cross-linked oligomers. The cross-linking reaction had a broad pH optimum between 5.5 and 6.5. An approach to substrate saturation of the enzyme required extensin monomer concentrations of 20 to 40 milligrams per milliliter. Preincubation with catalase completely inhibited the cross-linking reaction, which was highly dependent on hydrogen peroxide and optimal at 15 to 50 micromolar. We therefore identified the cross-linking activity as extensin peroxidase.

Since the original discovery of the structural cell wall protein extensin (12, 23, 34) and speculation about its role, the gap between fact and fancy has steadily narrowed. Early suggestions (20, 26) of its covalent attachment to the wall polysaccharides became unlikely in view of experiments with anhydrous hydrogen fluoride. Liquid HF, a protein solvent, solubilized the wall polysaccharides (by solvolysis) but not the protein (37). Thus, a covalent extensin network seemed likely, and we suggested the possibility of 'two semi-independent but interacting networks' of cellulose and extensin, respectively (28).

The isolation of putative precursors which were readily soluble (4) with an extensin-like composition (43) supported the network hypothesis. And the involvement of an unknown tyrosine derivative (27) as an intermolecular cross-link seemed possible after the identification of IDT[2] in cell wall hydrolysates (15), and hence a new protein cross-linking amino acid.

Demonstration of IDT as a short intramolecular cross-link in two extensin tryptic peptides (13) made the suggested involvement of peroxidase in extensin cross-linkage (9, 29) more plausible and led to the discovery of a monomeric extensin pool *in muro* readily eluted from intact cells of rapidly growing suspension cultures (40). Pool turnover kinetics were consistent with the status of these monomers (P1 and P2) as soluble precursors to an insoluble network (40). Moreover, peptide mapping and amino acid sequencing identified the hexapeptide Val-Lys-Pro-Tyr-His-Pro of P1 as a putative cross-link domain, occurring about five times in the monomer (41).

Our extensin precursor preparations routinely yield substrate (mg) amounts, which have enabled us to develop a quantitative assay for *in vitro* cross-linking. We describe here methods for the isolation and assay of extensin precursor cross-linking activity. This involved $CaCl_2$ elution of extensin cross-linking activity from intact cells and assay of the enzymic activity as the conversion rate of extensin monomers to oligomers, measured by FPLC on Superose-6. We confirmed cross-linking by direct TEM visualization of the oligomeric products.

The essential enzymic characterization of the cross-linking activity included: pH optimum, substrate saturation, inhibitors, and dependence on exogenous hydrogen peroxide. We therefore propose the trivial name 'extensin peroxidase' for the isolated cross-linking activity, which we have previously described briefly in progress reports over the past 2 years (21, 30).

MATERIALS AND METHODS

Elution of Crude Extensin Peroxidase. We grew tomato (*Lycopersicon esculentum*) cell cultures on MET medium at 27°C for 8 d as described previously (40), harvested the cells from 1 L culture medium on a coarsely sintered funnel, washed the cells briefly with distilled water, and then eluted the cell pad with two 250 ml aliquots of 50 mM $CaCl_2$ by percolation for about 5 min. The eluates were then concentrated (× 50 yielding 10 ml final volume) by ultrafiltration at 40 to 50 psi in an Amicon ultrafiltration apparatus (YM-10 membrane) in the cold room. After overnight dialysis against 2 L 50 mM (pH 6.3) sodium citrate buffer and centrifugation for 15 min at 9000 rpm Sorvall SS-34 rotor), we filtered the supernatant through an 0.22 μm Millipore membrane. This gave a sterile enzyme preparation which could be used immediately or alternatively stored frozen at −20°C.

[1] Work funded by grant No. DCB 8315 901 from the United States National Science Foundation, with additional support from the United States Department of Energy, contract No. DE-AC02-76ERO-1338, and a fellowship from the North Atlantic Treaty Organization Science Committee through the Deutscher Academischer Austauschdienst to S. K.

[2] Abbreviations: IDT, isodityrosine; P1, P2, monomeric extensin precursors P1 and P2; FPLC, fast protein liquid chromatography; CAPS, chromatographic applications package software; TEM, transmission electron microscopy; ABTS, 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid); PCV, packed cell volume; Pt/C, platinum/carbon.

Assay for Cross-Linking by Extensin Peroxidase. The routine assay involved the addition in the following order of: 10 μl (pH 6.5) McIlvaine buffer, 10 μl P1 extensin precursor (10 mg/ml in HPLC water), 5 μl 90 μM hydrogen peroxide (freshly prepared from 30%), and 5 μl crude enzyme preparation (appropriately diluted) for a total incubation volume of 30 μl.

For inhibitors, the order of additions was: buffer, inhibitor, enzyme, followed by a 2 min preincubation, then P1 precursor, and hydrogen peroxide.

After incubation for 1 to 7 min in the dark at 23°C, we added 10 μl 50 mM mercaptoethanol as a stop reagent, then added 10 μl (10 mg/ml) BSA as an internal standard, and injected 40 μl onto a Superose-6 FPLC column (Pharmacia 30 × 1 cm 6% cross-linked agarose beads) using a Rheodyne 7125 zero dead volume injector. The column was equilibrated with 0.2 M (pH 7.0) sodium phosphate buffer (containing 0.005% sodium azide) at a flow rate of 530 μl/min and was monitored at 220 nm.

We integrated peak areas via IBM CAPS software and expressed results as the difference in monomer peak area before and after incubation. Finally, we calculated initial rates of precursor (substrate) conversion, using the first order rate equation $A = A_0 \cdot e^{-kt}$ where A is the monomer remaining after t seconds; A_0 is the amount of monomer at zero time; k is the first order rate constant:

$$k = \frac{\ln(A_0/A)}{t}.$$

A Lotus 1-2-3 spreadsheet was convenient for data entry and calculation. We entered t in seconds and A in μg; then $A_0 \cdot k$ gave the initial rate in terms of μg P1 cross-linked/sec.

Extensin Precursor P1 Preparation. Extensin precursor P1 preparation was by salt-elution of intact cells as described earlier (40). These preparations generally contained about 10% endogenous lower order oligomers. For experiments involving TEM visualization of oligomers formed during *in vitro* monomer cross-linking, we prepared an oligomer-free substrate by a final purification step on Superose-6.

Amino Acid Analyses. We used a continuous pH gradient system using buffers from the Pickering Co. (Mountain View, CA), as previously described (40).

Peroxidase Assays. Peroxidase assays involved the following substrates at the given concentration in 0.1 M (pH 6) phosphate buffer containing 5.8 mM H_2O_2 and monitored continuously at the appropriate wavelength: 8 mM guaiacol (470 nm), 0.018 syringaldazine (1 mM stock in DMSO) (530 nm), 0.4 mM ABTS (405 nm), and 10 mM 4-aminoantipyrene (510 nm).

Transmission Electron Microscopy. We prepared rotary-shadowed extensin monomers and oligomers for TEM by spraying samples (1–30 μg/ml) in 50% aqueous glycerol onto freshly cleaved mica chips and then shadowed with Pt/C at an angle of 5°. After backing with carbon at 90°, we floated the replicas on distilled water, collected them on 300 mesh copper grids, and examined them in a JEOL 100 CX II transmission electron microscope operated at 100 kV (18).

RESULTS

Cross-Linking Rates at Low P1 Substrate Concentrations (0–10 mg/ml). The FPLC assay depended on accurately measuring the decrease in peak area of monomeric extensin as the cross-linked reaction products accumulated in the oligomeric peak fractions (Fig. 1). Thus, monomer peak areas of zero time controls, minus the monomer peak area after an appropriate reaction time (1–7 min at 23°C), showed the extent of monomer to oligomer conversion. However, as the quantitative assay relied on substrate depletion (rather than product appearance; see "Discussion") and the enzyme was generally far from substrate saturation, we adopted a standard assay whose conditions (3.3 mg P1/ml) were essentially first order. Hence we calculated initial reaction rates from the first order rate equation: $A = A_0 \cdot e^{-kt}$.

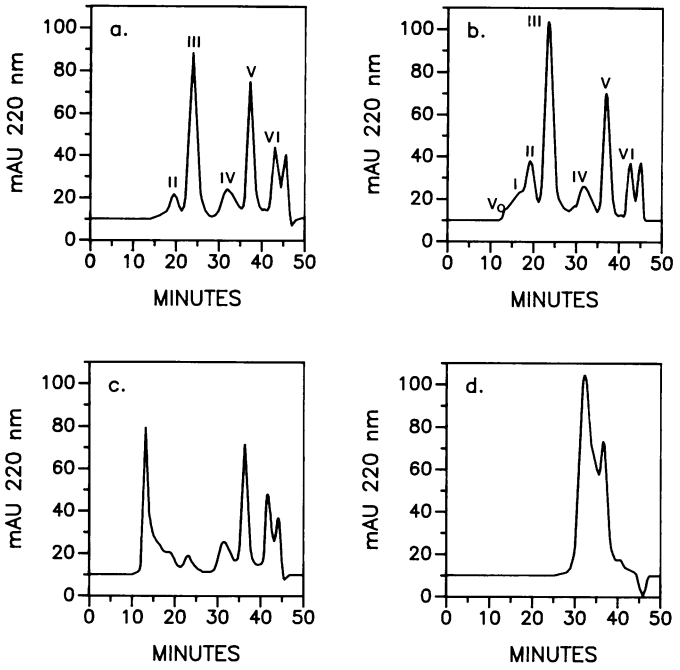

FIG. 1. Superose-6 FPLC profiles of P1 extensin monitored at 220 nm (a) before and (b) after cross-linking for 1 min (c), for 5 min (d) 25 μl (1.2 mg/ml) crude enzyme alone. a, Peak II represents a small amount of lower order endogenous oligomers invariably present in extensin precursor preparations, even isolated from cells in the presence of 2-mercaptoethanol (noncross-linking conditions). Peak III is monomeric extensin. Peak IV is crude enzymic protein. Peak V is contributed by the citrate component of McIlvaine's buffer, and peak VI is 2-mercaptoethanol of the stop reagent. b, Three size classes of cross-linked P1 appear progressively with time: a void peak (V_0) (note that Superose-6 has a nominal exclusion limit of 40 mD for globular proteins), a large-oligomer fraction (I), and a small-oligomer fraction (II).

These assays enabled us to choose the correct enzyme activity so that all kinetic experiments were in the linear region of the plot of reaction rate against enzyme concentration (Fig. 2). The crude enzyme protein content generally ranged from 2 to 9 μg in each 30 μl assay. However, the range was from 2 to 27 μg for the experiment shown in Figure 2.

Under the above standard conditions, concentrated crude cell eluates typically gave specific activities of approximately 3.7 mg P1 cross-linked/min/mg crude enzyme, based on initial rates. Incubation times of 5 to 15 min generally gave a greater than 95% conversion of monomer to oligomer.

Substrate Specificity and Controls. The crude activity specifically cross-linked the extensin monomers P1a, P1b, and P2 isolated from tomato cell suspension cultures. However, for most experiments we used P1a, although P1b showed no obvious differences. Other proteins, namely bovine serum albumin, apomyoglobin, trypsin inhibitor, ribonuclease, thyroglobulin, and aldolase were not cross-linked under the standard assay conditions. Horseradish peroxidase (Sigma type I) in the microgram range did not cross-link extensin monomers. The crude cross-linking enzyme active in Na acetate, citrate, and phosphate buffers was inactivated after 1 min at 100°C and also oxidized typical peroxidase substrates such as guaiacol, syringaldazine, ABTS, and 4-aminoantipyrene. However, the crude enzyme showed no catalase activity; there was no decrease in the 230 nm absorbancy of 2 mM hydrogen peroxide incubated with the crude

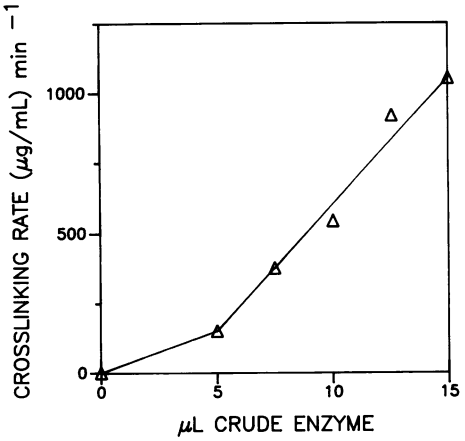

FIG. 2. Cross-linking rate as a function of enzyme concentration. Standard conditions were 3.3 mg/ml P1 and 15 μM H_2O_2 in pH 6.5 buffer at 23°C and a 5 min reaction time. We measured the initial rate as described in "Materials and Methods." The amount of crude enzyme varied from 2 to 27 μg protein in a 30 μl reaction mixture.

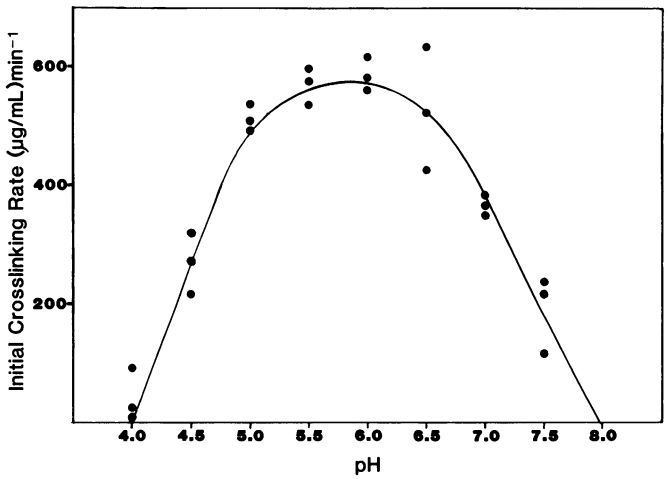

FIG. 3. Optimum pH for extensin peroxidase catalyzed cross-linking. Standard conditions were 3.3 mg/ml P1 and 500 μM H_2O_2 at 23°C. McIlvaine buffer varied from pH 4.0 to 7.5, and at each pH we calculated the initial cross-linking rate after three different reaction times (3–7 min) chosen to ensure that the extent of monomer conversion was between 10 and 40% for greatest precision. The three-point time-course aliquots were stored in liquid nitrogen until injection. Each 30 μl reaction mixture contained 2.5 μg crude enzyme protein.

enzyme alone. Crude enzyme gave a Superose-6 protein profile spanning the 30 to 60 kD range, but contained negligible amounts of endogenous extensin precursors (Fig. 1d) when isolated from stationary phase cells grown on MET medium.

pH Optimum. Under standard assay conditions (3.3 mg/ml P1 and 500 μM H_2O_2), and using the wide range McIlvaine citrate-phosphate buffer, the pH activity curve was classically bell-shaped with a broad optimum between pH 5.5 and pH 6.5 (Fig. 3).

Optimum Peroxide Concentration. The reaction rate was highly dependent on hydrogen peroxide levels. At a monomeric substrate concentration of 3.3 mg/ml, the optimum peroxide concentration was between 15 and 50 μM (Fig. 4). There was no cross-linking activity in the absence of hydrogen peroxide or after brief preincubation of the reaction mixture (minus cross-linking enzyme) with catalase (Sigma C-100). The enzyme is therefore a peroxidase.

Substrate Saturation. We determined an approximate saturation curve, with pH 6.5 and 2 mM H_2O_2 as standard conditions. The enzyme needed high levels of monomeric extensin substrate (20–40 mg/ml) to approach saturation (Fig. 5), making these experiments indeed expensive when using 0.6 to 1.2 mg extensin for each assay! Also, at high substrate levels (10–40 mg/ml), where the reaction was no longer first order, the rates were only approximate.

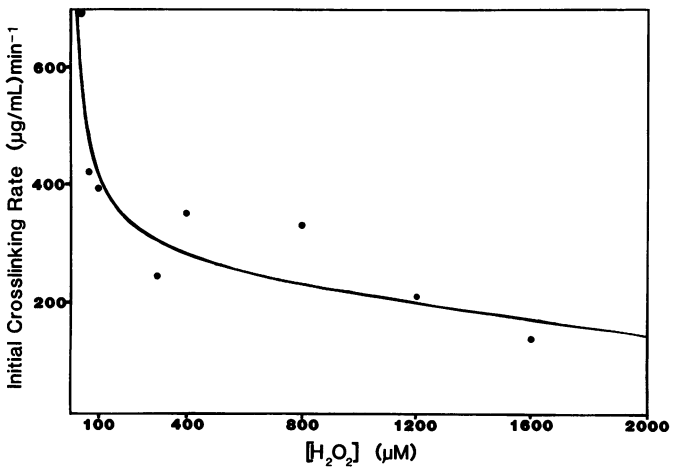

FIG. 4. Optimum hydrogen peroxide concentration. Standard conditions were 3.3 mg/ml P1 and 5 to 2000 μM H_2O_2 at 23°C, in pH 6.5 buffer, with a 5 min reaction time, measuring the initial rate according to "Materials and Methods." Each 30 μl reaction mixture contained 2.4 μg crude enzyme protein.

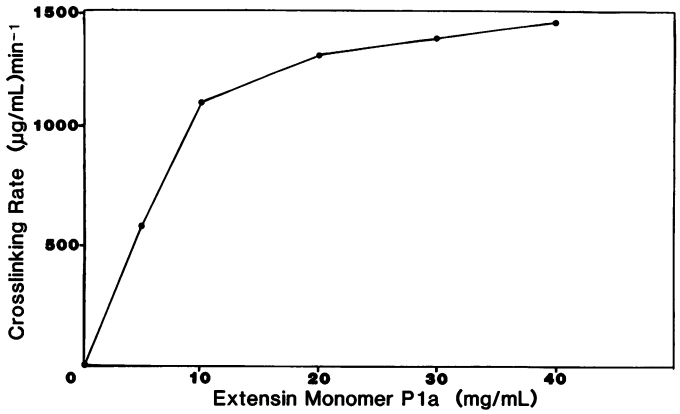

FIG. 5. Cross-linking rate dependence on P1 concentration. Standard conditions were 5 to 40 mg/ml P1 and 2 mM H_2O_2 at 23°C, in pH 6.5 buffer. The reaction time was 5 min, and the 30 μl reaction mixture contained 9 μg crude enzyme. At high substrate levels (30–40 mg/ml), we diluted the reaction mixture 1:1 with buffer before injecting the usual 40 μl, to avoid overloading the Superose column.

Inhibitors of Cross-Linking Activity. Generally, we used the standard assay with low substrate levels at pH 6.5 and 200 μM H_2O_2, but after preincubating the enzyme with inhibitor for 2 min, followed by a 5 min cross-linking reaction. Addition of 1 μg catalase to a 30 μl reaction mixture effectively inhibited the appearance of cross-linked products, thus demonstrating peroxide dependence of the reaction. Mercaptoethanol, at concentrations higher than the added peroxide, completely inhibited the cross-linking reaction. Thus, we routinely used 10 mM 2-mercaptoethanol (final concentration) as a stop reagent. Potassium cyanide and sodium azide also inhibited the cross-linking reaction; under standard assay conditions we observed 48% inhibition at 100 μM KCN and 32% at 25 μM NaN_3.

Enzyme Stability and Partial Purification. Cross-linking activ-

ity stored at 4°C was stable for several days but was unstable when slowly frozen in a −20°C freezer or slowly thawed. However, quick-freeze quick-thaw conditions retained all the activity even after multiple freeze-thaw cycles, *e.g.* we routinely froze 0.5 ml aliquots in glass vials in liquid nitrogen and thawed them under the cold tap as needed. At protein concentrations lower than about 1 mg/ml, the enzyme lost some activity (Fig. 2). Crude enzyme chromatographed on Superose-6 yielded cross-linking activity only in the region corresponding to well-retarded protein (Fig. 1d). There was no cross-linking activity in the region corresponding to P1 extensin monomers.

Yield of Crude Enzyme from 8-d Cells. Crude concentrated eluates, assayed under the standard conditions described in "Cross-linking Rates at Low P1 Substrate Concentrations" gave very high cross-linking activities. Typically, the eluate from 100 g (fresh weight) 8-d cells yielded 21.6 mg crude enzymic protein, corresponding to an initial cross-linking rate of 80 mg P1/min.

Cross-Linked Products. We applied four criteria of covalent cross-linkage between extensin oligomers: First, the decreased retardation on FPLC gel filtration (Fig. 1) implied an increased size consistent with oligomerization. Second, the amino acid composition of putative oligomers was similar to that of extensin monomers (Table I). Third, treatment with reagents such as 10 mM 2-mercaptoethanol and 10 mM EGTA did not dissociate the oligomers, as judged from the gel filtration profiles. And fourth, TEM of (Pt/C) rotary shadowed oligomers showed branched assemblies with occasional pores (Fig. 6).

A time-course of the cross-linking reaction observed by HPLC gel filtration on Superose-6 (Fig. 1) showed three product classes, namely, a very high mol wt void, an intermediate oligomeric peak, and a peak of small oligomers. At low concentrations of extensin monomers (1–5 mg/ml), the void peak rapidly dominated the reaction profile, but at high monomer concentrations (20–40 mg/ml) the small-oligomer peak was the major reaction product.

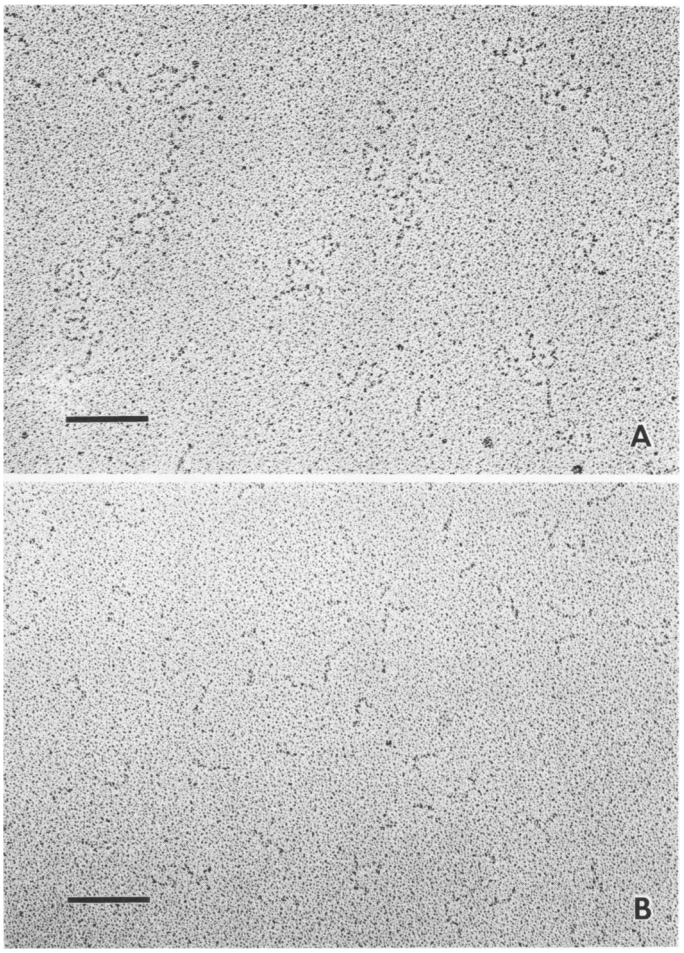

FIG. 6. Transmission electron microscopy of rotary-shadowed oligomers prepared from pure monomers. Gel filtration on Superose-6 yielded a pure fraction confirmed as monomeric by rechromatography, which gave a single peak. After concentrating the purified monomers in a Centricon microconcentrator (10 kD cut-off), an aliquot was cross-linked by the crude enzyme and then rechromatographed on Superose-6. We dialyzed the fraction corresponding to the small oligomers and shadowed samples of the dialysate as described in "Materials and Methods" and shown in (a) above and as compared with the monomer controls shown in (b).

DISCUSSION

Extensin monomers become cross-linked *in muro*, an inference based on much previous work, is also consistent with the primary cell wall as a true organelle (24) with its own abundant complement of enzymes ranging from phosphatase (33) to peroxidase (36). De Jong (11) first showed that peroxidase localization and lignification were not correlated, implying additional roles for wall peroxidases, currently summarized (13, 29) as general catalysts of matrix supramolecular assembly, by cross-linking wall polysaccharides (14), protein, and lignin. Presumably *in muro* cross-linking enzymes must be mobile (in order to seek out cross-link sites) and therefore amenable to the intact cell elution technique (25), which elutes only cell surface proteins; these account for about 2% of the total protein (in 8-d cells) and therefore represent a highly effective first purification step. Thus, we eluted suspension-cultured tomato cells with 50 mM $CaCl_2$ essentially as described for preparing extensin precursors (40) but at a later (early stationary) phase of growth corresponding to depletion of the *in muro* extensin precursor pool of 8-d cells (*cf.* Fig. 4 of Ref. 40) when we inferred more cross-linking activity.

The simplest and most direct assay of cross-linking activity depends on increased size of the oligomeric reaction products. A light scattering or turbidimetric approach was unsuccessful, and polyacrylamide gel electrophoresis was limited by the large size of the products, although agarose remains a possibility for future testing. The availability of a new FPLC column, Superose-6, capable of resolving a high mol wt range led to a rapid, sensitive, and quantitative assay of extensin monomer cross-linking, which also allowed the recovery of oligomeric products for further analysis. We measured the disappearance of monomers with time as this was somewhat more precise than measuring the appearance of the oligomeric reaction products, although there was generally a good correspondence between disappearance of monomers and appearance of oligomers (Fig. 1, a and c). At low monomeric substrate concentrations, the reaction rate was directly proportional to their concentration, so we used the first ll ◊der rate equation to determine initial reaction rates. For greater accuracy, we used a three-point reaction time-course for each pH, as shown in Figure 3. We avoided running the reaction longer than required for a 30% loss of monomer, as the very high mol wt products tended to stick to and plug the column and column filter. However, the reaction was extremely facile showing a remarkable 90% monomer to oligomer conversion within 5 min under optimal conditions (Fig. 1c).

The crucial question arises: does the *in vitro* cross-linking activity represent a reaction which also occurs *in muro*, or does the cross-linking merely result from the artifactual propensity of heme proteins for promoting nonspecific cross-linking (35, 38)?

Classical biochemistry assumes that an enzyme and its substrate in the same organelle will react. Earlier, we demonstrated an *in muro* pool of salt-elutable monomeric extensin precursors

Table I. *Amino Acid Composition of Extensin Oligomers after Superose*

	Void Oligomers	Medium Oligomers	Small Oligomers	Monomers
		mol %		
Hyp	28.7[a]	33.9	33.8	33.5
Asp	3.1	1.6	1.6	1.8
Thr	5.8	5.5	5.8	7.2
Ser	9.1	9.3	9.3	9.5
Glu	5.2	2.3	2.1	1.9
Pro	7.0	7.5	7.9	8.3
Gly	3.6	2.9	2.3	1.6
Ala	2.5	1.4	1.2	2.0
Cys	0	0	0	0
Val	8.8	7.3	8.8	5.0
Met	0.5	0	0.3	0
Ilu	1.3	1.2	1.0	0.9
Leu	2.1	0.8	1.0	0.8
Tyr	7.1	8.6	8.1	8.9
Phe	0.9	0.6	0.6	0.6
Lys	8.2	9.1	8.0	10.1
His	4.9	7.3	7.0	7.1
Arg	1.2	0.7	1.4	0.7

[a] These are representative amino acid analyses of Figure 1 fractions: void and peaks I, II, and III.

to network extensin (40), while here we have shown that a similar brief wash of intact cells with $CaCl_2$ elutes a specific extensin cross-linking activity. We conclude that both enzyme and substrate are in the same organelle and therefore probably react *in muro*. On the other hand, if the *in vitro* cross-linking activity were artifactual we would need to explain how such a facile reaction *in vitro* is inactive *in muro*! We would also have to assume that true cross-linking activity was either nonelutable or highly labile. Thus, we propose the presence of a specific cross-linking enzyme. The following observations are consistent with our suggestion that the *in vitro* cross-linking activity represents a specific extensin peroxidase responsible for *in muro* cross-linking:

(a) The 8-d cells gave very active preparations more than sufficient to meet the *in muro* cross-link demand. For example, we calculated (40) that 1 g (dry weight) growing cells added 44 μg Hyp/h to the cell wall. This amounts to 2.2 mg extensin precursor/h per 100 g cells fresh weight, based on cells of 5% dry weight and extensin precursors containing 10% w/w Hyp. Now, the cross-linking activity eluted from 100 g cells fresh weight gave an initial cross-linking rate of 80 mg P1/min under standard conditions using 3.3 mg P1/ml ("Results"). Thus, even at *in muro* precursor concentrations as low as 33 μg/ml, the corresponding initial cross-linking rate of about 0.8 mg/min (or 48 mg/h) would comfortably exceed the minimum requirement of 2.2 mg/h.

(b) The cross-linking activity did not cross-link other proteins tested but was highly specific for extensin. The extent of the specificity must await purification of the enzyme and further experiments with interspecific extensins, deglycosylated extensins, and defined peptide substrates. Mixed isozymes of horseradish peroxidase did not cross-link extensin under similar conditions. Thus, cross-linking is not a property of plant peroxidases in general, but involves a specific enzyme tentatively identified as extensin peroxidase in view of its absolute requirement for peroxide.

(c) The cross-linking enzyme needed high levels of extensin monomers for saturation (Fig. 5). This is consistent with the relatively high *in muro* levels of monomeric substrate during rapid growth (40). One can predict a need for rapid monomer cross-linking under physiologically stressful conditions, such as invasion by pathogens, where rapid polymerization could create an effective barrier.

(d) The cross-linking enzyme required low levels of hydrogen peroxide for optimal activity, again physiologically consistent with the need to generate only low levels of such a highly diffusible and toxic molecule. There is good evidence for *in muro* H_2O_2 generation in other systems (16).

(e) An optimum enzymic activity around pH 6 means minimal cross-linking at lower pHs, consistent with the 'acid-growth' hypothesis (7). On the other hand, increased extensin cross-linking would be consistent with increased wall pH and concomitant slower growth. We assume that the *in muro* pH (buffered by a high level of pectic polyanions) is lower than the pH of a weakly buffered surrounding medium. Thus, during rapid growth the pH *in muro* is probably about two units below the cross-linking pH optimum; indeed, around pectic polyanions, the local 'micro' pH may even become low enough to cleave sensitive linkages, such as the single highly labile (19) Asp-Pro present in the postulated 'brace' extensin P3 (39, 41). This may reflect a mechanism for loosening a preexisting extensin network during rapid growth.

(f) An extensin peroxidase cross-linking activity elutable from the cell surface is also consistent with the well known correlation between the cessation of hypocotyl growth and increased levels of firmly bound hydroxyproline, presumably network extensin (8). Indeed, recent work (5) shows that, in pea epicotyls exposed to specific peroxidase inhibitors, the elongation rate and inhibitor K_d are correlated; *i.e.* the smaller the K_d (more effective inhibitor) the greater the inhibition of elongation rate.

(g) Although we have not chemically identified the intermolecular cross-link generated, the known intramolecular isodityrosine cross-link (13) might suggest its possible intermolecular involvement, especially as the occurrence of tyrosine-cross-linked networks is a general biological phenomenon including resilin (1), collagen (22), yeast cell walls (3), insect cuticle (17) and, of particular interest, the sea urchin fertilization envelope cross-linked by a specific ovoperoxidase (10).

However, the precise significance of extensin peroxidase to wall models and plant response to stress must await cross-link identification, further enzyme purification, and cDNA cloning, in progress. Nevertheless, the isolation of a soluble cross-linking system is consistent with major postulates (31, 32) of the 'warp-weft' model: extracellular assembly of two interpenetrating polymeric networks (42), where cross-link periodicity creates an extensin network of defined porosity which mechanically couples the major load-bearing cellulosic polymer. In the language of materials science, such a micro-composite might approximate to an 'angle-interlock' type of construction (6) with additional covalent cross-links. Remarkably small amounts of one component can influence the overall properties of a composite (2).

In summary, we have placed our earlier postulates of extensin cross-linking on a firmer biochemical footing, using a well-defined macromolecular substrate and a soluble enzymic fraction containing a putative extensin peroxidase.

Acknowledgments—We thank Ms. Virginia Boone for expert help with the cultures, Dr. John Heckman for the electron microscopy, Ms. Karen Taraszka for assaying activity against various peroxidase substrates, Ms. Marcia Kieliszewski, and Mr. Greg Rorrer for helpful discussions, and Mr. David Plass (MSU-High School Honors Science Program 1985) who first observed the extensin monomer to oligomer conversion. Dr. P. M. Dey thanks the Fulbright commission for a travel grant.

LITERATURE CITED

1. ANDERSEN SO 1964 The crosslinks in resilin identified as dityrosine and trityrosine. Biochim Biophys Acta 93: 213–215
2. BAER E 1986 Advanced polymers. Sci Am 255: 179–190
3. BRIZA R, G WINKLER, H KALCHHAUSER, M BREITENBACH 1986 Dityrosine is a prominent component of the yeast ascospore wall. A proof of its structure. J Biol Chem 261: 4288–4294

4. BRYSK MM, MJ CHRISPEELS 1972 Isolation and partial characterization of a hydroxyproline-rich cell wall glycoprotein and its cytoplasmic precursor. Biochim Biophys Acta 257: 421–432
5. BYL T, BJ TALLER, GR SCHONBAUM 1987 Benzohydroxamic acid-induced cell elongation in Pisum sativum. Plant Physiol 83: S-135
6. CHOU T-W, RL MCCULLOUGH, RB PIPES 1986 Composites. Sci Am 255: 193–203
7. CLELAND RE 1986 The role of hormones in wall loosening and plant growth. Aust J Plant Physiol 13: 93–103
8. CLELAND RE, AM KARLSNES 1967 A possible role of hydroxyproline-containing proteins in the cessation of cell elongation. Plant Physiol 42: 669–671
9. COOPER JB, JE VARNER 1984 Cross-linking of soluble extensin in isolated cell walls. Plant Physiol 76: 414–417
10. DEITS T, M FARRANCE, ES KAY, L MEDILL, EE TURNER, PJ WEIDMAN, BM SHAPIRO 1984 Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchin egg. J Biol Chem 259: 13525–13533
11. DE JONG DW 1966 Speculation on the mechanism of ion transport in roots based upon indirect evidence from histochemical studies. Bot Gaz 127: 17–26
12. DOUGALL DK, K SHIMBAYASHI 1960 Factors affecting growth of tobacco callus tissue and its incorporation of tyrosine. Plant Physiol 35: 396–404
13. EPSTEIN L, DTA LAMPORT 1984 An intramolecular linkage involving isodityrosine in extensin. Phytochemistry 23: 1241–1246
14. FRY SC 1979 Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. Planta 146: 343–351
15. FRY SC 1982 Isodityrosine, a new cross-linking amino acid from plant cell wall glycoprotein. Biochem J 204: 449–455
16. GROSS GG, C JANSE, EF ELSTNER 1977 Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (Armoracia lapathifolia Gilib.) Planta 136: 271–276
17. HASSON C, S MANICKAM 1987 Protein crosslinking by peroxidase: possible mechanism for sclerotization of insect cuticle. Arch Insect Biochem Physiol 5: 13–28
18. HECKMAN JW, BT TERHUNE, DTA LAMPORT 1988 Characterization of native and modified extensin monomers and oligomers by electron microscopy and gel filtration. Plant Physiol 86: 848–856
19. JAUREGUI-ADELL J, J MARTI 1975 Acidic cleavage of the aspartyl-profile bond and the limitations of the reaction. Anal Biochem 69: 468–473
20. KEEGSTRA K, KW TALMADGE, WD BAUER, P. ALBERSHEIM 1973 The structure of plant cell walls. Plant Physiol 51: 188–196
21. KIEFER S, D EVERDEEN, DTA LAMPORT 1987 *In vitro* cross-linking of extensin precursors. Plant Physiol 83: S-108.
22. LABELLA F, P WAYKOLE, G QUEEN 1968 Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. Biochem Biophys Res Commun 30: 333–338
23. LAMPORT DTA 1963 Oxygen fixation into hydroxyproline of plant cell wall protein. J Biol Chem 238: 1438–1440
24. LAMPORT DTA 1964 Cell suspension cultures of higher plants, isolation and growth energetics. Exp Cell Res 33: 195–206
25. LAMPORT DTA 1965 The protein component of the primary cell wall. Adv Bot Res 2: 151–218
26. LAMPORT DTA 1970 Cell wall metabolism. Annu Rev Plant Physiol 21: 235–270
27. LAMPORT DTA 1974 The role of hydroxyproline-rich proteins in the extracellular matrix of plants. *In* ED Hay, TJ King, J Papaconstaninou, eds, Macromolecules Regulating Growth and Development. The 30th Symposium of the Society for Developmental Biology. Academic Press, New York, pp 113–130
28. LAMPORT DTA 1978 Cell wall carbohydrates in relation to structure and function. *In* TA Thorpe, ed, Frontiers of Plant Tissue Culture, Proceedings of the 4th International Congress-Plant Tissue and Cell Culture. Calgary, Canada, pp 235–244
29. LAMPORT DTA 1980 Structure and function of plant glycoproteins. *In* J Preiss, ed, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp. 501–541
30. LAMPORT DTA 1986 Roles for peroxidases in cell wall genesis. *In* H Greppin, C Penel, Th Gaspar, eds, Molecular and Physiological Aspects of Plant Peroxidases. International Symposium, University of Geneva, Switzerland, 1985, pp 199–208
31. LAMPORT DTA 1986 The primary cell wall: a new model. *In* RA Young, RM Rowell, eds, Cellulose, Structure, Modification and Hydrolysis. John Wiley & Sons, New York, pp 77–90
32. LAMPORT DTA, L EPSTEIN 1983 A new model for the primary cell wall: a concatenated extensin-cellulose network. Curr Top Plant Biochem Physiol 2: 73–83
33. LAMPORT DTA, DH NORTHCOTE 1960 The use of tissue cultures for the study of plant cell walls. Biochem J 76: 52P
34. LAMPORT DTA, DH NORTHCOTE 1960 Hydroxyproline in primary cell walls of higher plants. Nature 188: 665–666
35. LEATHAM GF, V KING, MA STAHMANN 1980 In vitro protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. Phytopathology 70: 1134–1140
36. LIU EH, DTA LAMPORT 1974 An accounting of horseradish peroxidase isozymes associated with the cell wall and evidence that peroxidase does not contain hydroxyproline. Plant Physiol 54: 870–878
37. MORT AJ, DTA LAMPORT 1977 Anhydrous hydrogen fluoride deglycosylates glycoproteins. Anal Biochem 82: 289–309
38. RICE RH, YM LEE, WD BROWN 1983 Interactions of heme proteins with hydrogen peroxide: protein crosslinking and covalent binding of benzopyrene and estradiol. Arch Biochem Biophys 221: 417–427
39. SHOWALTER AM, JE VARNER 1988 Biology and molecular biology of plant hydroxyproline-rich glycoproteins. *In* A Marcus, ed, The Biochemistry of Plants, Vol 15. In press
40. SMITH JJ, EP MULDOON, DTA LAMPORT 1984 Isolation of extensin precursors by direct elution of tomato cell suspension cultures. Phytochemistry 23: 1233–1239
41. SMITH JJ, EP MULDOON, JJ WILLARD, DTA LAMPORT 1986 Tomato extensin precursors P1 and P2 are highly periodic structures. Phytochemistry 25: 1021–1030
42. SPERLING LH 1986 History and development of interpenetrating polymer networks. *In* RB Seymour, GS Kirschenbaum, eds, High Performance Polymers: Their Origins and Development. Elsevier, Amsterdam, pp 225–231
43. STUART DA, JE VARNER 1980 Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. Plant Physiol 66: 787–792