

Cellulase Occurs in Multiple Active Forms in Ripe Avocado Fruit Mesocarp

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ABSTRACT

The existence of multiple forms of avocado (*Persea americana* Mill. cv Hass) cellulase in crude protein extracts of ripe avocado fruit is reported. Cellulase was separated into at least 11 multiple forms by native isoelectric focusing in the pH range between 4 and 7 and visualized by both activity staining using Congo red and immunostaining. The enzyme components were acidic proteins with isoelectric points in the range of pH 5.10 to 6.80, the predominant forms having isoelectric points of 5.60, 5.80, 5.95, and 6.20. All 11 forms were immunologically related with molecular masses of 54 kilodaltons.

Ripening of avocado fruit is accompanied by softening of the fruit which is believed to be due to an extensive breakdown of its cell wall (20). Fruit softening is associated with an increase in the activities of hydrolytic enzymes such as cellulase and polygalacturonase (1, 6, 14, 19). Although an increase in avocado cellulase activity correlates well with softening (1) and changes in the structural integrity of the cell wall (19, 20), elucidation of its role *in vivo* is far from complete (12).

The increase in cellulase activity during avocado fruit ripening (1, 14, 19) is due to the *de novo* synthesis of its protein correlating with an increase in the steady-state amount of cellulase mRNA (8, 15, 26).

2-D¹ gel electrophoresis of *in vitro* translation of poly(A⁺) RNA from ripe fruit suggested the existence of multiple forms of cellulase protein that appear during ripening of avocado fruit (26). Subsequently, immunoprecipitation with avocado cellulase antibody of the translation products of poly(A⁺) RNA from ripe fruit and hybrid-select translation with a near full-length cDNA cellulase clone from avocado confirmed the existence of five to six cellulase isoforms (10). A similar pattern of antibody-reactive cellulase isoforms was shown after immunoblotting avocado crude protein extracts following 2-D PAGE (urea IEF/SDS-PAGE), (10). Thus, based on these results and on primer extension experiments, it was suggested that the multiplicity of cellulase isoforms arise from a message heterogeneity of cellulase mRNA (10). This message heterogeneity was also supported by the number of bands on Southern blots probed with cellulase cDNAs which was an

indication of the occurrence of a small cellulase gene family in avocado (25). However, only one cellulase cDNA and one gene, *cell*, has been identified to date that are expressed during avocado ripening (7, 8, 25). Because of this, arguments may arise suggesting that the multiple forms of cellulase protein after 2-D PAGE of the *in vitro* translation products of Poly(A⁺) RNA are artifacts of extraction or electrophoresis. Moreover, it is not known whether these multiple cellulase polypeptides correspond to a cohort of active forms of cellulase enzyme *in vivo*.

In this report, we present evidence that cellulase occurs in multiple active forms in crude protein extracts of ripening avocado fruit that are not artifacts of extraction or electrophoresis. Isoforms of cellulase were separated by native IEF and visualized by activity staining and by immunostaining.

MATERIALS AND METHODS

Total Protein Extraction

Frozen lyophilized tissue powder (1 g) was thawed in 3 mL of buffer (14) containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM MgSO₄, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 μM leupeptin, and 10% (v/v) glycerol. The mixture was allowed to stand on ice for 15 min with occasional stirring and then centrifuged at 20,000g for 20 min. The supernatant was passed through Miracloth (Calbiochem) and used for fractionation by native PAGE and native IEF.

PAGE, Native IEF, and 2-D Gels

Native PAGE was performed by the system of Laemmli (16) except that the SDS was omitted. The resolving gel consisted of 7.5T2.5C and 0.1% CMC (24), and the stacking gel was 5.0T2.5C. Electrophoresis was conducted at 4°C with a constant current of 20 mA until the dye reached the bottom of the slab gel.

Native IEF was carried out in a vertical minigel system (Bio-Rad) as described previously (23). The gels (1.5 mm thick) were prepared from the following mixture: 5.05 mL water, 1.8 mL acrylamide mixture (30% [w/v] acrylamide, 0.8% [w/v] bis-acrylamide), 1.8 mL 50% (v/v) glycerol, and 0.45 mL ampholyte mixture of 3:1 ratio of pH range 5 to 7 to 4 to 6. Gels were polymerized with the addition of 40 μL of 10% (w/v) ammonium persulfate and 10 μL TEMED. The cathode solution was 25 mM NaOH, and the anode solution was 20 mM acetic acid. Protein samples were mixed with 4% (v/v) ampholyte of the same pH range used for the gel preparation and electrophoresed for 1.5 h at a constant 180

¹ Abbreviations: 2-D, two-dimensional; poly(A⁺) RNA, polyadenylated RNA; CMC, carboxymethylcellulose; IEF, isoelectric focusing; pI, isoelectric point; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

V and then for an additional 1.5 h at a constant 300 V. The temperature during electrophoresis was kept low by placing the minigel in ice.

The pI of cellulase isoforms was determined by excision of a series of equal segments from the gel and by subsequent elution in 10 mM KCl and measurement of the pH of the solution with a conventional electrode. These values were verified by using LKB pI protein markers.

2-D PAGE (native IEF, first dimension; SDS-PAGE, second dimension) was carried out as described by O'Farrell (18). Following IEF, one lane from the native IEF slab gel was cut out and fixed in 50% methanol:15% acetic acid for 20 min. Subsequently, the lane was placed in sample buffer (17) for 20 min. Next, the lane was positioned in a stacking gel using the Laemmli system (16) and electrophoresed at 200 V for 45 min in a Bio-Rad minigel system. For the 2-D gels (native IEF/native IEF), one lane was cut out from the first dimension native IEF gel and placed in a second dimension native IEF slab gel, pH range between 4 and 7. Electrophoretic conditions were as described earlier.

Activity Staining

Cellulase activity on CMC-PAGE was detected as follows. The gel was incubated in 0.1 M sodium acetate (pH 5) for 40 min and then stained in 1% Congo red (2, 4) for another 40 min. Next, the gel was destained in 1 M NaCl until clear bands in the red background were obtained. Cellulase activity on native IEF gels was stained according to the overlay technique (2, 4, 21). Following IEF, the gel was incubated in 0.1 M sodium acetate (pH 5) for 10 min with mild agitation. IEF gels were then layered carefully on CMC-containing agarose-gel bonds and incubated at 30°C for 1 to 4 h, depending on the specific activity of the enzyme. After incubation, gels were separated, and CMC-agarose gels were placed in 1% (w/v) Congo red solution for 20 to 30 min and then destained in 1 M NaCl. For photographic purposes (better contrast), the agarose overlays were rinsed in 5% (v/v) acetic acid which turned the red background into dark blue. The overlay gels for enzyme detection were cast between two glass plates separated by 0.5-mm spacers. On one of these plates, a gel support film for agarose was placed so that the agarose overlay was affixed permanently (21). The overlays contained 0.8% (w/v) agarose, 0.1 M sodium acetate (pH 5), and 0.1% (w/v) CMC.

Protein Gel Blotting

Electrophoretic blotting of IEF gels was carried out in 12.5 mM Tris-96 mM glycine buffer. Immunological detection methods were as described before (15). Biotinylated proteins were used as mol wt standard markers (11).

Protein Determination

Protein concentration was measured by the method of Bradford (5) using BSA as a standard.

RESULTS

To establish the presence of cellulase isoforms *in vivo*, we attempted to activity stain the cellulase isoforms following

resolution by native PAGE. When the substrate for cellulase was incorporated in the native separating gel, seven active, but not well-resolved, forms could be seen (data not shown). It should be mentioned that the incorporation of 0.1% (w/v) CMC in the IEF gel resulted in the degradation of the substrate during electrophoresis, indicating that the cellulase forms were active during electrophoresis as well as near their pIs.

To resolve this complication, we utilized an agarose overlay after IEF to detect enzyme activity. Native IEF in the pH range between 4 and 7 resolved cellulase protein into 10 to 11 active forms which degraded CMC on the agarose overlay (Fig. 1). Native IEF coupled with the overlay technique gave better separation and sharper bands than those resolved by native PAGE. The pIs of cellulase forms ranged from pH 5.10 to 6.80 with the more abundant forms having pIs of 5.60, 5.80, 5.95, and 6.20 (Fig. 1).

A possibility exists that these 11 forms resulted from ampholyte-protein interactions. Although native PAGE resolved cellulase in multiple active forms, the possibility of a possible artifact due to IEF was minimized by the following steps which were done to elucidate the situation.

First, equal amounts of sample were applied at either end of the gel. Figures 1 and 2 show that similar patterns of cellulase forms were obtained regardless of the position of sample application, *i.e.* in the alkaline or in the acetic environment. Protein samples (equivalent to 20 μ g protein) were applied in the wells of the IEF gels filled with 25 mM NaOH (Fig. 1) and 20 mM acetic acid (Fig. 2). Second, the immunoblot of Figure 3 which resulted from 2-D gel electrophoresis (native IEF, first dimension; native IEF, second dimension)

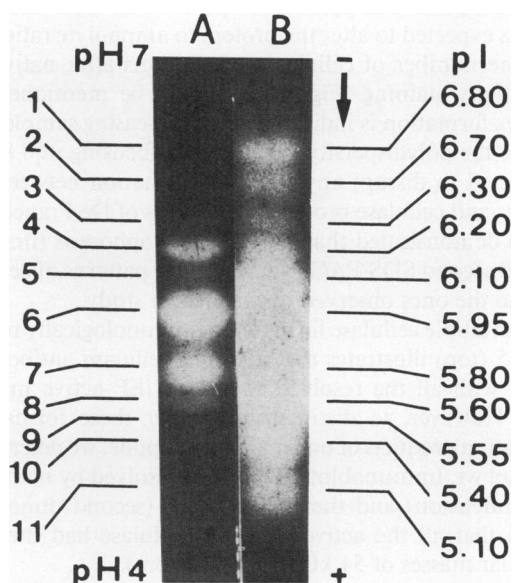


Figure 1. Native IEF of avocado fruit cellulase. To distinguish the multiple active forms, gel A was incubated on CMC-agarose gel for 2 h, whereas gel B was incubated for 4.5 h. Numbers on the left indicate the cellulase active forms starting from the cathode end. Numbers on the right show the pI values of cellulase isoforms. Protein samples (equivalent to 20 μ g protein) were applied in the wells of the IEF gel filled with 25 mM NaOH (cathode). The anode was 20 mM acetic acid.

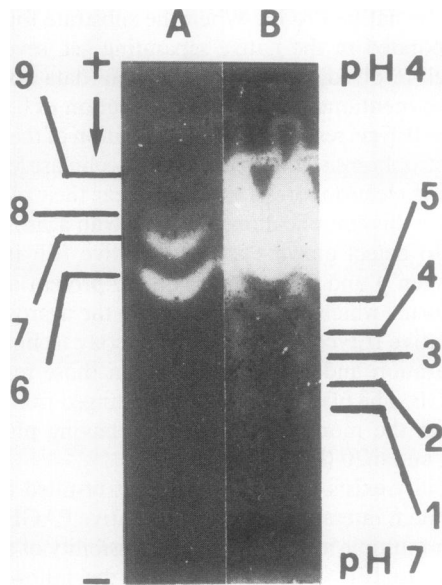


Figure 2. Native IEF of ripe avocado fruit cellulase. To distinguish the multiple active forms, gel A was incubated on CMC-agarose gel for 30 min, whereas gel B was incubated for 1.5 h. Numbering and conditions were the same as in Figure 1 except that protein samples were loaded into the acidic end (anode, 20 mM acetic) rather than into the basic end (cathode, 25 mM NaOH). The electric poles were reversed.

reveals that each band of cellulase in the first dimension gave, again, one band in the second dimension. Third, serial dilution of the protein sample from ripe avocado fruit mesocarp, which is expected to alter the protein to ampholyte ratio, gave the same number of cellulase active forms after native IEF and activity staining (Fig. 4). It should be mentioned that complex formation is indicated when increasing sample loads increase the polydispersity (13). Fourth, focusing in 8 M urea is expected to disrupt any complex formation between ampholytes and cellulase protein. The results of De Francesco *et al.* (10) demonstrated that 2-D gel electrophoresis (first urea IEF and second SDS-PAGE) gave similar patterns of cellulase forms to the ones observed in the present study.

The multiple cellulase forms were immunologically related. Figure 5 (top) illustrates that avocado cellulase antibody (3) reacted with all the resolved by native IEF active multiple forms. However, to distinguish whether these forms were degradation products of one main polypeptide, we determined their mol wt. Immunoblots of proteins resolved by native IEF (first dimension) and then SDS-PAGE (second dimension) showed that all the active forms of cellulase had the same molecular masses of 54 kD (Fig. 5, bottom).

DISCUSSION

The progress in avocado fruit softening closely correlates with an increase in activity, protein, and mRNA levels of cellulase (1, 6–8, 10, 14, 15, 19, 25, 26). Despite the recent knowledge obtained concerning the synthesis, processing (3, 9), and gene expression of cellulase during avocado ripening (7, 8, 15, 25, 26), there is no available information regarding

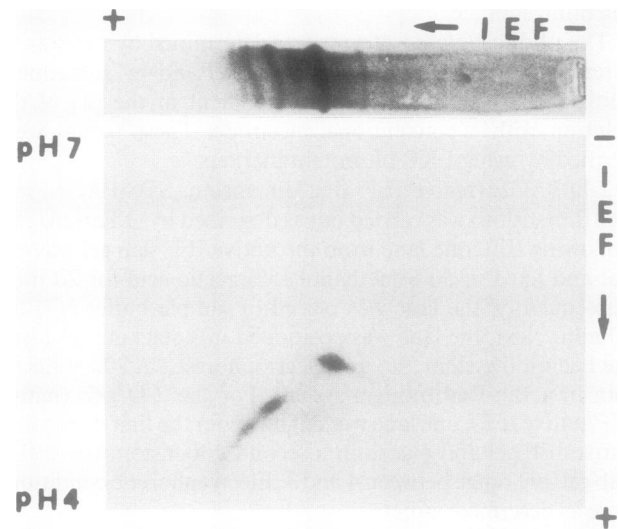


Figure 3. Protein immunoblot analysis of ripe avocado cellulase after 2-D gel electrophoresis (native IEF, first dimension; native IEF, second dimension). Top, Avocado proteins were fractionated by native IEF and one lane was cut, blotted onto the nitrocellulose membrane, and reacted with polyclonal cellulase antibody. Bottom, A lane was cut from the same IEF gel as the top panel and was subjected to native IEF again in the second dimension; it was subsequently blotted and reacted with the same cellulase antibody.

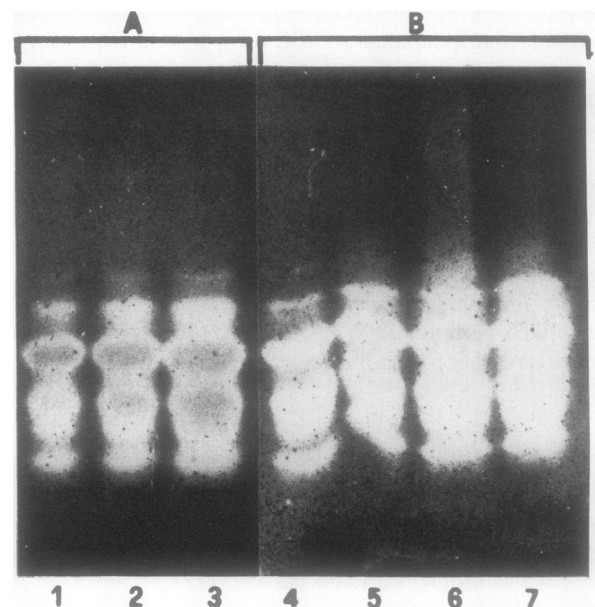


Figure 4. Activity staining of serial dilutions of avocado fruit cellulase after native IEF. Aliquots of serially diluted crude avocado protein extracts were applied in the wells of the IEF gel. Lanes 1 to 7 (μg of protein): 1, 7.5; 2, 15; 3, 30; 4, 60; 5, 90; 6, 120; 7, 180. A, Gel was incubated on CMC-agarose gel for 2 h; B, gel was incubated for 1 h. Conditions of gels were the same as in Figure 1.

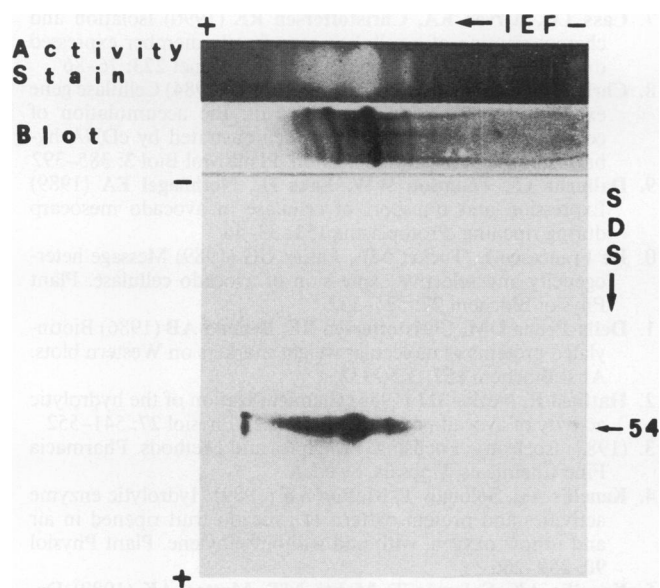


Figure 5. 2-D gel electrophoresis of cellulase (native IEF, first dimension; SDS-PAGE, second dimension). Top, Avocado proteins were analyzed by native IEF and either activity stained or immunovisualized by cellulase antibody. Bottom, A lane was cut from the native IEF and was subjected to SDS-PAGE; it was subsequently blotted and reacted with the same cellulase antibody.

number, electrophoretic separation, and active forms of avocado fruit cellulase.

The present results demonstrate that avocado cellulase can be resolved into at least 11 distinct forms by native IEF (Fig. 1), which degraded CMC on the CMC-agarose gels. Instead of the approximately seven isoforms that were separated by native PAGE, 11 protein zones showing cellulase activity were recognized under the refined conditions of IEF and the overlay technique. The enzyme component with the pI of 5.95 was the major form in all our preparations and the first to appear during ripening (ref. 10 and manuscript in preparation).

The heterogeneity of cellulase protein is based mainly on charge properties, and this can explain the difference in resolution between native PAGE and IEF. These results combined with those of Figure 5, which show that all of the cellulase isoforms had the same mol wt and few differences in pIs and, therefore, a relatively small difference in the ratios of charge to mass, would account for the poor separation and resolution of cellulase in native PAGE. It should be mentioned that the migration of proteins in native gels and IEF is determined by the ratio of charge to mass and charge, respectively.

Heterogeneity is possibly caused by one or more of the following: (a) artifact due to IEF (protein-ampholyte interactions) (13, 22), (b) artifact due to extraction procedure, (c) posttranslational modification (variations in a carbohydrate or lipid moiety of the enzyme or differences in their sulfation, phosphorylation, or acetylation pattern), and (d) existence of a cellulase gene family and/or message heterogeneity.

Our results indicate that the heterogeneity of cellulase isoforms was not the result of protein-ampholyte interactions (Figs. 2, 3, and 4). First, when equal amounts of protein

samples were applied at either alkaline or acidic end of a IEF gel, similar patterns were obtained indicating that complex formation is unlikely, because the protein samples have been in contact with different species of ampholytes (Fig. 2). Second, a focused lane gave single spots on protein blots when refocused in the second dimension (Fig. 3). Resplitting into several bands is an indication of artifact formation (13). Third, different amounts of protein extract ranging from 7.5 to 180 μ g of protein, which effectively alters the protein to ampholyte ratio, gave similar patterns of focused isoforms. Complex formation is indicated when increasing sample loads increase the polydispersity (13). Finally, combining the results of De Francesco *et al.* (10) with those of Figure 5 suggests that electrofocusing in either 8 M urea (10) or in native conditions (Fig. 5) gave similar patterns on protein blots derived from 2-D gels (IEF, first dimension; SDS-PAGE, second dimension). It should be mentioned that any complex formation would be disrupted in 8 M urea.

The possibility of artifact formation during extraction procedure seems improbable, because 2-D gels of *in vitro* translation products of immunoprecipitated with cellulase antibody of poly(A)⁺ RNA and hybrid-selected mRNA (10) gave similar patterns to protein blots derived from 2-D gels using a crude protein preparation solubilized by different extractions procedures (10) (Fig. 5).

Heterogeneity caused by variations in a carbohydrate or lipid moiety of the enzyme and/or in their sulfation, phosphorylation, and acetylation pattern can not be ruled out. Avocado cellulase is a glycoprotein and four molecular forms of avocado cellulase that represent processing intermediates of the protein have been demonstrated (3). However, 2-D gels showed that all of the native IEF forms had similar mobilities in SDS-polyacrylamide gels. It should be noted, however, that some lack of accurate resolution in minigel systems is expected. Moreover, *in vitro* translation products from both polysomal poly(A)⁺ RNA and hybrid-selected cellulase mRNA (10), which did not undergo posttranslational modifications, showed similar patterns in 2-D gels to those presented here (Fig. 5).

The multiplicity of cellulase active forms can be, most probably, explained by the presence of a heterogeneous population of cellulase mRNA that is expressed during avocado ripening. Indeed, De Francesco *et al.* (10) suggested the existence of such message heterogeneity of cellulase based on (a) 2-D gel analysis of translation products of ripe avocado mRNA immunoprecipitated with the same cellulase antibody used in this study and hybrid selected with cellulase cDNA clones and (b) primer extension experiments. It is noteworthy that the 2-D gel analysis of De Francesco *et al.* (10) resembled that presented in Figure 5.

This message heterogeneity of avocado cellulase can be explained by the existence of a small cellulase gene family. The evidence for the existence of such a gene family is not conclusive. Tucker *et al.* (25) proposed the presence of a small cellulase gene family in avocado, based on multiple bands on Southern blots that reacted with pAV363 a full cDNA cellulase clone. In support of this observation, as cited in ref. 10, Clegg and his colleagues believe that cellulase may be encoded by a small multigene family in avocado. They analyzed cellulase genes in 17 varieties and six species of

avocado by probing Southern blots with cellulase cDNA probes, and in all cases, they noticed a consistent presence of multiple bands on Southern blots. On the other hand, Cass *et al.* (7), without ruling out the possibility of the existence of a small cellulase gene family, proposed that the gene designated *cell* is responsible for a major portion, if not all, of the cellulase transcripts in ripe fruit. In addition to the *cell* gene, they also cloned and characterized a gene designated *cel2* which exhibited extensive nucleic acid and amino acid sequence similarity with *cell*, 81 and 95%, respectively (7). However, none of the known characterized cellulase cDNAs derived from ripe fruit represent *cel2* transcripts. They postulated that *cel2* may be expressed in low levels in ripe fruit. Therefore, the development of gene-specific probes for *cel2* will allow them to study the expression of *cel2* during fruit ripening. In this context, the product of *cel2* might well be one or more of the multiple forms analyzed by native IEF that degraded CMC *in vitro* in our experiments. Alternatively, if we assume that the *cell* (7) is responsible for all the cellulase transcripts in ripe fruit, then the message multiplicity would be explained by posttranscriptional processing, *i.e.* exon shuffling as suggested by De Francesco *et al.* (10).

In conclusion, cellulase occurs in multiple active forms in ripe avocado fruit mesocarp based on native IEF gels and visualized by enzyme activity staining and immunodetection.

ACKNOWLEDGMENTS

We thank Dr. G. Thireos for reading the manuscript, Dr. A.B. Bennett for generously providing the avocado cellulase antibody, and Dr. M.L. Tucker for his constructive comments on the manuscript. We are grateful to Prof. K.A. Roubelakis-Angelakis for her continuous support and for providing laboratory space.

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