Synthesis and Processing of Cellulase from Ripening Avocado Fruit

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ABSTRACT

The biosynthesis and processing of cellulase from ripening avocado fruit was studied. The mature protein is a glycoprotein, as judged by concanavalin A binding, with a molecular weight of 54,200. Upon complete deglycosylation by treatment with trifluoromethane sulfonic acid the mature protein has a molecular weight of 52,800 whereas the immunoprecipitated *in vitro* translation product has a molecular weight of 54,000. This result indicates that cellulase is synthesized as a large molecular weight precursor, which presumably possesses a short-lived signal peptide. A membrane-associated and heavily glycosylated form of the protein was also identified. This putative secretory precursor was enzymically active and the carbohydrate side chains were sensitive to endoglycosidase H cleavage. Results of partial endoglycosidase H digestion suggest that this precursor form of the mature glycoprotein possesses two high-mannose oligosaccharide side chains. The oligosaccharide chains of the mature protein were insensitive to endoglycosidase H cleavage, indicating that transport of the membrane-associated cellulase to the cell wall was accompanied by modification of the oligosaccharide side chains. The presence of a large pool of endoglycosidase H-sensitive membrane-associated cellulase (relative to an endoglycosidase H-insensitive form) suggest that transit of this protein through the Golgi is rapid relative to transit through the endoplasmic reticulum.

Cellulase has been shown to be induced to a high level during ripening of avocado fruit (4, 30). Recent work has demonstrated, using a cellulase cDNA probe, that the increase in cellulase protein results from an increase in cellulase mRNA at the onset of ripening (14). Although the induction of cellulase is temporally associated with fruit softening (4, 30), the substrate of this enzyme *in vivo* remains unclear (19). In spite of our incomplete understanding of the role of cellulase in fruit softening, its high level of induction suggests that it plays an important role in fruit ripening, presumably by contributing to cell wall degradation.

In addition to its possible importance in determining textural properties of ripe avocado fruit, the basic regulatory mechanisms involved in cellulase gene expression and secretion are of great interest. Indeed, mechanisms of protein secretion from plant cells have received only scant attention. Two notable exceptions are studies of hydroxyproline rich protein (9, 13) and of α-amylase secretion from cereal aleurone cells (23, 25, 28, 29, 31). In both cases the presence of a short-lived signal peptide at the N-terminus of the protein has been demonstrated (9, 28, 29). In addition to an N-terminal signal peptide, glycosylation and post-translational modifications of proteins may play a role in target-

ing secreted proteins to the correct cellular compartment (17). Studies of intracellular transport of legume storage proteins have demonstrated that intracellular targeting may be accompanied by co-translational glycosylation (5) and posttranslational processing of either the carbohydrate (11) or polypeptide moiety (6) of the transported protein. Considerably less is known about the pathway and processing events associated with secretion of proteins to the cell wall. As a first step in describing the molecular basis of secretion of ripening-associated proteins we have characterized the molecular forms of avocado cellulase following synthesis, processing, and secretion to the cell wall.

MATERIALS AND METHODS

Plant Material. Avocados (*Persea americana* Mill. cv Hass) were obtained from the University of California South Coast Field Station within 48 h after harvest. The avocados were placed in individual respiration jars at 20°C with continuous air flow (6 L/h). Production of ethylene and CO_2 was monitored and ripe fruit selected and used for cellulase extractions approximately 2 d after peak ethylene production.

Cellulase Purification. Cellulase was purified by first homogenizing 100 g of ripe avocado fruit tissue in 1 L of ice-cold 40 mM Na acetate, 100 mM NaCl (pH 5.0). The homogenate was centrifuged at 11,000g for 20 min at 4°C in a GSA rotor (Sorvall). The middle soluble layer was collected (820 ml) kept at 4°C and brought to 30% $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$. After 2 h at 4°C the precipitate was pelleted at 11,000g for 20 min in a GSA rotor (Sorvall) and the supernatant brought to 55% $(NH_4)_2SO_4$. The resulting precipitate was collected as above, dissolved in 40 ml of 40 mM Na acetate (pH 5.0) and dialyzed overnight against 4 L of the same buffer. Precipitated material formed during dialysis was pelleted by centrifugation at 8,500g for 10 min in a HB-4 rotor (Sorvall) and discarded. Ten ml of the soluble enzyme extract was applied to a 2.5 × 18 cm column of CF-11 cellulose (Whatman). The cellulose column was eluted with 4 column volumes of 40 mM Na acetate (pH 5.0) followed by 2 column volumes of 40 mM Na acetate, 100 mM NaCl, and 100 mM cellobiose (pH 5.0) as described previously by Awad and Lewis (3). Protein-containing fractions eluted with cellobiose buffer were pooled and concentrated in an ultrafiltration pressure cell (Amicon; YM-10 membrane) to 10 ml. Two buffer changes to 50 mM Na acetate, 500 mM NaCl, 1 mM DTT, 1 mM Ca acetate, 1 mM Mn acetate (pH 6.0) (ConA[2] buffer) were made during ultrafiltration. This concentrated fraction was applied to a 1.6 × 16 cm column of concanavalin A-Sepharose 4B (Pharmacia) and eluted with 4 column volumes of ConA buffer followed by 1 column volume of the same buffer supplemented with 100 mM α-methylmannoside. Protein-containing fractions eluted with α-methylmannoside were concentrated, as described

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above, to 7 ml and the buffer changed to 40 mM Na acetate (pH 5.0). This fraction was applied to a 1.6 × 30 cm column of Sephacryl S-300 (Pharmacia) and eluted with the same buffer. Cellulase-containing fractions were concentrated to 10 ml and stored at −70°C.

The purification of cellulase was monitored by 'dot-blotting' (20) of fraction aliquots and immunodetection of cellulase using cellulase antibody and horseradish peroxidase-conjugated second antibody (20), or by monitoring of cellulase activity (see below).

Cellulase Antibody. Antiserum to avocado cellulase was generously provided by Dr. L. Lewis and M. Durbin (3). Serum IgG was purified by chromatography on protein A-Sepharose 4B (18). The IgG fraction was further purified by passage over a column to which protein extracted from preclimacteric avocado fruit was covalently bound to CNBr-activated Sepharose 4B (Pharmacia). Because cellulase is not present in preclimacteric avocado fruit, this step absorbed minor contaminating antibodies to other avocado proteins. The resulting antibody was highly specific for cellulase as judged by immunoblot analysis (see below).

Membrane Isolation. Ripe avocado fruit tissue (18 g) was homogenized in 30 ml of 200 mM sucrose, 150 mM KCl, and 25 mM Tris/Mes (pH 7.0). The homogenate was centrifuged at 5000g for 20 min in a SW 28 (Beckman) rotor. The soluble middle layer was collected. Membrane and soluble proteins were separated by chromatographing 10 ml aliquots on a 1.6 × 20 cm column of Sepharose 4B (Pharmacia). Alternatively, a microsomal membrane fraction was isolated from the avocado extract by collecting a 10,000 to 80,000g membrane pellet. A greater yield of membrane-associated cellulase, was obtained by the latter method.

Gel Electrophoresis, Protein Blotting, and Immunodetection. Proteins were analyzed by SDS-PAGE using 16 cm 10% polyacrylamide gels and the buffer system of Laemmli (24). For immunodetection of cellulase, proteins were electroblotted to nitrocellulose (Bio-Rad) essentially as described by Burnette (7). The transfer buffer contained 150 mM Tris, 20 mM glycine, 20% methanol, and 0.01% SDS and transfer was carried out at 1 A for 75 to 90 min. Protein blots were first incubated in 20 mM Tris, 150 mM NaCl (pH 7.5) (TBS) supplemented with 3% BSA. Cellulase protein was detected by incubation in TBS with cellulase antiserum (1:15,000 dilution) followed by two washes in TBS supplemented with 0.05% Tween-20. The blot was then incubated in TBS with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution) (Bio-Rad) and, following two washes in TBS supplemented with 0.05% Tween-20, developed in TBS with 0.5 mg/ml 1-chloro-4-naphthol (Bio-Rad) and 0.01% H_2O_2. Biotinylated proteins were used as mol wt markers on protein blots as previously described (15).

Protein Deglycosylation. Deglycosylation of membrane-associated and mature (soluble) cellulase was carried out enzymically and chemically. Prior to enzymic deglycosylation, protein samples were treated with either 0.2% Triton X-100 or by heating to 100°C in the presence of 0.2% SDS. Following this pretreatment the sample was diluted 3-fold in 50 mM Na acetate (pH 5.75). Enzymic deglycosylation with endoglycosidase H (Miles Laboratories) was carried out in 150 μl containing 50 mM Na acetate (pH 5.75), 7.5 milliunits endoglycosidase H, and 10 μg of pure cellulase or 150 μg of membrane-associated cellulase protein. The reaction was carried out at 35°C for 10 h. Chemical deglycosylation of mature (soluble) cellulase was carried out by anhydrous treatment with trifluoromethane sulfonic acid as described (16).

Trypsin Treatment. Membrane-associated (200 μg) or purified soluble (2 μg) cellulase was incubated for 1 h at 28°C in 200 μl of 250 mM sucrose, 25 mM Hepes, (pH 7.5), and 50 units trypsin (Behring Diagnostics). Before addition of trypsin, membrane-

associated cellulase was either untreated, or pretreated with 0.05% Triton X-100 or by sonication in a bath sonicator for 5 min. Trypsin digestion was stopped by the addition of electrophoresis sample buffer (2% SDS, 5% 2-mercaptoethanol, 60 mM Tris, 10% glycerol [pH 6.8]) and boiling. Products were analyzed by SDS-PAGE and immunoblotting.

In Vitro Translation. RNA was isolated from ripe avocado fruit by the method of Cathala et al. (8) and poly (A)⁺ RNA purified by oligo-dT cellulose (Pharmacia) chromatography (26). In vitro translation of poly-(A)⁺ RNA was carried out in the presence of [³⁵S]methionine, using a wheat germ extract prepared according to Anderson et al. (2). Immunoprecipitation of in vitro translation products was carried out using heat killed, formalin fixed Staphyloccous aureus cells (Miles Laboratories) as described (22).

Assays. Cellulase activity was assayed viscometrically using carboxylmethylcellulose (Sigma) as substrate as described by Awad and Lewis (3) in the presence of 0.1% Triton X-100. Phospholipids were assayed according to Stewart (33). Protein was assayed according to Markwell et al. (27). Enzyme linked immunosorbent assays of cellulase in column fractions were carried out by binding protein (antigen) to microtiter plate wells and quantifying cellulase protein by reaction with cellulase antiserum and horseradish peroxidase conjugated goat anti-rabbit IgG (32). The peroxidase substrate used was 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS; Sigma) and 0.01% H_2O_2. Absorbance was measured with a microtiter plate reader (Flow Laboratories).

RESULTS

Cellulase Purification. Our purification scheme represents an extension of the purification of avocado cellulase previously reported by Awad and Lewis (3). We begin by making a two step $(NH_4)_2SO_4$ precipitation that results in an extract enriched in cellulase (Fig. 1, lane 1). Our first purification step employed a cellulose column in an affinity purification step originally used by Awad and Lewis (3). This results in a high degree of purification (Fig. 1, lane 2). However, less than 50% of cellulase binds to the cellulose column, resulting in low recovery. Rather than repeating this low yielding step, as described previously (3), we substituted two other chromatographic purification steps. The first step was lectin affinity chromatography using ConA-Sepharose 4B. All of the applied cellulase bound to ConA-Sepharose 4B column and was quantitatively eluted with 100 mM α-methylmannoside. A final gel filtration chromatographic step (Sephacryl S-300) was employed to yield essentially pure cellulase (Fig. 1, lane 4). This purified mature form of cellulase was used for comparison with other forms of cellulase on SDS polyacrylamide gels.

Membrane-Associated Cellulase. Chromatography of an avocado extract on Sepharose 4B revealed two peaks of cellulase protein (Fig. 2A) and of cellulase activity (Fig. 2C). The first peak eluted in the void volume of the column. Previous studies have suggested that Sepharose 4B void volume eluants represent membranous components of the tissue extract (25). This interpretation is supported by the elution profiles of phospholipid and protein (Fig. 2B). Essentially all of the phospholipid, but only 9% of the protein, eluted in the void volume. Approximately 5% of cellulase activity and cellulase protein was present in the void volume fraction.

To quantify the relative levels of cellulase activity in the two Sepharose 4B column peaks it was necessary to ensure that the enzyme effect on carboxylmethyl cellulose viscosity ($N^{-3.66}$) was linear with time (1). Figure 3 shows the time course of the cellulase activity for equal volume aliquots taken from fraction 4 or 13 of the Sepharose 4B column elution (Fig. 2). In both cases the activity was linear over a 6 h time period and the
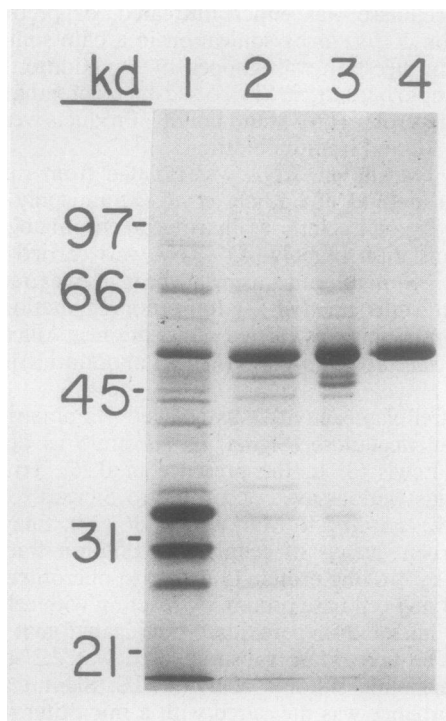
FIG. 1. SDS-polyacrylamide gel of proteins at stages of cellulase purification. Lane 1, (NH₄)₂SO₄ fractionated (30–55%) proteins (40 μg); lane 2, proteins eluted from cellulose column with cellobiose-containing (20 μg) buffer; lane 3, proteins eluded from ConA-Sepharose 4B column with α-methylmannoside containing (10 μg) buffer; lane 4, proteins in fractions containing peak cellulase activity following gel filtration chromatography on Sephacryl S-300 (5 μg). The gel was stained with Coomassie blue G-250.

relative cellulase activity in each fraction could be determined from the slopes of the lines (1). Based on these slopes and on the quantitation of cellulase protein by enzyme-linked immunosorbent assay it was possible to estimate the relative specific activities of cellulase in fractions 4 and 13 (Table I). Although this activity is not expressed in absolute terms, the similarity in relative values indicates that the membrane-associated cellulase possesses enzymic activity of similar magnitude as the soluble form of cellulase.

We next examined whether the membrane-associated cellulase was adsorbed to membrane surfaces or present within membrane vesicles. The accessibility of the membrane-associated cellulase to trypsin digestion was used as a criterion to identify exposed portions of the cellulase protein. In the absence of membrane-disruptive agents cellulase protein was protected from proteolysis (Fig. 4, lane 4). In the presence of 0.05% Triton X-100, or following sonication, cellulase became exposed to the proteolytic action of trypsin (Fig. 4, lanes 6 and 7). This result suggests that membrane-associated cellulase is not simply absorbed to the membrane surface and may be present in a soluble form within membrane vesicles.

Deglycosylation of Membrane-Associated Cellulase. The membrane-associated cellulase was approximately 2500 D larger than the mature (soluble) cellulase, as judged by SDS-PAGE (Fig. 2A). To determine whether this apparent size difference was due to differential glycosylation of the two cellulase forms we treated the proteins with endoglycosidase H, an enzyme that cleaves high-mannose oligosaccharide side chains between the two proximal GlcNAc residues (34). Before endoglycosidase H treatment the cellulase protein was pretreated with either 0.2% Triton X-100 or by heating to 100°C in the presence of 0.2%
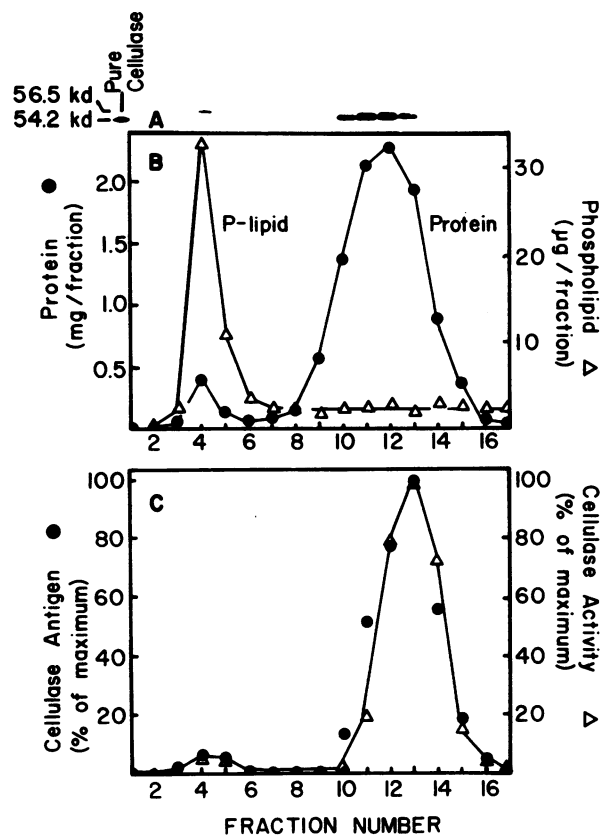

FIG. 2. Separation of membrane-associated and soluble cellulase by Sepharose 4B chromatography. A, Immunoblot of proteins from each column fraction detected with cellulase antibody and horseradish peroxidase conjugated second antibody. Mol wt were determined by reference to biotinylated protein standards (12). B, Elution profile of phospholipid and protein; C, elution profile of cellulase antigen (determined by ELISA) and of cellulase activity.
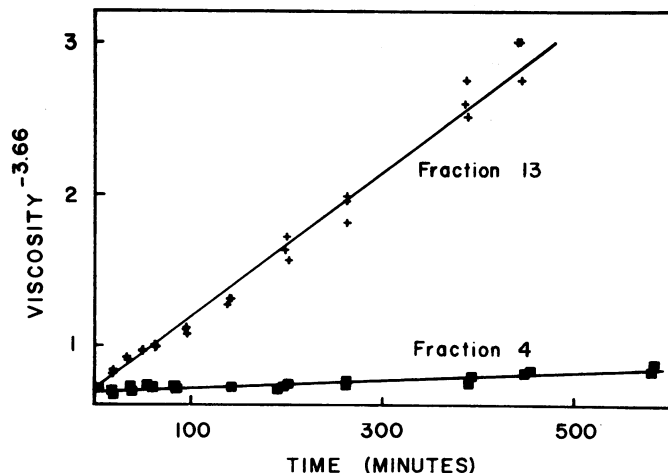

FIG. 3. Time course of cellulase activity associated with fractions 4 and 13 of the Sepharose 4B elution. Viscosity was calculated and raised to the power of 3.66 according to Almin et al. (1). The slopes of the lines provide a relative estimate of cellulase activity in each fraction (1).

SDS. The membrane-associated cellulase was sensitive to endoglycosidase H treatment whereas the mature cellulase was not (Fig. 5). When the membrane-associated cellulase was pretreated with Triton X-100, endoglycosidase H treatment yielded three distinct cellulase bands of approximately 56,500, 54,800, and

Table I. *Relative Specific Activities of Membrane-Associated and Soluble Cellulase*

Relative cellulase activity was determined from the slopes of the lines in Figure 3. The relative amount of cellulase in each fraction was determined by ELISA (see "Materials and Methods") using serial dilutions of aliquots from fractions 4 and 13. The values shown were determined from a linear region of the assay and corrected to represent the absorbance developed from a 10 μl aliquot of each fraction. The relative specific activity was calculated as the ratio of activity per unit absorbance from the ELISA.

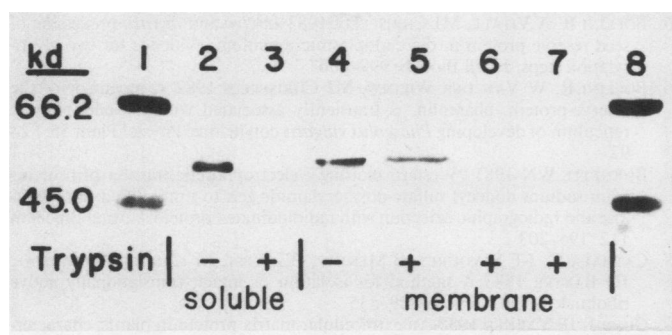| Fraction	Relative Cellulase Activity	ELISA Absorbance	Relative Specific Activity
	slope × 10^3	A/10 μl	slope/A
4	0.29	0.08	3.62
13	4.97	1.40	3.55

FIG. 4. Immunoblot of soluble and membrane-associated cellulase following trypsin treatment. Lanes 1 and 8, biotinylated mol wt markers (12); lane 2, purified soluble cellulase; lane 3, purified soluble cellulase following trypsin treatment; lane 4, membrane-associated cellulase; lane 5, membrane-associated cellulase following trypsin treatment; lane 6, membrane-associated cellulase following trypsin treatment in the presence of 0.05% Triton X-100; lane 7, membrane-associated cellulase following 5 min sonication and trypsin treatment.
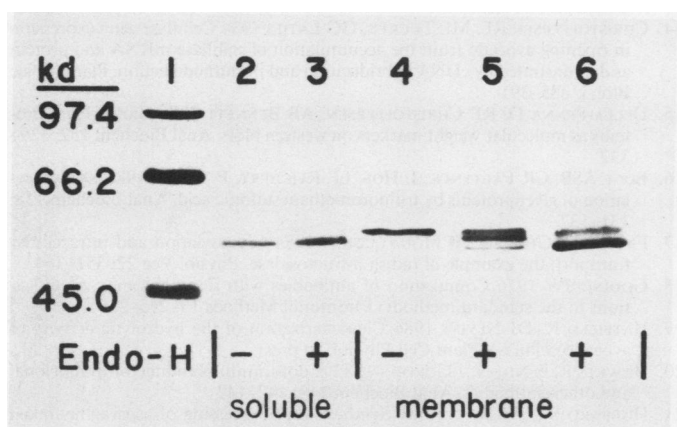

FIG. 5. Immunoblot of soluble and membrane-associated cellulase following endoglycosidase H treatment. Lane 1, biotinylated mol wt markers (12); lane 2, purified soluble cellulase; lane 3, purified soluble cellulase, pretreated with 0.2% SDS and boiling, following endoglycosidase H treatment; lane 4, membrane-associated cellulase; lane 5, membrane-associated cellulase, pretreated with 0.2% Triton X-100, following endoglycosidase H treatment; lane 6, membrane-associated cellulase, pretreated with 0.2% SDS and boiling, following endoglycosidase H treatment. All endoglycosidase treatments were for 10 h at 37°C.

52,800 D (Fig. 5, lane 5). When the membrane-associated cellulase was pretreated with SDS at 100°C, endoglycosidase H treatment predominantly yielded the 52,800 D form of cellulase (Fig. 5, lane 6). Because SDS treatment increases the reactivity of glycoproteins to endoglycosidase H digestion (34) we interpret the digestion pattern with Triton X-100 pretreatment to represent a partial digestion of the membrane-associated cellulase and digestion following SDS treatment to represent a more complete endoglycosidase H digestion. One interpretation of the endoglycosidase H digestion pattern following Triton X-100 pretreatment is that the membrane-associated cellulase possesses two high mannose oligosaccharide side chains and that the three forms represent molecular species possessing none (52,800 D), one (54,800 D), or two (56,500 D) of the oligosaccharide side-chains intact.

In Vitro **Translation.** To determine whether cellulase is synthesized as a large mol wt precursor we examined the size of *in vitro* translated cellulase. For size comparison of the *in vitro* translation product with mature cellulase protein it was necessary to deglycosylate the mature protein. Because we had already observed that this protein was resistant to endoglycosidase H action we used a chemical agent, trifluoromethane sulfonic acid, to deglycosylate the mature protein (16). This treatment decreased the apparent mol wt relative to the untreated mature protein (Fig. 6, lane 3). The deglycosylated mature protein also appeared slightly smaller (1200 D) than the *in vitro* translation product immunoprecipitated with cellulase antibody (Fig. 6, lane 4). Interestingly, trifluoromethane sulfonic acid treatment resulted in about 50% loss of protein as judged by Coomassie blue staining on SDS polyacrylamide gels (not shown), but an even greater loss of antibody reaction on the protein immunoblot (Fig. 6). This suggests that a significant fraction of the cellulase antibodies are directed toward carbohydrate epitopes.

DISCUSSION

In this study we identified four molecular forms of avocado cellulase that we propose represent processing intermediates of the protein. (a) By immunoprecipitation of *in vitro* translation
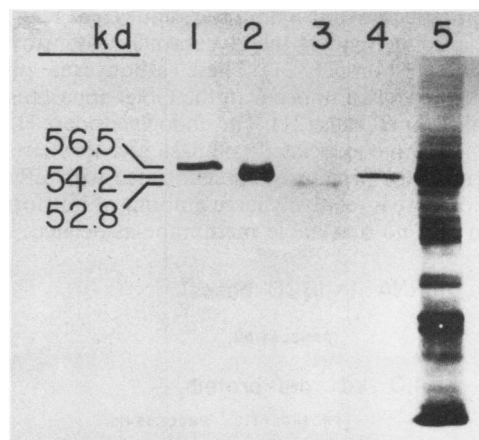

FIG. 6. Immunoblot and autoradiograph of molecular forms of avocado cellulase. All lanes were run on the same gel and blotted to nitrocellulose. Proteins in lanes 1 to 3 were immunodetected and radioactive proteins in lanes 4 and 5 were detected by autoradiography of the nitrocellulase blot. This allowed direct registration of lanes without correcting for gel shrinkage during processing for autoradiography of dried gels. Lane 1, membrane-associated cellulase (50 μg); lane 2, purified soluble cellulase (0.5 μg); lane 3, purified soluble cellulase following deglycosylation with trifluoromethane sulfonic acid (0.5 μg); lane 4, *in vitro* translation product immunoprecipitated with cellulase antibody; lane 5, total *in vitro* translation products.

products we identified a 54,000 D polypeptide. (b) Chemical deglycosylation of mature cellulase or endoglycosidase H digestion of membrane-associated cellulase yielded a 52,800 D polypeptide. We propose that forms 1 and 2 differ in size by virture of a signal peptide which is proteolytically removed in vivo. (c) A 56,500 D membrane-associated cellulase was identified and shown to be a glycosylated form of the enzyme. The association of this glycosylated cellulase with membranes suggests that this is a secretory form of the protein. (d) Mature cellulase was purified and identified as a 54,200 D glycoprotein. The relationship that we propose between these four forms of cellulase is summarized in Figure 7.

The presence of an ephemeral signal peptide indicated by the difference in mol wt of the in vitro translated and deglycosylated protein is consistent with the signal hypothesis of protein secretion in eukaryotic cells. We have now confirmed the presence of the signal peptide by comparison of the nucleotide sequence of a full length cellulase cDNA with the N-terminal amino acid sequence of the mature protein (RE Christoffersen, AB Bennett, unpublished data). Based on sequence data, the size of the signal peptide is approximately 2500 D rather than 1200 D as deduced on the basis of electrophoretic mobility. This discrepancy may result from lack of accurate resolution on SDS polyacrylamide gels.

An enzymically active, membrane-associated cellulase was identified which we propose is a secretory precursor form of the mature protein. The difference in mol wt between the membrane-associated and mature cellulase was due to the presence of endoglycosidase H-sensitive carbohydrate residues. Partial endoglycosidase H digestion yielded three forms of the protein that could be distinguished on SDS-polyacrylamide gels, suggesting the presence of two glycosylation sites, each linked to a high mannose oligosaccharide (34).

The subcellular localization of the membrane-associated cellulase was not determined. Proteins targeted to either the cell wall or protein bodies are generally thought to be synthesized on ribosomes bound to ER. The ER has also been shown to be the site of glycosylation of pea storage proteins (12). It is therefore likely that cellulase is synthesized and glycosylated in the ER. Modification of glycoprotein high-mannose oligosaccharides by the removal of glucose and mannose residues and readdition of other sugar residues renders the glycoprotein insensitive to endoglycosidase digestion (11, 21). These carbohydrate processing events have been shown to occur in the Golgi apparatus in both plant (11) and animal cells (21). The endoglycosidase H sensitivity of the membrane-associated cellulase glycoprotein suggests that this form of the protein is still associated with ER vesicles. Because we observe a relatively large amount of this form of the glycoprotein and no detectable membrane-associated, endogly-
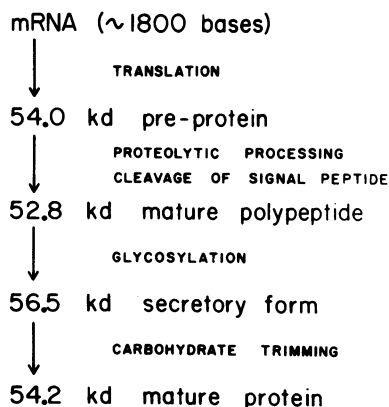
cosidase H insensitive cellulase, this further indicates that processing of cellulase through the Golgi is relatively fast compared to the transit time of this protein through the ER. A similar conclusion regarding transport of phytohemagglutin through the ER and Golgi of bean cotyledons has been reported (10).

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FIG. 7. Schematic diagram illustrating our proposal describing the relationship between the various molecular forms of avocado cellulase.

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