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# Preparation and Purification of Glucanase and Chitinase from Bean Leaves[1]

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## ABSTRACT

**Glucanase (endo-β-1,3-glucan 3-glucanohydrolase, EC 3.2.1.6, laminarinase, callase) and chitinase (poly-β-1,4-[2-acetamido-2-deoxy]-D-glucoside glycanohydrolase, EC 3.2.1.14) were extracted from ethylene-treated bean (*Phaseolus vulgaris* L. cv. Red Kidney) leaves and purified on hydroxyapatite and carboxymethyl Sephadex columns. The glucanase prepared was homogeneous as judged by analytical centrifugation data, electrophoresis, and antibody-antigen reactions. On the basis of gel filtration, antibody-antigen reactions, and amino acid analysis, the molecular weight was estimated to be between 11,500 and 12,500. However, ultracentrifugation gave a higher estimate of 34,000. The glucanase had an isoelectric point near pH 11 and was specific for β-1,3-linkages. The chitinase was only partially purified as judged by electrophoretic behavior.**

Enzyme systems capable of generating (13) and degrading (8) β-1,3-glucans have been described. The β-1,3-glucans themselves are widespread and have been associated with callose, leaf and stem hairs, root hairs, cystoliths, pollen mother cells, laticifers, pollen grains, pollen tube walls, wounded parenchyma cells (10), ovules (12), development of microspores from tetrads (15), and cell walls (17). β-1,3-Glucans are also important components of carbohydrate reserves of cereals, algae, and fungi (5).

A number of roles have been proposed for glucanase (β-1,3-glucanase, laminarinase, callase) in plants. These include degradation of seed glucans (11), control of cell elongation (17, 20) (see however 9, 26), regulation of pollen tube growth (25), cell expansion of yeast (27), fertilization (12, 15), and removal of phloem callose (8).

In an earlier paper (1), we described an increase in glucanase in ethylene-treated bean leaves that was associated with protein synthesis *de novo* and correlated with the removal of callose from the phloem. In order to characterize the enzyme more fully and to evaluate the role of the enzyme in the normal physiology of the plant, we have selected a purification procedure that resulted in the preparation of electrophoretically pure glucanase.

## MATERIALS AND METHODS

**Preparation of Glucanase.** Bean (*Phaseolus vulgaris* L. cv. Red Kidney) leaves (1,300 g) treated for 3 days with 10 μl/liter of ethylene (2) were homogenized with 1,400 ml of water in a Waring Blendor. Except where noted, this and subsequent steps were performed at 5 C. The homogenate was filtered through cheesecloth and centrifuged at 10,000*g* for 10 min, and the precipitate was discarded (step 1). The supernatant was heated to 60 C for 10 min by placing the flask containing the homogenate in a boiling water bath, stirring rapidly, and monitoring the temperature rise until 60 C was reached. The flask was then removed from the bath, cooled slowly at room temperature, and, after 10 min, placed in an ice bath to lower the temperature to 5 C. The denatured protein was removed by centrifuging at 10,000*g* for 10 min (step 2).

The 60 C supernatant was mixed with diethylaminoethyl cellulose (Cellex-D, Bio-Rad Laboratories, Richmond, Calif.) at the rate of 1 g of DEAE[3]-cellulose to 30 ml of 60 C supernatant and filtered through filter paper on a Buchner funnel. The pad of DEAE-cellulose was washed once with 100 ml of water (step 3).

The glucanase was precipitated from the DEAE-cellulose filtrate by adding 36 g of $(NH_4)_2SO_4$ per 100 ml of filtrate, letting the mixture stand for 30 min at 0 C and centrifuging at 10,000*g* for 10 min. The supernatant fraction was discarded and the pellet was dissolved in 0.05 M K phosphate, pH 7, using one-fourth the original volume of DEAE-cellose filtrate (step 4). The 60% saturated $(NH_4)_2SO_4$ fraction was then dialyzed against three changes of distilled water and the protein lyophilized (step 5).

The lyophilized enzyme was then dissolved in 5 ml of water, and the insoluble material was removed by centrifuging at 10,000*g* for 10 min. The glucanase was then purified on a 28- × 2-cm hydroxyapatite (Bio-Gel HT, Bio-Rad Laboratories) column. All column chromatography was done at room temperature. The column was washed with 100 ml of 0.005 M Na phosphate, pH 6.8, before adding the 200-mg protein sample. After the addition of the 5-ml sample, the protein was eluted with a linear gradient consisting of 200 ml of 0.005 M Na phosphate, pH 6.8, and 200 ml of 0.2 M Na phosphate, pH 6.8 (see Fig. 1). The column effluent was monitored for protein, glucanase, chitinase, peroxidase, and RNase activity. The presence of peroxidase was used in subsequent purifications as an indication of purity.

[1] In conducting the research reported herein, the investigators adhered to "Guide for Laboratory Animal Facilities and Care" established by the committee on the guide for laboratory animal facilities and care of the Institute of Laboratory Animal Resources, NAS-NRC.

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[3] Abbreviations: CM: carboxymethyl; DEAE: diethylaminoethyl; NAG: N-acetyl-D-glucosamine.

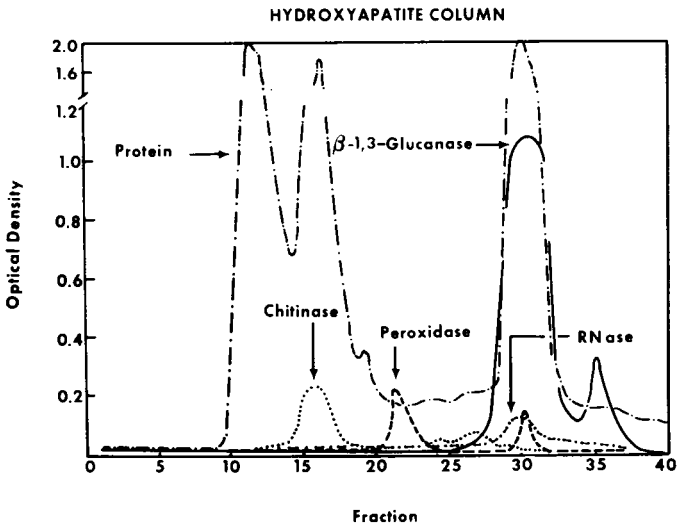


FIG. 1. Separation of β-1,3-glucanase, peroxidase, RNase, and chitinase on hydroxyapatite with a 0.005 M to 0.2 M sodium phosphate, pH 6.8, linear gradient.

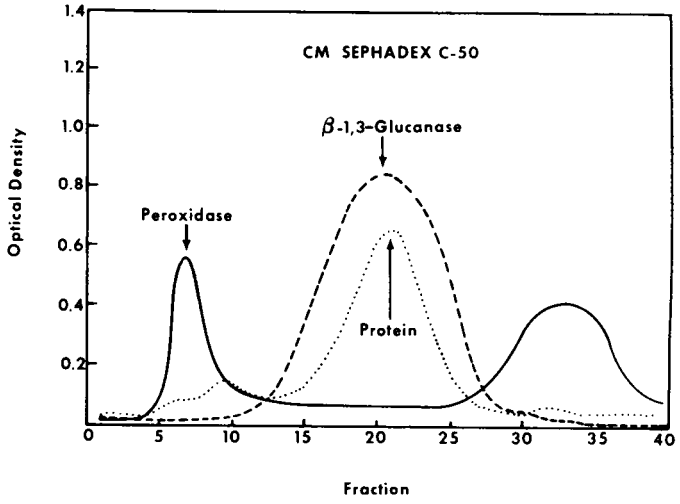


FIG. 2. Chromatography of β-1,3-glucanase on CM-Sephadex C-50.

The glucanase in the tubes was pooled, dialyzed against three changes of water, and lyophilized (step 6).

The glucanase from the hydroxyapatite column was then dissolved in 12.5 ml of 0.05 M K phosphate, pH 6, and separated on a 30- × 2.5-cm CM-Sephadex C-50 (Pharmacia Fine Chemicals, Piscataway, N. J., 4.5 meq g, 40 to 120 μ particle size) column. The column was prepared according to the manufacturer's directions, and the 60-mg sample, in 12.5 ml of 0.05 M K phosphate, pH 6, was loaded on the column. The enzyme was eluted with 100 ml of 0.05 M K phosphate, pH 6, followed by a linear gradient of 150 ml of 0.05 M K phosphate, pH 6, and 150 ml of 0.05 M K phosphate, pH 6, plus 0.25 M NaCl. Figure 2 shows that the majority of protein eluted from the column consisted of glucanase, and that peroxidase occurred in the two adjacent peaks. The glucanase in the tubes was then pooled, dialyzed against three changes of distilled water, and lyophilyzed (step 7). Table I shows that this procedure recovered 12% of the glucanase originally present and resulted in a 15.5-fold increase in activity. Assuming that the glucanase obtained by this procedure is pure, 6.6% of the soluble protein of ethylene-treated bean leaves was glucanase.

**Protein.** Protein was measured by either the Lowry (19) method for specific activity measurements or by recording the absorbance at 280 nm for column chromatography effluents.

**Glucanase.** Endo-β-1,3-glucan 3-glucanohydrolase (EC 3.2.1.6) was measured according to the method described earlier (1). A unit of glucanase is represented by the production of 1 mg of glucose equivalent per hour at 50 C.

**Chitinase.** Poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolase (EC 3.2.1.14) activity was measured by the release of N-acetyl-D-glucosamine (NAG) from colloidal chitin according to the method of Reissig *et al.* (24). The formation of N-acetyl-D-glucosamine was verified by chromatography on cellulose thin layer plates with 1-butanol-pyridine-water (10:10:5, v/v) as the solvent and *p*-dimethylaminobenzaldehyde (23) as the developing reagent. A unit of chitinase activity is represented by the production of 1 μg of NAG per hour at 45 C.

**Peroxidase.** Peroxidase (EC 1.11.1.7) was measured by incubating 0.1 ml of enzyme solution with 4 ml of guaiacol for 15 min at 25 C and observing the absorbance at 470 nm. The guaiacol solution consisted of 3 ml of 0.05 M K phosphate, pH 7, 0.5 ml of 2% guaiacol, and 0.5 ml of 0.3% $H_2O_2$.

Table I. *Purification of Glucanase*

| Step | Purification | Glucanase Units[1] | Units Recovered | Protein Recovered | Protein Recovered | Specific Activity |
|---|---|---|---|---|---|---|
| | | | % | mg | % | Units glucanase mg protein |
| 1 | Crude Homogenate | 122,000 | 100 | 2,730 | 100 | 45 |
| 2 | 60 C for 10 min | 93,100 | 76 | 1,515 | 55 | 62 |
| 3 | DEAE-cellulose | 78,400 | 64 | 700 | 25 | 112 |
| 4 | 60% $(NH_4)_2SO_4$ | 76,000 | 62 | 427 | 16 | 178 |
| 5 | Lyophilized | 70,000 | 58 | 200 | 7.3 | 350 |
| 6 | Hydroxyapatite | 31,800 | 31 | 60 | 2.2 | 530 |
| 7 | CM-Sephadex | 15,200 | 12 | 22 | 0.8 | 695 |

[1] One unit = 1 mg glucose equivalent/hr at 50 C.

**RNase.** Polyribonucleotide-2-oligonucleotide transferase (cyclizing) (EC 2.7.7.16) was measured according to the method of Abeles *et al.* (1).

**Electrophoresis of Proteins on Cellulose Polyacetate.** The purity of glucanase and chitinase prepared by the above methods was examined by electrophoresis on cellulose polyacetate (Sepraphore III, Gelman Instrument Co., Ann Arbor, Mich.). The electrode buffer was 0.135 M tris and 0.043 M citric acid, pH 7, prepared by adding 16.4 g of tris and 9.04 g of citric acid to water to make 1 liter. The cellulose polyacetate was soaked in diluted buffer (67 ml of electrode buffer to 933 ml of water) for 30 min before applying a 3-μl sample containing 10 μg of protein/μl. Electrophoresis was run at 190 v for 75 min. The strips were stained in 7% acetic acid containing 0.25% Coomassie brilliant blue R-250 (Mann Research Laboratories). For best results the stain must be filtered before use. After a 3-min staining period the strips were cleared in three successive rinses of 7% acetic acid. The strips were then air-dried, cleared with mineral oil, mounted between glass microscope slides, and photographed.

**Preparation of Rabbit Antibodies.** A specific antibody response was elicited in rabbits by injecting 2 mg of purified glucanase in Freund's complete adjuvant (Difco) at multiple sites followed by an additional subcutaneous injection of 0.8 mg one week later. The rabbits were bled out by cardiac puncture 8 days later when the precipitating antibody level was sufficiently high. The serum was sterilized by filtering through a 0.22-μ membrane

filter. The serum was used directly for double diffusion analysis on 1.5% Noble agar (Difco) plates according to the method of Ouchterlony (19, p. 85).

Prior to use in quantitative precipitin studies, the rabbit serum was further fractionated to prevent serum carbohydrates from interfering with the glucanase assay. The globulin-containing fraction was prepared by mixing equal amounts of saturated $(NH_4)_2SO_4$ and rabbit serum. After centrifuging at 10,000*g* for 10 min, the pellet was washed once with 50% saturated $(NH_4)_2SO_4$, centrifuged again, and dissolved in 0.15 M NaCl. Material prepared this way was shown to be free of reducing material using the dinitrosalicylic reagent described earlier (2).

**Quantitative Precipitin Assays.** Quantitative precipitin assays were carried out using a constant quantity of antibody and 2-fold dilutions of purified glucanase. This procedure, which was opposite to the normal procedure in which antigen is kept constant, was necessitated because we wished to assay the enzymatic activity remaining in the supernatant as well as protein in precipitate. One milliliter of glucanase (6.4 μg/ml) was mixed with various antibody dilutions and incubated for 1 hr at 37 C and then for 16 hr at 4 C. The antibody-glucanase complex was then centrifuged at 2,000*g* for 5 min, and the pellet was washed with 3 ml of cold 0.15 M NaCl. The first supernatant was retained for glucanase assays, and the pellets were dissolved in 0.8 ml of 1 N NaOH and protein was estimated by measuring the absorbance at 280 nm.

## RESULTS

**Sedimentation Velocity.** Molecular weight was estimated by determining the sedimentation velocity in a Spinco Model E analytical ultracentrifuge. Figure 3 shows a schlieren profile for glucanase (3 mg/ml 0.15 M NaCl), taken 80 min after centrifugation at 48,000 rpm. The sedimentation constant for glucanase was 3.1 S and, assuming the protein was a globular protein, is equivalent to a molecular weight of 34,000.

**Gel Filtration.** A second estimation of molecular weight was made by comparing the elution of glucanase from Sephadex G-200 with a series of known standards. The molecular weight standards and the procedures followed were obtained from Pharmacia Fine Chemicals. Figure 4 shows that the glucanase eluted after the RNase A, which has a molecular weight of 13,700.

**Antibody-Antigen Reactions.** Figure 5 shows the results of an experiment with glucanase from step 5 of the purification summarized in Table I. Glucanase (0.1 mg/ml) was placed in the center well and 2-fold dilutions of antiserum in the outer wells. The presence of a single precipitin band over a wide range of antibody-antigen ratios is evidence of the homogeneity of the glucanase preparation. When pure (step 7) and impure (step 5) preparations were examined, a single line of identity was noted, indicating that the antiserum was specific for glucanase.

**Quantitative Precipitin Experiment.** The results of a typical quantitative precipitin experiment are shown in Figure 6. Initial experiments had revealed an anomalous enzymatic activity profile. This was due to the instability of the enzyme at dilute concentrations in the absence of other proteins. Furthermore, most of the decrease in activity occurred during the assay at 50 C rather than during the incubation with antibody. The curve with albumin was obtained by adding 0.1 mg of egg albumin/ml during the incubation period. Assuming a molecular weight of 160,000 for the antibody and 12,500 for the glucanase, the molar ratio of antibody to antigen at equivalence was estimated as 1.7. This is close to a value of 1.5 for the equivalence ratio of RNase (molecular weight 13,400) (18, p. 26).

The results of the quantitative precipitin experiment, particularly the fact that the location of the area of maximal precipitation was the same area of least glucanase activity, further

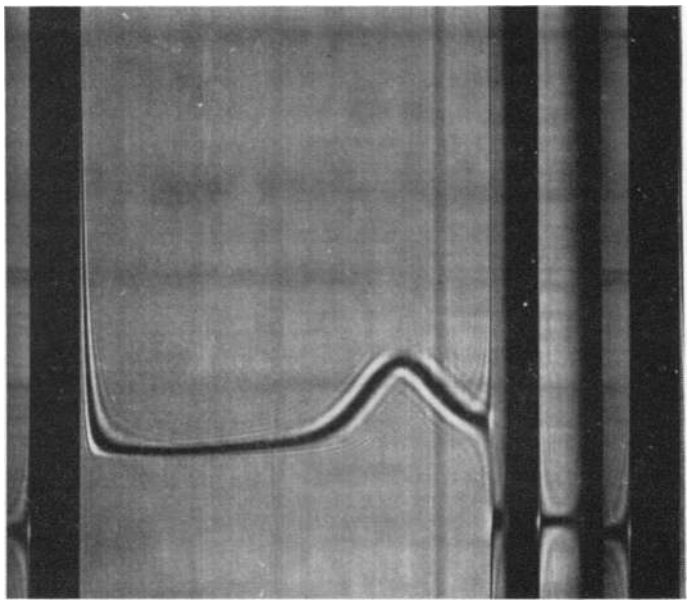

FIG. 3. Ultracentrifugal pattern of purified glucanase. The protein (3 mg/ml in 0.15 M NaCl) was centrifuged in a 4° single sector cell in an An-D rotor at 48,000 rpm at 20 C. Photograph was taken 80 min after reaching speed. Sedimentation from right to left.

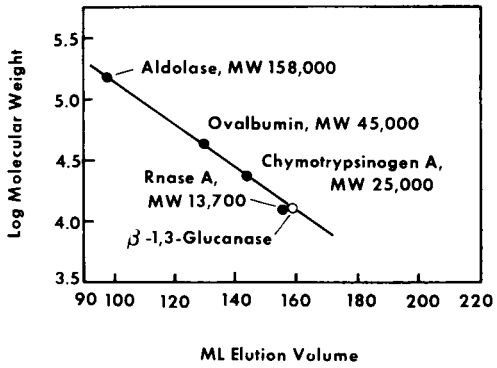


FIG. 4. Determination of molecular weight of glucanase by gel filtration on Sephadex G-200. Column dimensions were 2.5 × 45 cm. Elution buffer was 0.15 M NaCl, 0.005 M potassium phosphate, pH 7.0.

indicated that the antigen is homogeneous. It is known that soluble antibody-antigen complexes exist in the region of antibody excess. Similar to the results with phosphatase, the supernatant fluid in this region possessed enzymatic activity, indicating that antibody complexing does not preclude enzymatic activity of the glucanase (18, p. 40).

**Amino Acid Composition of Glucanase.** The amino acid composition of glucanase is shown in Table II. The glucanase was hydrolyzed at 105 C in 6 N HCl for 24 hr in an evacuated sealed ampoule, and the amino acids were separated on an automatic amino acid analyzer. Another estimation of minimal molecular weight was made on the basis of number of residues per 11,439 g of protein. Because the isoelectric point of the protein was near pH 11 we assume that most, if not all, of the aspartic acid and glutamic acid was in the form of the amides.

**Isoelectric Point.** Samples of glucanase were placed on cellulose polyacetate that had been soaked in buffers of various pH. At pH 11, the glucanase did not move from the origin, but at pH 12 it moved toward the anode, and at pH 10 it migrated toward the cathode. At increasingly lower pH values the rate of migration

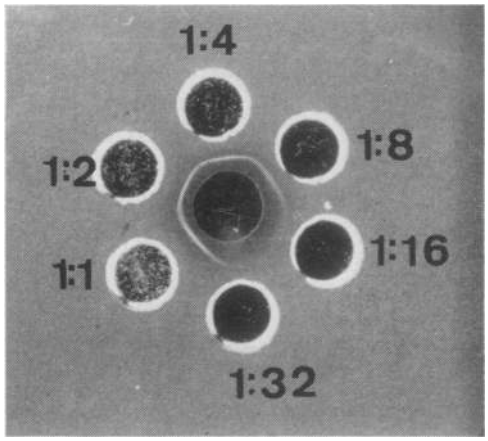


FIG. 5. Double diffusion analysis of glucanase. Center well contains glucanase (0.1 mg protein/ml) from purification step number 5 (see Table I). Outer wells contain twofold dilutions of rabbit antiserum.

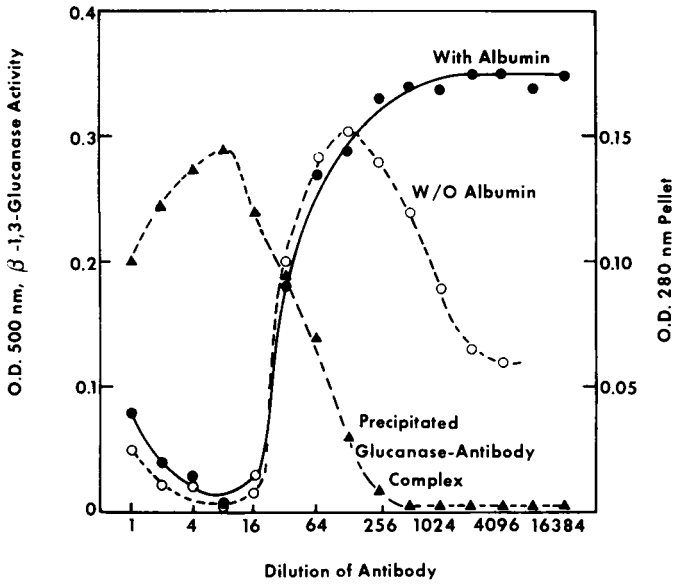


FIG. 6. Quantitative precipitin analysis of glucanase.

increased. From these observations we estimated the isoelectric point was near pH 11.

**Specificity of Glucanase.** Reducing groups were produced from glucans containing β-1,3-linkages such as pachyman (β-1,3 only) and laminarin (a mixture of β-1,3 and β-1,6 linkages). The enzyme had no effect on β-1,2, β-1,4, β-1,6, α-1,4, or α-1,6 linked glucans. Addition of EDTA ($10^{-7}$ to $10^{-3}$ M) had no effect on the activity of the glucanase.

**Chitinase.** During the course of these experiments we examined the possibility that glucanase might represent a protective enzyme capable of attacking the cell walls of invading fungal pathogens. A number of workers have shown that fungal cell walls contain β-1,3-glucans and chitin and can be attacked by a combination of these enzymes (21, 28). Using the chitinase assay described by Reissig *et al.* (24), we detected an increase in chitinase activity after ethylene treatment of bean leaves parallel to the increase in glucanase reported earlier (Table III). The enzyme was able to reduce the viscosity of chitosan (deacylated chitin), had a temperature optimum of 45 C, was denatured by 70 C treatment for 10 min, and had a pH optimum of 4. The enzyme appears to be an exoenzyme because N-acetyl-D-glucosamine was the only amino sugar identified on thin layer chromatography of the

Table II. *Amino Acid Composition—Integral Number of Amino Acid Residues*

The sample analyzed was 1.358 mg.

| Amino Acid | Amino Acid Residues | Amino Acid Residues | Amino Acid Residues per 11,439 g Protein | Nearest Integral Number of Residues per 11,439 g Protein |
|---|---|---|---|---|
| | *μmoles/sample* | *g/100 g protein*[1] | | |
| Aspartic acid | 1.158 | 13.79 | 13.69 | 14 |
| Threonine | 0.285[2] | 3.14 | 3.56 | 4 |
| Serine | 0.670[3] | 6.74 | 8.86 | 9 |
| Glutamic acid | 0.758 | 10.12 | 8.97 | 9 |
| Proline | 0.537 | 5.40 | 6.36 | 6 |
| Glycine | 0.773 | 4.56 | 9.15 | 9 |
| Alanine | 0.647 | 4.76 | 7.66 | 8 |
| Valine | 0.749 | 7.69 | 8.87 | 9 |
| Methionine | 0.171 | 3.32 | 2.02 | 2 |
| Isoleucine | 0.450 | 5.27 | 5.32 | 5 |
| Leucine | 0.732 | 8.57 | 8.66 | 9 |
| Tyrosine | 0.581 | 9.81 | 6.88 | 7 |
| Phenylalanine | 0.348 | 5.29 | 4.11 | 4 |
| Lysine | 0.244 | 3.24 | 2.89 | 3 |
| Histidine | 0.088 | 1.25 | 1.04 | 1 |
| Arginine | 0.498 | 8.05 | 5.90 | 6 |
| | | 100.00 | | 105 |

[1] Based on 966.004 μg recovered

[2] Corrected for destruction during hydrolysis assuming 94.7% recovery

[3] Corrected for destruction during hydrolysis assuming 89.5% recovery

Table III. *Effect of Ethylene on Glucanase, Chitinase, and Peroxidase Activity of Bean Leaves*

Plants were treated with 10 μl/liter ethylene and removed after the times indicated. The leaves were homogenized in water (1 g leaf tissue/ml water) with a Waring Blendor, filtered through Miracloth, and then centrifuged at 10,000*g* for 10 min. The supernatant fluid was incubated with either laminarin, colloidal chitin, or guaiacol.

| Days of Ethylene Treatment | Protein | Glucanase | Chitinase | Peroxidase |
|---|---|---|---|---|
| | *mg/ml* | *mg glucose $ml^{-1}$ $hr^{-1}$* | *μg NAG $ml^{-1}$ $hr^{-1}$* | *Δ 470 nm $(ml \cdot 2\ min)^{-1}$* |
| 0 | 3.0 | 0.89 | 0.21 | 1.4 |
| 1 | 2.6 | 28.0 | 4.5 | 1.6 |
| 2 | 2.1 | 34.0 | 7.5 | 1.8 |
| 3 | 1.9 | 42.0 | 10.0 | 2.0 |

reaction products. (See "Materials and Methods" for details.) The chitinase had properties similar to those of the glucanase since it occurred in the material (step 5) placed on the hydroxyapatite column (see Fig. 1). It could be further purified on CM-Sephadex using the same elution solvents used for glucanase (see Fig. 7). The specific activity of the chitinase purified on CM-Sephadex was 480 units/mg protein. This represents a 7.7-fold increase in activity compared with the crude homogenate which contained 62 units/mg protein. The glucanase and chitinase were found to have extremely long half lives. We found that the levels of these enzymes in leaf tissue remained unchanged for 3

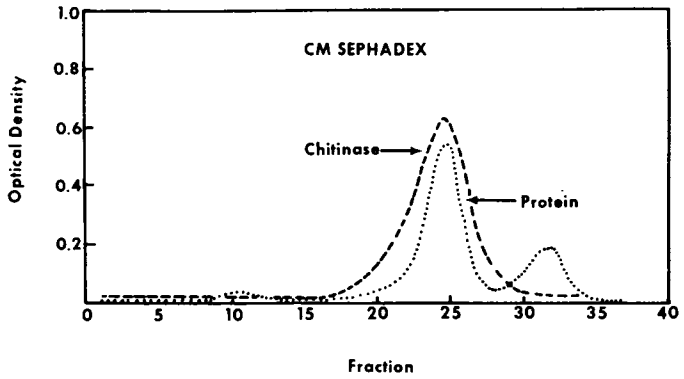


FIG. 7. Chromatography of chitinase on CM-Sephadex C-50.

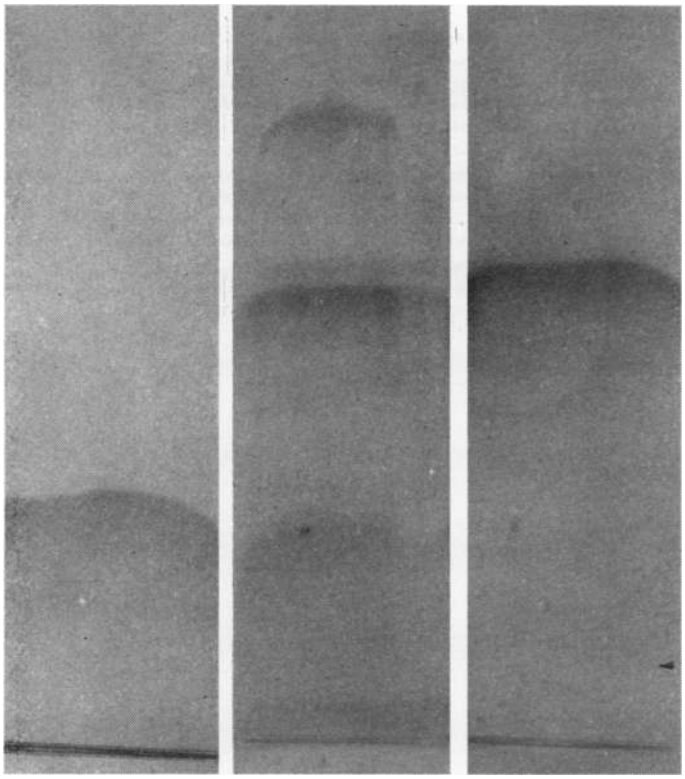

FIG. 8. Electrophoretic separation of glucanase, chitinase, and protein from step 5 of the purification procedure on cellulose polyacetate. The strip on the left is chitinase, the center strip is step 5 of the purification procedure, and the right hand strip glucanase.

days after a 24-hr 10 μl/liter ethylene exposure was used to increase enzyme activity.

A comparison of the electrophoretic properties of glucanase, chitinase, and the protein mixture before hydroxyapatite chromatography is shown in Figure 8. These results indicate a single band for the glucanase, but the chitinase fraction from the CM-Sephadex column appears as a diffuse set of three bands. On the assumption that two-thirds of the chitinase fraction are contaminating proteins, we estimate that about 4% of the total soluble leaf protein was chitinase.

## DISCUSSION

**Purity.** The results of analytical centrifugation, electrophoresis, and antibody-antigen reaction indicate that the purification scheme used resulted in the preparation of glucanase free of significant amounts of contaminating substances.

**Molecular Weight.** Estimates of molecular weight by gel filtration, antibody-antigen reactions, and amino acid analysis gave approximately similar values of 11,500 to 12,500. However, analytical centrifugation indicated a higher molecular weight of 34,000. We obtained similar results (2.7–3.1 S), using a more dilute preparation in a sucrose density gradient (centrifugation was 47,000*g* for 16 hr). The reason for the higher value is not known, but one reason may be aggregation of protein during the sedimentation.

**Functions of the Glucanase and Chitinase.** The reasons for the increase in glucanase, chitinase, and other enzymes in ethylene-treated leaves are not clear. There are two possibilities that appear reasonable at this point. Other functions of glucanase such as control of growth and fertilization discussed earlier are probably not involved, because mature leaves represent potentially senescing organs.

First, these hydrolytic enzymes and others like them such as RNase and protease represent essentially degrading or catabolic enzymes, concerned with solubilizing cellular components prior to translocation from the leaf into other parts of the plant. If so, then the presence of large quantities of chitinase is hard to understand, since there is little information to suggest that higher plants contain significant quantities of chitin. However, this may be due to the difficulty in locating and identifying polymers of NAG. Chitin has been reported in algae (14, 30). However, it should be noted that there are a number of other reports of enzymes capable of hydrolyzing N-acetyl-D-glucosamine linkages in plants. A chitinase was reported to occur in the almond enzyme complex emulsin (32), and a β-N-acetylglucosaminidase was found in the cotyledons of germinating pinto beans (3). This enzyme was thought to be involved in the degradation of hemagglutinins that contain covalently linked N-acetyl-D-glucosamine in their carbohydrate chains. If the presence of chitinase is common in plants, then this raises the possibility that N-acetyl-D-glucosamine polymers play a role, structural or otherwise, in the plant.

A second explanation for chitinase and glucanase activity is that these enzymes have an antibiotic role in plants. That is, they do not attack endogenous carbohydrates but rather protect plants from fungal pathogens by digesting the invading fungal cell walls. We know from earlier reports that fungi contain significant amounts of β-1,3-glucans and chitin (14, 30), and that these walls are readily attacked by glucanase and chitinase (28). We also know that an increase in ethylene production is associated with viral (4, 22), bacterial (16), and fungal diseases (31). It is conceivable that increased rates of ethylene production play a role in the host's response to pathogens by controlling the synthesis of phytoalexins (7) or protective enzymes (29). Stahmann *et al.* (29) reported that ethylene caused sweet potato tubers to become resistant to black rot disease (*Ceratocystis fimbriata*) by increasing peroxidase activity. However, Chalutz and DeVay (6) were unable to observe an increase in disease resistance when they tried to confirm these observations. Data in Table III indicate that ethylene caused a small increase in peroxidase in bean leaves which paralleled the rise in glucanase and chitinase activity.

In conclusion, we have been able to characterize and purify two enzymes in ethylene-treated tissue on whose roles we can only speculate. It appears unreasonable that 10% of the soluble protein of these leaves would be in the form of enzymes that did not play some essential physiological role.

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## LITERATURE CITED


1. Abeles, F. B. and L. E. Forrence. 1970. Temporal and hormonal control of β-1,3-glucanase in *Phaseolus vulgaris* L. Plant Physiol. 45: 395–400.
2. Abeles, F. B., R. E. Holm, and H. E. Gahagan. 1967. Abscission: Induction of degradative enzymes during aging. *In:* F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, pp. 1515–1523.
3. Bahl, O. P. and K. M. L. Agrawal. 1968. Glycosidases of *Phaseolus vulgaris*. I Isolation and characterization of β-N-acetylglucosaminadse. J. Biol. Chem. 243: 98–102.
4. Balazs, E., R. Gaborjanyi, A. Toth, and Z. Kiraly. 1969. Ethylene production in *Xanthi* tobacco after systemic and local virus infections. Acta Phytopathol. Acad. Sci. Hung. 4: 355–358.
5. Bull, A. T. and C. G. C. Chesters. 1966. The biochemistry of laminarin and the nature of laminarinase. Advan. Enzymology 28: 325–364.
6. Chalutz, E. and J. E. DeVay. 1969. Production of ethylene *in vitro* and *in vivo* by *Ceratocystis fimbriata* in relation to disease development. Phytopathology 59: 750–755.
7. Chalutz, E. and M. A. Stahmann. 1969. Induction of pisatin by ethylene. Phytopathology 59: 1972–1973.
8. Clarke, A. E. and B. A. Stone. 1962. β-1,3-Glucan hydrolases from the grape vine (*Vitis vinifera*) and other plants. Phytochemistry 1: 175–188.
9. Cleland, R. 1968. Auxin and wall extensibility: Reversibility of auxin-induced wall loosening process. Science 160: 192–194.
10. Currier, H. B. 1957. Callose substances in plant cells. Amer. J. Bot. 44: 478–488.
11. Dillon, T. and P. O'Colla. 1951. Enzymatic hydrolysis of 1,3 linked polyglucosans. Chem. Ind: 111.
12. Esser, K. 1963. Bildung und Abbau von Callose in den Samenanlagen der Petunia Hybrida. Zeit. Bot. 51: 32–51.
13. Fiengold, D. S., E. F. Neufeld, and W. Z. Hassid. 1958. Synthesis of a β-1,3-linked glucan by extracts of *Phaseolus aureus* seedlings. J. Biol. Chem. 233: 783–788.
14. Foster, A. B. and J. M. Webber. 1960. Chitin. Advan. Carbohyd. Chem. 15: 371–393.
15. Frankel, R., S. Izhar, and J. Nitsan. 1969. Timing of callase activity and cytoplasmic male sterility in petunia. Biochem. Genet. 3: 451–455.
16. Freebairn, H. T. and I. W. Buddenhagen. 1964. Ethylene production by *Pseudomonas solanacearum*. Nature 202: 313–314.
17. Heyn, A. N. J. 1969. Glucanase activity in coleoptiles of *Avena*. Arch. Biochem. Biophys. 132: 442–449.
18. Kabat, E. A. and M. M. Mayer. 1961. Experimental Immunochemistry, Ed. 2. Charles C. Thomas, Springfield, Illinois.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265–275.
20. Masuda, Y. and S. Wada. 1967. Effect of β-1,3-glucanase on the elongation growth of oat coleoptile. Bot. Mag. 80: 100–102.
21. Mitchell, R. 1963. Addition of fungal cell-wall components to soil for biological disease control. Phytopathology 53: 1068–1071.
22. Nakagaki, Y., T. Hirai, and M. A. Stahmann. 1970. Ethylene production by detached leaves infected with tobacco mosaic virus. Virology 40: 1–9.
23. Partridge, S. M. 1948. Filter-paper partition chromatography of sugars. Biochem. J. 42: 238–248.
24. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217: 959–966.
25. Roggen, H. P. J. R. and R. G. Stanley. 1969. Cell-wall-hydrolysing enzymes in wall formation as measured by pollen-tube extension. Planta 84: 295–303.
26. Ruesink, A. W. 1969. Polysaccharidases and the control of cell wall elongation. Planta 89: 95–107.
27. Shimoda, C. and N. Yanagishima. 1968. Strain dependence of the cell-expanding effect of β-1,3-glucanase in yeast. Physiol. Plant. 21: 1163–1169.
28. Skujins, J. J., H. J. Potgieter, and M. Alexander. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-1,3-glucanase. Arch. Biochem. Biophys. 111: 358–364.
29. Stahmann, M. A., B. G. Clare, and W. Woodbury. 1966. Increased disease resistance and enzyme activity induced by ethylene and ethylene production by black rot infected sweet potato tissue. Plant Physiol. 41: 1505–1512.
30. Tracey, M. V. 1955. Chitin. Modern methods of plant analysis. 2: 264–274.
31. Williamson, C. E. 1950. Ethylene, a metabolic product of diseased and injured plants. Phytopathology 40: 205–208.
32. Zechmeister, L., G. Toth, and M. Balint. 1938. Über die chromatographische Trennung einiger Enzyme des Emulsins. Enzymologia 5: 302–306.