Chitinase from Soybean Seeds: Purification and Some Properties of the Enzyme System

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Chitinase was isolated and purified from soybean seed extract for the first time by ammonium sulfate precipitation followed by chromatography on ground chitin. Five protein peaks containing chitinase activity were eluted, with three peaks containing chitobiase activity as well. Purification was up to 258-fold. The procedure is simple, is inexpensive, and yielded chitinase of higher specific activity than two out of three commercial enzymes tested. The average molecular weight of the chitinase was 31 600 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Preliminary results indicate the enzyme acts as an endochitinase and that isoenzymes may be present. The enzyme exhibited a relatively low pH optimum in the range of 3.3–4.0. Activity on regenerated chitin was considerably greater than on more crystalline native substrates.

Recent interest in the commercial development of chitin [poly-β-(1→4)-*N*-acetyl-D-glucosamine] and its derivatives has demonstrated the potential need for inexpensive, reliable sources of chitinase (EC 3.2.1.14). Chitinases are currently being produced commercially from bacterial cultures and cost over $200/g. The level of activity of these enzymes varies between suppliers, from near zero to over 280 ImU/mg, and can vary substantially among different batches from the same supplier as observed in our laboratory. Ohtakara et al. (1978) have investigated a number of microorganisms as potential sources of chitinase for use in the commercial production of chitin degradation products. Cosio et al. (1982) have described the use of bacterial chitinase in a process for the conversion of shrimp processing waste into yeast single-cell protein. The use of bacteria as a source of the enzyme is attractive because large cultures can be developed and it is possible to obtain chitinase-overproducing strains through genetic manipulation. However, as noted by Smirnoff (1978), microbial fermentation can be time consuming, somewhat risky, and expensive. In addition, Berkeley (1978) has pointed out the potential role of chitinolytic microorganisms in the contamination and spoilage of chitinous products if they are used as an on-site source of enzyme for the modification of chitinous materials.

While most of the interest in the commercial production of chitinase has been focused on microorganisms, the enzyme is produced by a variety of plant and animal species as well (Muzzarelli, 1977; Jeuniaux and Cornelius, 1978). Smirnoff (1978) has described a method for the extraction of chitinase from the gastric juice and intestinal chyme of chickens on a semiindustrial scale. The presence of chitinase in bean seeds was initially described by Powning and Irzykiewicz (1965). They proposed that the enzyme serves as a defense mechanism against the invasion of fungal pathogens whose cell walls contain chitin.

The purpose of this investigation was to provide an easily obtained, inexpensive source of purified chitinase for use in our ongoing studies on the use of chitin in animal feed (Zikakis et al., 1982) and studies of chitin structure. Soybeans were chosen as the source material because of their low cost and availability. To the best of our knowledge, this is the first report on the presence of chitinase in soybeans.

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MATERIALS AND METHODS

Materials. Soybeans, *Glycine max* (L) Merrill cv. Ware, were obtained from the University of Delaware farm and stored at 0–4 °C until use. Technical-grade ground chitin from tanner crab shells was purchased from Bioshell, Inc., Albany, OR. Purified chitin from crab shells was purchased from Sigma Chemical Co., St. Louis, MO. Purified shrimp chitins, chitosan from blue crab shells, and microcrystalline chitin (Austin et al., 1981) were obtained through Hercules, Inc., Wilmington, DE, and the College of Marine Studies, University of Delaware. Practical-grade chitosan, *N'*-acetyl-D-glucosamine (GlcNAc), *N,N'*-diacetylchitobiose, β-*N*-acetylglucosaminidase (chitobiase), and chitinase from *Streptomyces griseus* were purchased from Sigma Chemical Co. Chitinase from *Streptomyces antibioticus* was purchased from Calbiochem-Behring Corp., La Jolla, CA. Chitinase from *Serratia marcescens* was obtained from United States Biochemicals (USB), Cleveland, OH, as well as from the Department of Health and Human Services, U.S. Public Health Service, National Institutes of Health (NIH), Bethesda, MD. All other reagents and solvents were of reagent grade. Unless otherwise stated, glass distilled, deionized water was used throughout the experiments.

Soybean Extraction. Soybean seeds were extracted by a modification of the procedure used by Powning and Irzykiewicz (1965) for the extraction of chitinase from plant seeds. Soybeans were ground in a Wiley mill to pass a 20-mesh sieve. The ground beans (40–60 g) were then extracted with 0.05 M citrate buffer, pH 4.5 (10 g of beans/100 mL of buffer), for 2 h at 4 °C on a magnetic stir plate. The mixture was then centrifuged for 20 min at 12500*g* and the residue discarded. The supernatant was passed through Schleicher & Schuell No. 595 filter paper. Solid ammonium sulfate was added to 50% saturation and the solution placed on a magnetic stir plate at 4 °C for 1 h. The suspension was then centrifuged for 20 min at 12500*g* and the supernatant decanted. The precipitate was redissolved in 0.05 M citrate buffer (pH 4.5) at 4 °C overnight on a magnetic stir plate. The suspension was then centrifuged and filtered as above, and the filtrate (400–600 mL) was applied to a ground chitin chromatography column.

Chitin Chromatography. The chromatography column consisted of a glass tube (1.9 cm i.d. × 50 cm) equipped at both ends with rubber stoppers. The top fitting was a No. 2 stopper fitted with a glass Pasteur pipet. The bottom fitting was a No. 3 stopper fitted with a glass

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Pasteur pipet and a nylon mesh screen. The column inlet was connected to a 1-L buffer bottle and the column outlet connected to a Buchler Fractomette Alpha 2000 fraction collector. Head pressure was 2 m.

Ground chitin (Bioshell, Inc.) was prepared by the method of Bloch and Burger (1974). The chitin was washed successively with tap water, 0.05 N HCl, and 1% sodium carbonate and then packed into the column to give a bed volume of about 70 mL. The column was equilibrated with 0.05 M citrate buffer (pH 4.5) with a final flow rate of 2 mL/min.

Following the application of the soybean extract, the column was successively eluted with 1 L of 0.01 M Tris-HCl- 1 M NaCl (pH 7.4), 1 L of 0.01 M Tris-HCl (pH 7.4), 2 L of 0.05 N HCl adjusted to pH 3.3, and 1 L of 0.05 M citrate buffer (pH 4.5) as modified from Bloch and Burger (1974). Chromatography was carried out at room temperature (25 °C), and 8-mL fractions were collected. All fractions were analyzed individually for protein at 280 nm on a Gilford 250 spectrophotometer. Fractions containing protein were stored at 4 °C until used.

Substrates. Regenerated chitin was prepared by the reacetylation of chitosan (Sigma Chemical Co.) according to the method of Molano et al. (1977), using acetic anhydride. When the resulting chitin suspension was dried, it tended to form intractable particles that could not be resuspended. Therefore, the following procedure was used to obtain the desired concentration. The suspension was placed on a magnetic stir plate to ensure a uniform distribution of material and 1 mL removed with an automatic pipettor (the suspension tended to clog glass pipets). This aliquot was vacuum filtered through a preweighed piece of Schleicher & Schuell No. 595 filter paper and the paper dried overnight at room temperature (25 °C). The paper was then weighed, the initial weight subtracted, and the remainder considered to be the weight of chitin present in 1 mL of the original suspension. The volume of the suspension was then adjusted to achieve a concentration of 15 mg/mL (Molano et al., 1977).

All other enzyme substrates were used as they were obtained, without modification.

Chitinase Assay. Enzyme (250 μL) was incubated in 15-mL test tubes with 1 mL of 0.05 M citrate buffer (pH 3.5), 50 μL of chitobiase (as obtained from Sigma Chemical Co.), and 0.5 mL of regenerated chitin suspension (15 mg/mL). Volume was adjusted to 2 mL with water, and the tubes were placed in a magnetic stirring table (Tri-R Instruments, Inc., Rockville Centre, NY). This appartus was capable of stirring up to eight test tubes simultaneously. The mixtures were incubated for 30 min at room temperature with constant stirring and then centrifuged for 3 min at 900*g*. The supernatants were then assayed for GlcNAc by a modification of the method of Reissig et al. (1955).

Samples (0.5 mL) of each supernatant were mixed with 0.25 mL of 0.27 M potassium tetraborate solution, followed by heating in a boiling water bath for exactly 8 min. The solutions were cooled with tap water, 3 mL of a 1% (dimethylamino)benzaldehyde solution was added, and the solutions were vortexed. After 20 min at 37 °C, the absorbance of the samples was measured at 585 nm on a Gilford 250 spectrophotometer. Appropriate assay blanks were treated in the same fashion. The concentration of GlcNAc in solution was determined from a standard curve prepared with four GlcNAc reference solutions (0.1, 0.4, 0.7, and 1.0 μmol/mL) treated as above. All enzyme activities were expressed in International milliunits (ImU) per milliliter, with 1 ImU defined as the production of 1 nmol of GlcNAc/min at 25 °C.

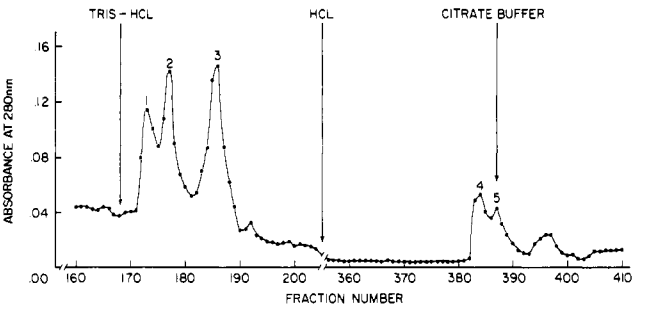

Figure 1. Elution profile from the chitin column. Arrows indicate buffer changes. Numbers indicate active peaks. Fraction volume was 8 mL.

Determination of chitinase activity on native chitins was carried out as described, with the substitution of 2.5 mg of chitin and 0.5 mL of water for the 0.5 mL of regenerated chitin suspension.

Chitobiase Assay. Enzyme solution (250 μL) was incubated with 125 μL of chitobiose solution (2.5 mg/mL) and 125 μL of 0.05 M citrate buffer (pH 3.5) for 30 min at room temperature, as modified from Ohtakara (1963). The entire volume (0.5 mL) was then assayed for GlcNAc as described.

Protein Concentration. Protein was determined by the protein-dye binding method of Bradford (1976), as described by Bio-Rad Laboratories, Richmond, CA.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis (DISC-PAGE) was performed according to the technique of Maurer (1971), using 5 mm i.d. × 12.5 cm gel tubes. The medium consisted of a 2.5% stacking gel (2 cm) and a 7.5% separating gel (7.5 cm). From 10–50 μg of protein were applied, with a running time of about 2.5 h. The gels were fixed in 12.5% trichloroacetic acid and stained with Coomassie Brilliant Blue R-250 for protein.

Polyacrylamide gel electrophoresis was also carried out in the presence of sodium dodecyl sulfate (SDS-PAGE) by the method of Weber and Osborn (1969), as modified by the Sigma Chemical Co. (1982). Samples (10–50 μg of protein) were run on 10% gels (10 cm) for about 5 h. The gels were fixed in 40% methanol and 7% acetic acid and stained with Coomassie Brilliant Blue R-250 for protein.

Results

Enzyme Purification. The chitin column consistently yielded five protein peaks containing chitinase activity (Figure 1). The majority of the activity was recovered in peaks 1–3. These peaks also contained from 1.7 to 2.2 ImU/mL chitobiase activity. The initial region of the profile (fractions 1–50) contained a very large peak of unbound material. A small percentage of the total chitinase activity of the extract was eluted with these fractions, indicating that the binding capacity of the column was slightly exceeded. The deleted region of the profile contained no major protein peaks but did contain a small amount of chitinase activity. This may have been an artifact caused by the leaching of a small amount of GlcNAc from the column, as a result of degradation of the chitin packing material. Although the elution profile shown in Figure 1 was reproducible, the amount of activity recovered in each peak varied between column runs due to continual refinements in the purification and assay techniques. Therefore, the values listed in Table I are the averages of five separate purifications and are considered to be conservative.

Table I. Purification of Soybean Chitinase

fraction	protein, mg/mL	chitinase act., ImU/mL	sp. act., ImU/mg	x-fold purifcn	% yield
crude extract	2.73	1.42	0.46	0.0	100
ammonium sulfate precipitate	1.53	1.46	0.85	1.8	85
chitin column					
peak 1	0.05	1.07	26.3	64.6	2.3
peak 2	0.06	0.95	14.6	31.9	1.1
peak 3	0.04	2.17	97.4	258.6	7.0
peak 4	0.02	0.09	5.4	13.6	0.3
peak 5	0.02	0.38	17.1	42.9	1.4
					total: 12.1

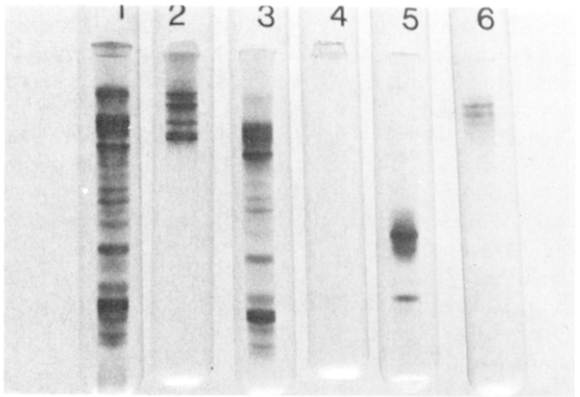

Figure 2. DISC–PAGE of various chitinase preparations: 1, crude soybean extract; 2, supernatant from the ammonium sulfate cut; 3, redissolved ammonium sulfate precipitate; 4, chitinase from peak 1 of the elution profile; 5, chitinase from United States Biochemicals; 6, chitinase from the National Institutes of Health.

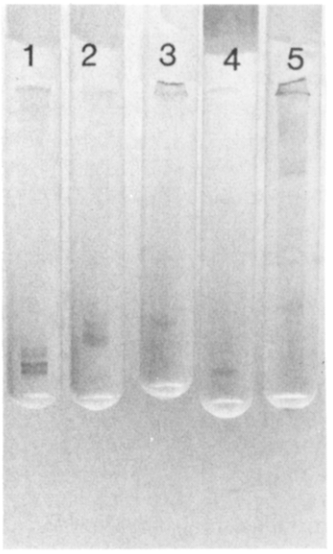

Figure 3. DISC–PAGE of purified chitinase fractions: 1, chitinase from the deleted region of the elution profile; 2, chitinase from peak 2; 3, chitinase from peak 1; 4, chitinase from peak 3. 5, same sample as gel 2, electrophoresed after 1 week at 4 °C.

Electrophoresis. The electrophoretic patterns of various chitinase preparations are shown in Figures 2 and 3. A sample from peak 1 of the elution profile yielded a single band on DISC-PAGE (gel 4, Figure 2). This sample appeared to be purer than the commercial chitinase (gel 5), although the protein concentration of the soybean sample may have been too low to reveal any additional bands. We attempted to concentrate our purified enzyme samples but were unable to do so. The enzyme consistently passed through several ultrafiltration membranes designed to separate molecules on the basis of molecular weight. Filters with exclusion limits of 10 000–50 000 daltons were tried, without success.

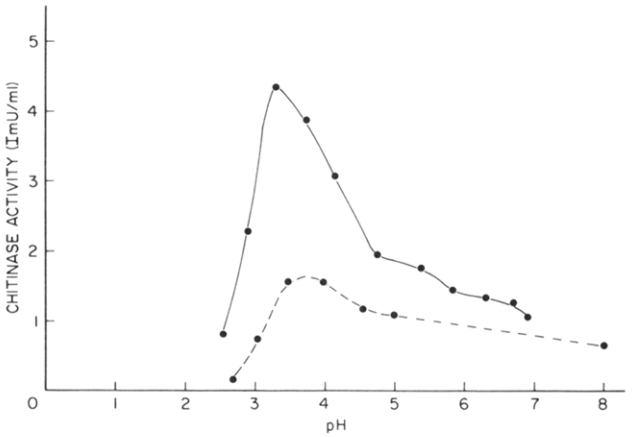

Figure 4. Effect of pH on chitinase activity: (—) chitinase from peak 1; (- - -) chitinase from peak 2.

Table II. Comparison of Soybean Chitinase with Commercial Chitinases (1982 Prices)

enzyme source	cost/g, $	sp act., ImU/mg
United States Biochemicals	198	283.9[a]
Calbiochem-Behring	240	60.6[a]
Sigma Chemical Co.	232	17.2[a]
soybeans	?	89.0

[a] Enzyme incubated in 0.05 M citrate buffer, pH 6.0.

It is interesting to note that the USB and NIH chitinases were both isolated from the same source, *Serratia marcescens*, yet their migration patterns were quite different. The migration patterns for soybean chitinase samples from different active peaks were also different and were reproducible (Figure 3). For example, the two proteins seen on gel 4 (Figure 3) do not correspond to any of the proteins seen on gels 2 and 3. As illustrated by gels 2 and 5 (Figure 3), the pattern could also change after storage of the enzyme. Each of the bands seen in gel 4 (Figure 3) was sliced from the gel and eluted overnight in 0.05 M citrate buffer (pH 4.5), and the eluates were assayed for enzyme activity. Each band contained a small amount of chitinase activity, but no chitobiase activity was detected. This procedure was repeated on additional gels with negative results, apparently due to the lower protein concentration of the samples applied to these gels.

Molecular Weight. The average molecular weight of soybean chitinase was 31 600 as determined by SDS–PAGE. In addition to this chitinase band, a variable number of higher molecular weight bands were observed for each of the samples. The predominant pattern was for two bands in the molecular weight range of 82 000–94 500 to appear in addition to the chitinase band. From one to three bands in the range of 43 000–62 000 were also observed in some cases. As was observed with DISC-PAGE, the same sample often yielded a different pattern after storage for 1–3 weeks at 4 °C. In addition, the sample shown on gel 2 (Figure 3), from peak 2 of the elution profile, exhibited only three bands on SDS–PAGE, in contrast to the four bands present on DISC-PAGE. This result, together with the variation seen in the electrophoretic patterns of different purified fractions, suggests the possible presence of more than one form of the enzyme.

Properties. Optimum pH for chitinase from peak 1 was 3.3, while chitinase from peak 2 was most active in the range of pH 3.5–4.0 (Figure 4). The specific activity of

Table III. Activity of Soybean Chitinase on Various Native Chitins after 30-min and 24-h Incubations

substrate	chitinase act., ImU/mL 30 min	24 h
chitosan (blue crab)	0.00	0.01
shrimp chitin (20 mesh)	0.00	0.02
shrimp chitin (40 mesh)	0.61	0.01
shrimp chitin (60 mesh)	0.31	0.03
shrimp chitin (micronized)	0.61	0.01
microcrystalline chitin (M_r 10 000)	0.92	0.14
crab chitin (Sigma Chemical Co.)	0.00	0.19
crab chitin (Bioshell, Inc.)	0.00	0.05
regenerated chitin	7.40	

soybean chitinase was greater than the specific acitivity of two out of three commercial enzymes tested (Table II). The sample used for this comparison was a representative sample from peak 3 of the elution profile (the peak containing the highest level of activity). The assays were performed simultaneously, with the commercial enzymes incubated at pH 6.0, the optimum pH as given by the suppliers.

As shown in Table III, the soybean enzyme was much less active on native substrates than on the less crystalline regenerated chitin. Microcrystalline chitin was the preferred native substrate overall. The results obtained for the shrimp chitins demonstrated a weak relationship between activity and particle size. The apparent activity of chitosan after 24 h is probably due to the hydrolysis of residual GlcNAc residues in the polymer and not to the presence of a chitosanase. It should be noted that the concentration of native chitin used for these assays was one-third of the concentration used for regenerated chitin. This may have had an adverse effect on the activity measured on the native substrates, although the results of the 24-h incubation also indicate very low activity.

The purified chitinase preparations retained about 75% of their activity after 1 month at 4 °C. The effect of freezing on activity was more drastic, with an average loss of 61% after 1–6 days in the freezer (−20 °C).

Chitobiase. As stated, peaks 1–3 of the elution profile also contained chitobiase activity, when assayed by using chitobiose as the substrate. However, when samples from these peaks were assayed on regenerated chitin with no additional chitobiase in the reaction mixture, no GlcNAc was released. It was also found that the chitobiose solution used as a substrate blank for the chitobiase assay had a positive absorbance when assayed for GlcNAc. This may reflect contamination of the commercial chitobiose with GlcNAc or interference in the assay by chitobiose itself. The absorbance of the chitobiose solution was subtracted from the total absorbance of the incubated samples via the use of a substrate blank for each chitobiase assay.

Attempts were made to identify chitobiase (M_r 100 000; Li and Li, 1970) in the electrophoretic patterns observed for the purified chitinase preparations. No standard was available for comparison, as the commercial chitobiase did not stain on DISC-PAGE gels despite the application of more than adequate amounts of protein. Gels were also stained for chitobiase activity by using (4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide (Srivastava et al., 1974), with negative results. The chitobiase in peaks 1–3 is apparently present in only very low concentrations.

DISCUSSION

To the best of our knowledge, this is the first communication on the presence of chitinase in soybeans. The purification procedure described is simple and inexpensive and yielded chitinase of a purity comparable to that of commercial enzymes based on specific activity measurements. Although the enzyme was purified up to almost 260-fold, the results of DISC- and SDS–PAGE indicate the presence of residual contaminants. Therefore, we do not claim to have produced a homogeneous product by this procedure. The column packing material was a technical-grade chitin that presumably contained relatively large amounts of contaminating proteins, salts, and pigments (Muzzarelli, 1977). This may have resulted in the binding of additional proteins to the column and their subsequent coelution with the chitinase fractions. However, the electrophoresis results may also indicate the presence of chitinase isoenzymes. The presence of two active protein bands in different regions of gel 4 (Figure 3) suggests a difference in charge properties between two forms of the enzyme. The observed differences in the migration patterns of samples from different active peaks, on both DISC- and SDS–PAGE gels, also suggest the possible presence of isoenzymes. Further experiments designed to resolve this question are planned for the future.

The purification of multiple chitinases has been reported by Jeuniaux (1966), Kimura (1976), and Bade (1978) among many others. Correa et al. (1982) have purified a mannan-associated endochitinase from yeast that exhibited an unusual and variable pattern of active bands upon polyacrylamide gel electrophoresis. The number of bands remained unchanged on SDS–PAGE, and only a single band was observed after storage of the enzyme. It was proposed that the mannan played some role in the formation of chitinase polymers whose distribution could change over time. Although soybean chitinase did not stain with Alcian blue after DISC-PAGE, it is interesting to speculate on the possible association of the enzyme with carbohydrate.

The presence of chitobiase activity in peaks 1–3 was not surprising. Chitinase and chitobiase are often purified together and separation of the two enzymes can be difficult. However, the negative results obtained when the preparations were assayed without additional chitobiase were unexpected. Chitobiase can generally hydrolyze oligomers up to the hexamer at decreasing rates as the size of the chitin oligosaccharide increases (Muzzrelli, 1977). Molano et al. (1979) found that an endochitinase from wheat germ released only the pentamer and tetramer after a 0.5-h incubation, with significant amounts of the dimer and trimer being produced only after 25 h. Soybean chitinase appears to hydrolyze regenerated chitin in a similar fashion. The copurified soybean chitobiase is apparently incapable of hydrolyzing these initial oligomeric degradation products at a detectable rate, while the commercial chitobiase has a broader substrate specificity.

The much greater activity of soybean chitinase on regenerated chitin as compared to native substrates is consistent with the probable role of the enzyme in nature. Powning and Irzykiewicz (1965) were the first to suggest that plant chitinases served as a defense against the invasion of parasitic fungi whose cell walls contain chitin. The region of the apex of fungal hyphae is the area where all growth occurs (Burnett, 1976). Evidence suggests that growth depends on a delicate balance between cell wall synthesis and wall lysis. Chitinase could alter this balance (Muzzarelli, 1977). Molano et al. (1979) have suggested that the less organized cell wall structure in the apical region makes this area more susceptible to enzymatic attack. Regenerated chitin was chosen as the routine substrate for soybean chitinase due to its decreased chain length and greater homogeneity as compared to the more crystalline native chitins available. These properties were

considered to more closely approximate the fungal substrates the enzyme would naturally encounter.

The low activity of soybean chitinase on native substrates indicates that the enzyme may be of little value for the modification of commercial chitins. However, soybeans could prove to be a suitable source of chitinase for research activities involving more favorable substrates. As of the spring of 1983, soybeans cost about \$0.25/kg. Chitin of a higher grade than the chitin available for use as column packing material in this study was available for about \$18/kg, with discounts on orders over 45 kg (Bioshell, Inc.). Our procedure yielded about 200 mL of purified enzyme (25 8-mL fractions), having an overall average activity for the five purifications of 0.9 ImU/mL, for a total of 180 ImU. With an average of 50 g of beans as starting material, we estimate a potential yield of 3600 ImU/kg of soybeans. Further refinements in the purification and assay techniques, including the use of larger columns per volume of extract, could increase recovery. It is hoped that the simplicity and low cost of the procedure may be of value in the production of purified chitinase from other sources as well.

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