A new method of screening for chitinase inhibitors in crude fermentation broths as a means of discovering new insecticidal leads has been developed. In this procedure soluble Remazol brilliant violet 5R dye-coupled chitin degradation products released from insoluble chitin azure substrate by hydrolysis with *Streptomyces griseus* chitinase are filtered in 0.45 μm Millititer HA 96 well filtration plates and collected in 96 well microtiter plates. Inhibitors of this reaction are detected by a decrease in absorbance (570 nm) of the filtrate. A chitinase inhibitor, designated A82516, produced by culture A82516 was discovered using this screen. Purified A82516 was found to have an IC₅₀ of 3.7 x 10⁻⁶ M for *S. griseus* chitinase. At a test concentration of 0.27 mg/ml, A82516 was 100% effective in preventing development of house fly larvae to pupae. Allosamidin, a recently reported chitinase inhibitor in vitro, has spectral properties identical to A82516.

Chitin, a polymer of unbranched chains of N-acetyl-D-glucosamine units in β-1→4 linkages, is one of the main constituents of insect exoskeleton. Insects grow by rebuilding the exoskeleton following molts between larval or juvenile instars. At the beginning of the molting process exuvial fluid containing chitinase is secreted between the partly formed new cuticle and the old skeleton. Chitinase hydrolyzes chitin into oligosaccharides, the smallest of which is the disaccharide chitobiose, allowing the old exoskeleton to be shed. Inhibition of chitinase should interrupt insect molting and thus prevent maturation to the adult reproductive stage. We sought to develop a chitinase inhibition screen capable of accommodating large numbers of samples as a means of discovering novel fermentation-derived compounds with insect life cycle disrupting capabilities, but low probability of mammalian toxicity.

Older chitinase inhibition assays were cumbersome and unsuitable for rapid fermentation broth screening. Since the end product of chitinase digestion of chitin substrate is chitobiose or diacetylchitobiose, an additional incubation step was required to degrade the disaccharide to a product that would react with p-dimethylaminobenzaldehyde for photometric detection. More recent chitin degradation procedures utilizing radioactive chitin, glycol chitin, and colloidal chitin in agar were not adaptable to our large volume microbial screen due to the use of radioisotopes, the need for secondary colorimetric reactions, or our inability to measure zones of clearing in chitin agar. A recent procedure using γ-Chitin red substrate overcame these difficulties but was not designed for...
use as a high sample throughput screen. This paper describes a simple new procedure combining a related commercially available substrate (chitin azure), enzyme, and microtiter plate into a novel approach for screening fermentation broths for chitinase inhibitors. Unlike the previously described chitinase methods, the method we describe utilizes state of the art microtiter plate technology to process and analyze ninety-six samples (fermentations) at a time rather than manipulating and reading samples one at a time. The biological properties of one chitinase inhibitor detected by this method are described.

Materials and Methods

Reagents
Chitinase (EC 3.2.1.14; poly [1,4-β-(2-acetamido-2-deoxy-D-glucoside)]glycanohydrolase) from *Streptomyces griseus* (lyophilized crude powder) and chitin azure were both purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. Both the chitinase and the chitin azure were used without further purification.

Preparation of Microtiter Plates
The microtiter plates used were Millipore Millititer HA filtration plates with a 0.45 μm membrane sealed to the underside of the plate. Due to the insolubility of the substrate and the presence of mycelia we added a Whatman Glass Microfiber Filter (GF/C) to each well to prevent plugging of the membrane pores. The microfiber filters were cut slightly larger than the well with a one hole paper punch. Inside the well, the edges of the filter were gently pressed down with a wooden applicator stick to achieve a tight fit.

Microtiter Plate Assay
Fermentation broths (50 μl) were dispensed into the prepared Millititer plates (see above) using an automated dispenser. *Streptomyces griseus* chitinase EC 3.2.1.14 (0.42 units) dissolved in 50 μl of 100 mM acetate buffer (pH 5.0) containing 10% Merthiolate to prevent microbial growth was added to each well and allowed to incubate at room temp for 2 hours, after which 50 μl of chitin azure solution (4 mg/ml in 100 mM pH 5.0 acetate buffer containing 10% Merthiolate) was added. The Millititer plates were then incubated at 37°C overnight.

Supernatants of these reactions were collected in sterile Costar microtiter dishes after filtering with a Millipore Millititer Vacuum Holder. An additional 100 μl of 100 mM acetate buffer (pH 5.0) was added to each well after the first filtration, and filtered again to displace droplets hanging on the lower surface of the membrane.

Soluble Remazol brilliant violet 5R-coupled degradation products released by the hydrolysis of the insoluble chitin azure substrate were measured by absorbance at 570 nm, the λmax of Remazol brilliant violet 5R, using a Dynatech microtiter plate reader. Percent inhibition was calculated by the equation

\[
\text{% Inhibition} = \frac{(A - B)}{C} \times 100
\]

where A is the absorbance of the complete reaction mixture including enzyme and fermentation broth, B is the absorbance of the reaction mixture lacking enzyme (to account for absorption of the fermentation broth), and C is the absorption of the uninhibited enzymatic reaction. Fermentations with 50% or more inhibition of chitinase were of interest.

Semi-quantitative Spectrophotometric Assay for Isolation of Inhibitors
In order to assist in the isolation of pure chitinase inhibitors, a spectrophotometric assay was employed. Since organic solvents inhibit the enzyme, 500 μl samples (in quadruplicate) containing putative inhibitors were placed in 13 × 100 mm test tubes, dried under vacuum, and solubilized in 500 μl of 100 mM acetate buffer (pH 5.0) using sonication. Chitinase (8 units) in 1.0 ml of 100 mM acetate buffer (pH 5.0) containing 10% Merthiolate was added to duplicate tubes. One ml of 100 mM
acetate buffer (pH 5.0) containing 10% Merthiolate was added to duplicate controls. After a 2-hour incubation at room temp, 1.0 ml of chitin azure solution, 4 mg/ml in 100 mM acetate buffer (pH 5.0), containing 10% Merthiolate was added to each tube, vortexed thoroughly, and placed in a 37°C water bath overnight. Tubes were then centrifuged at 200 x g for 10 minutes. The absorbance of the supernatants was read at 570 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer. The percentage inhibition was calculated as in the microtiter plate assay.

**Quantitative Spectrophotometric Assay for Chitinase Inhibitors**

Enzyme reactions for IC₅₀ determinations of purified compounds included the addition of 8.6 units of S. griseus chitinase EC 3.2.1.14 dissolved in 1.2 ml 100 mM acetate buffer (pH 5.0) containing 10% Merthiolate, to 13 x 100 mm test tubes containing 600 μl of sample. The tubes were allowed to sit at room temp for 2 hours before adding 1.2 ml 100 mM acetate buffer (pH 5.0) containing 4.8 mg chitin azure and 10% Merthiolate. Tubes were vortexed thoroughly and incubated in a 37°C water bath. After a 15-minute incubation, 1.5 ml of each reaction mixture was removed and centrifuged at 200 x g for 10 minutes. Absorbances of the supernatants were read at 570 nm. This reaction is linear for 1 hour. After a total incubation time of 60 minutes, the remaining 1.5 ml reaction mixture was also centrifuged, and the absorbances of the supernatants were read at 570 nm. The changes in absorbance over the 45 minutes interval were plotted to determine IC₅₀ values.

**Producing Organism**

Chemical analysis of a whole cell hydrolysate of the producing culture, A82516, demonstrated the presence of L-LAP (diaminopimelic acid), and the sugars glucose, mannose and ribose. This represents a Type I cell-wall and a NC or no characteristic sugar pattern. This combination of cell-wall constituents is indicative of the genus *Streptomyces*. This organism was isolated from a soil collected in Bozeman, Montana. The culture was maintained as a lyophilized pellet and as a suspension under liquid nitrogen.

**Fermentation**

Slants were inoculated from the culture growing in seed medium and incubated at 30°C for 10 days. The seed medium was composed of glucose 1%, soluble starch 2%, yeast extract 0.5%, NZ-Amine A 0.5%, and CaCO₃ 0.1%. Seed medium (50 ml in a 250-ml Erlenmeyer flask) was inoculated from a mature slant and grown for 48 hours at 30°C on a rotary shaker (250 rpm). Two ml of the culture was mixed with an equal volume of suspension agent composed of 40 g glycerol and 20 g lactose per liter of deionized water. This mixture was maintained under liquid nitrogen as the stock culture.

One ml of the stock culture was used to inoculate 50 ml of seed medium in a 250-ml wide mouthed Erlenmeyer flask. The cells were incubated at 30°C for 48 hours on a rotary shaker at 250 rpm. An 0.8% vegetative seed inoculum (second stage) was used to inoculate 110 liters of fermentation medium consisting of soybean flour 0.5%, potato dextrin 2.5%, glucose 0.5%, lactalbumin 0.5%, CaCO₃ 0.2%, SAG471 0.01% and P2000 0.02%. The medium was adjusted to pH 7.0 prior to sterilization. The fermentation was carried out at 30°C for 114 hours with the DO₂ value maintained at 45%.

**Isolation of A82516 Chitinase Inhibitor**

Whole broth (100 liters) was filtered using 4% Hyflo filter aid. The methanol extract (40 liters) of the mycelial cake was concentrated under reduced pressure and lyophilized to give 200 g of brown solids. Crude extract (50 g) was subjected to reversed phase liquid chromatography (RP-HPLC) on a column (4.6 cm i.d. x 60 cm) of octadecylsilanized Whatman LP-1 silica gel. Activity eluted with HCOOH - H₂O (0.2 : 99.8) eluent was concentrated, then lyophilized to give a partially purified dried product (3.4 g). Purification was completed by two successive RP-HPLC steps using a column (2.2 cm i.d. x 61 cm) packed with DuPont Zorbax ODS resin. The activity was eluted with HCOOH - H₂O (0.2 : 99.8) and CH₃CN - HCOOH - H₂O (4.0 : 0.2 : 95.8) respectively and lyophilized to give pure A82516 chitinase inhibitor (73 mg).

**Fly Growth Regulator Test**

One-pint paper cans were lined with plastic bags. Twenty-five g of fly rearing medium (Purina)
was placed into each container. A82516 (8.21 mg) was added to 30 ml of distilled water yielding a final test concentration of 0.27 mg/ml. Control containers used water only.

A laboratory strain of the house fly, Musca domestica, was used in this test. Mixed sexes of adults were fed exclusively on fly food (sugar, powdered nonfat dry milk, and powdered egg yolk in the ratio 6:6:1). Freshly oviposited eggs were collected from the colony cages. Eggs were microscopically inspected and only those appearing viable were used. Twenty eggs per treatment were placed on 2×3 cm patches of black cloth which were placed on top of the rearing medium in each container on day 0.

On day 5 the containers were opened, the patch removed, and the percentage egg hatch was recorded. Also at this time the rearing medium was removed, teased apart, and the number of pupae were recorded.

The pupae were placed in a small isolation cage and on day 9 the number of successfully emerged adults was recorded. The results were then compared between numbers of flies produced from treated versus control regimens.

Chicken Feed-through Test

Two Hubbard broiler cockerels weighing approximately 800 g each were fed compound A82516 at a concentration of 25 μg/g in the diet for 3 weeks, then given nonmedicated ration for 1 week. Two comparable birds were fed nonmedicated ration for the 4 weeks test period. Feces (approx 60 g) were collected from the treatment and control pens once each week. House fly control was determined from development of 20 house fly eggs seeded onto fecal samples. The procedure was similar to that described in the fly growth regulator test except that manure rather than rearing medium was used.

Results and Discussion

Both qualitative and quantitative assays for chitinase inhibition were developed, based upon the colorimetric determination of soluble Remazol violet 5R-coupled saccharides hydrolyzed from insoluble chitin azure substrate. The microtiter plate procedure is a simple, convenient means of detecting chitinase inhibitors from fermentation broths. Release of the Remazol brilliant violet 5R dye-coupled hydrolysis products was measured directly by absorbance at 570 nm.

Since the rate of the chitinase reaction is slow, overnight incubation worked well for giving maximum dye release and increasing the difference in absorbance between uninhibited and inhibited reactions containing fermentation broths which absorb at 570 nm.

Approximately 3,000 fermentations were screened before A82516 was found. Using purified A82516, the spectrophotometric assay was shown to be semiquantitative in the concentration range from 5~100 μg/ml in the fermentation broth (see Fig. 1). Consequently, the sensitivity of this assay corresponds to levels of compounds commonly found in fermentation broths.

Chitinase inhibition activity was present in both the filtered broth and the mycelial methanol extract of culture A82516. A portion (1/4) of the mycelial methanol extract obtained from a fermentation of culture A82516 in a 165-liter stirred vessel was subjected to repetitive RPHPLC. Activity was monitored by the microtiter plate assay described above. Active fractions from the final HPLC step were combined, concentrated under reduced pressure, and lyophilized to give pure A82516 chitinase inhibitor (73 mg).

The molecular formula of A82516 was determined to be C_{25}H_{42}N_{4}O_{14} by high resolution fast atom bombardment mass spectra (HRFAB-MS). The 1H and 13C NMR spectral properties of A82516 are identical to those which were subsequently reported for allosamidin\(^1\), a chitinase inhibitor isolated from the mycelium of Streptomyces sp. Additional physico-chemical properties of A82516 are presented in Table 1.
Fig. 1. A82516 chitinase inhibitor concentrations versus percentage inhibition.

A82516 was found to have an IC₅₀ of 3.7 × 10⁻⁶ M in the quantitative spectrophotometric assay described above (see Fig. 2). When tested at a concentration of 0.27 μg/ml in the fly growth regulator test, A82516 did not affect egg hatch (95% hatch), but completely prevented development from larvae to pupae (0/20). A82516 was tested as a feed additive for chickens to determine whether the compound could disrupt the fly life cycle by feed through with feces. In this test A82516 fed in the diet at 25 μg/g was not effective in preventing fly development in the feces (fly control no greater than 35% at weeks 1~4). There was no bird mortality, nor was there any observable effect on feed consumption, final bird weight, or feed efficiency. Thus, although chitinase inhibitor A82516 (allosamidin) does interrupt the fly life cycle, it does not appear to be effective in applications requiring fly control through incorporation in poultry diets.

Addendum in Proof

Subsequent to submission of this manuscript for editorial review, the chitinase inhibition assay of Sakuda et al.¹⁰, utilizing a chitinase preparation derived from pupal silkworm (Bombyx mori) alimentary canals, appeared in print. Although they report only very weak inhibition of S. griseus chitinase by allosamidin, our method utilizing the readily available microbially produced enzyme was successfully used to independently discover and purify the same chitinase inhibitor. Our method enjoys the advantage of manipulating and assaying fermentation broth samples in multiples of ninety-six rather than individually.

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