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REVIEW PAPER

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Detection and characterization of chitinases and other chitin-modifying enzymes

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Abstract Multiple industrial and medical uses of chitin and its derivatives have been developed in recent years. The demand for enzymes with new or desirable properties continues to grow as additional uses of chitin, chitooligosaccharides, and chitosan become apparent. Microorganisms, the primary degraders of chitin in the environment, are a rich source of valuable chitin-modifying enzymes. This review summarizes many methods that can be used to isolate and characterize chitin-modifying enzymes including chitin depolymerases, chitodextrinases, chitin deacetylases, N-acetylglucosaminidases, chitin-binding proteins, and chitosanases. Chitin analogs, zymography, detection of reducing sugars, genomic library screening, chitooligosaccharide electrophoresis, degenerate PCR primer design, thin layer chromatography, and chitin-binding assays are discussed.

Keywords Chitin · Chitinase · Chitooligosaccharide · Chitosan · Chitosanase

Introduction

Chitin is composed of repeating N-acetyl-D-glucosamine (GlcNAc) residues and is a component of crustacean exoskeletons, diatoms, fungal cell walls, and squid pens. Several gigatons of chitin are thought to be produced annually in the biome, which makes it an abundant renewable resource [40]. Chitin is difficult to purify and modify chemically [40], so identification of microbial chitin-modifying enzymes and elucidation of their activities could facilitate the efficient production of specific chitin products. Chitin is a versatile and promising biopolymer with numerous industrial, medical, and commercial uses.

Bacteria and fungi have developed systems for the depolymerization, transport, and metabolism of chitin and chitooligosaccharides [16, 29]. Although chitin is ubiquitous in the marine environment, almost none can be found in marine sediments [67], demonstrating the efficiency of microbial chitin-degrading systems. In general, microbial degradation of chitin involves the activity of secreted chitin depolymerases {EC 3.2.1.14; poly[1,4-(N-acetyl-β-D-glucosaminide)] glycanohydrolase} that release GlcNAc, chitobiose, and chitooligosaccharides from the polymer (Fig. 1). These compounds then enter the periplasm where chitodextrinases (also EC 3.2.1.14) and N-acetylglucosaminidases (EC 3.2.1.52; β-N-acetyl-D-hexosaminide N-acetylhexosaminohydroase) act to form a pool of GlcNAc and, to a lesser extent, chitobiose [2, 29]. When transported into the cytoplasm, GlcNAc and chitobiose are metabolized or modified for use in cell wall biogenesis. The activity of each of these enzymes has the potential to be exploited to produce chitin-derived compounds of commercial interest. Some microorganisms, mostly fungi, are able to deacylate chitin by the activity of chitin deacetylases (EC 3.5.1.41; chitin amidohydrolase) to form chitosan. Chitosan can then be degraded by chitosanase (EC 3.2.1.132; chitosan N-acetylglucosaminohydrolase), and metabolized.

The medical and industrial uses of chitin, chitin derivatives, and chitinases are expanding and have been reviewed in detail. In industrial and applied fields, chitin and chitosan (deacylated chitin) are used in such areas as bioremediation of heavy metal contamination [5], paper and textile production [52], as additives in animal feed [1], and as a component of consumer products such as

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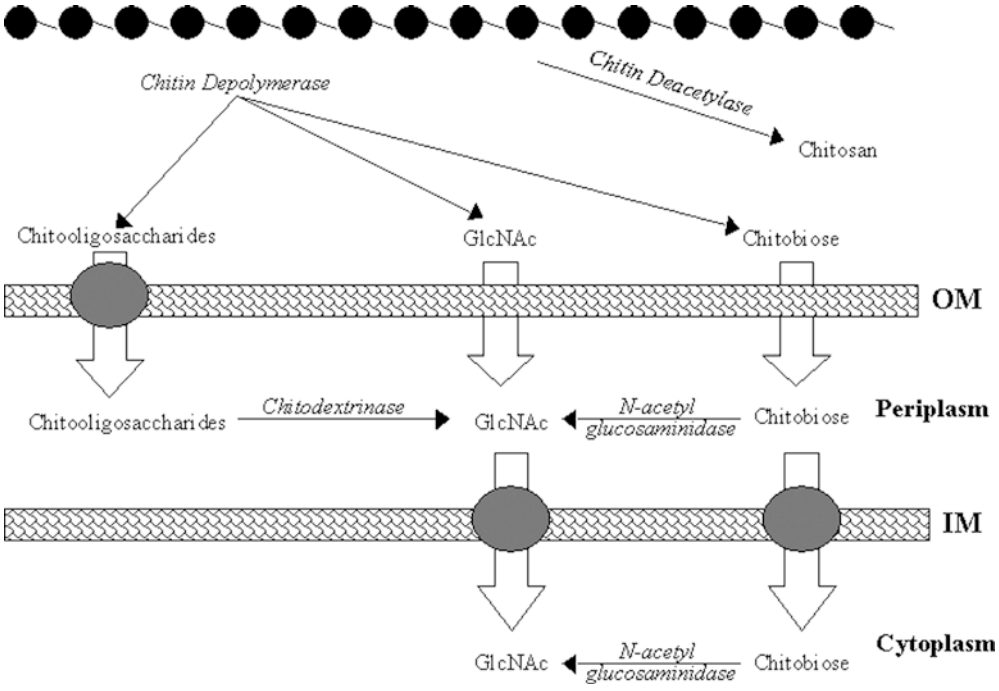

Fig. 1 Multiple enzymes are required for the degradation of chitin. The chitin polymer is degraded to chitooligosaccharides, N-acetyl glucosamine (GlcNAc), and chitobiose by the activity of chitin depolymerases. Alternatively, chitin can be deacylated by chitin deacetylases to form chitosan. Chitooligosaccharides are then transported to the periplasm, perhaps via a specific outermembrane porin, where they are degraded by chitodextrinases to form GlcNAc. Chitobiose, which enters the periplasm through non-specific porins, is degraded to GlcNAc by the activity of periplasmic N-acetylglucosaminidases, though some is transported to the cytoplasm. GlcNAc is subsequently transported to the cytoplasm where it is further metabolized or shunted to cell wall biogenesis. Cytoplasmic chitobiose is eventually degraded by cytoplasmic N-acetylglucosaminidases to form additional GlcNAc. *Shaded ovals* Transporters, *OM* outer membrane, *IM* inner membrane

cosmetics [60]. Uses for chitin-derived products in the medical field include drug and vaccine delivery systems [17, 27], wound and burn treatments [41, 53], blood cholesterol lowering agents [65], anti-clotting agents [13], antibacterial compounds [11, 21], and enhanced dissolution of some drugs such as ibuprofen [4, 26]. Chitinases have been shown to act as biocontrol and anti-fungal agents [12, 18, 33], as well as having anti-biofouling applications [38]. Chitinases can be used to create chitooligosaccharides of specific lengths, which have a variety of roles in the industrial and research applications mentioned above. Chitin-binding modules have been incorporated into protein expression vectors as fusion tags to facilitate the purification of recombinant proteins [7].

The diverse uses of chitin-derived products demand an equally diverse complement of enzymes that can be used to tailor them to specific needs. Chitinases and chitooligosaccharide-modifying enzymes have been isolated from a wide variety of organisms and characterized [16, 40]. Consequently, the known complement of these enzymes includes proteins with a range of pH and temperature optima, substrate specificities, and reaction end products. Because a large number of unculturable and/or unidentified bacteria, especially from the marine environment, are hypothesized to produce chitinases [9, 10], it is feasible that a large pool of uncharacterized chitin-degrading and -modifying enzymes has yet to be discovered. This review summarizes many of the current methods used to assay environmental isolates for chitinolytic activity, screen genomic libraries for genes encoding chitin-modifying proteins, and characterize the enzymatic properties of purified chitinolytic enzymes. While bacterial chitinases are the subject of most of the assays described here, some protocols can be easily modified for use in fungal and other eukaryotic systems.

Assays of culturable microorganisms for chitinolytic activity using solid media

In many chitinolytic systems, chitin is hydrolyzed by secreted chitin depolymerases. Because these enzymes are able to diffuse through agar, assays to identify chitinolytic bacteria or genomic clones encoding chitin-degrading enzymes can be performed by monitoring the degradation of polymeric chitin incorporated into an agar medium. Though these assays have a limited sensitivity, they represent a simple and inexpensive method to identify chitinolytic microorganisms. Initial screens for chitinase activity should include both alpha and beta chitin, as different forms of chitin may induce the expression of specific chitinases [55]. Alpha and beta chitin are incorporated into media at a concentration of 0.1% prior to heat-sterilization. Alpha chitin isolated from shrimp or crab shells can be purchased from Sigma

(C 8908 and C 9752, respectively; St. Louis, Mo.), while beta chitin from squid pens can be obtained from Industrial Research Chemicals (Lower Hutt, New Zealand). Squid pen chitin has a large particle size and a plastic-like consistency, but can be macerated before addition to agar or broth cultures to increase the available surface area and facilitate degradation.

When screening environmental isolates for chitinolytic activity, assay media should not contain glucose, as catabolite repression of microbial chitin depolymerase activity is well documented [16, 28, 42]. In the case of *Escherichia coli* transformants expressing a genomic library, Luria-Bertani agar supplemented with chitin is suitable. Halos or zones of clearing, usually 1–2 mm in diameter, will appear around colonies producing chitin depolymerase(s). Colonies should not be scored as negative until they have been incubated for several weeks; depolymerization of particulate chitin may take days or weeks to become visible, especially in the case of *E. coli* transformants expressing genomic libraries. Note that it is unlikely that the *E. coli* host is secreting the expressed proteins due to the specificity of type II secretion systems [47]; rather, enzymes are released due to cell leakage and/or lysis. Assay plates should be incubated in plastic bags with a damp paper towel to prevent drying during extended incubations.

Alternative substrates may be included in agar media to facilitate the visualization of chitin depolymerase activity. Chitin Azure (Sigma, C 3020) is prepared by covalently linking a soluble dye to colloidal chitin [54]. When Chitin Azure is depolymerized, clear zones appear around colonies that are easier to visualize than the halos formed on chitin plates. Chitin Azure should be incorporated in media at a final concentration of 0.08%, autoclaved, and incubated as described above for chitin plates. Alternatively, ethylene glycol chitin (EGC) (ICN157983-80; Fisher Scientific, Pittsburgh, Pa.) can be added to agar plates to screen for chitin depolymerase production [8]. EGC is a soluble form of chitin that can be prepared by the method of Yamada et al. [64] or purchased commercially (Fisher Scientific). EGC has the consistency of Styrofoam and must be macerated before it will dissolve in water. Grinding EGC with a mortar and pestle facilitates suspension in water. The suspension will appear somewhat cloudy. EGC agar contains 0.01% Trypan Blue and 0.4% EGC [8]. Solutions of Trypan Blue and EGC should be filter-sterilized prior to addition to sterile, molten agar. After culturing, clear halos will appear around colonies of chitinolytic bacteria. Note that ethylene glycol chitosan (Sigma, P 7364) can be utilized to assay for chitosanase production in the same manner.

Use of analogs for screening culturable bacteria or genomic libraries for chitinolytic activity

A variety of chitin analogs can be used to screen for the production of chitin-degrading enzymes. These analogs are especially effective for screening genomic libraries expressed in *E. coli*. Chitin depolymerase activity can be difficult to detect on chitin/agar plates because the production and secretion of foreign proteins may not be efficient in an *E. coli* host. In addition, chitin/agar screens identify only chitin depolymerase enzymes and not other chitin or chitooligosaccharide-modifying proteins. The *E. coli* strains EC300 (Epicentre Technologies, Madison, Wis.), DH5α, and DH5αE (Invitrogen, Carlsbad, Calif.) are suitable hosts for genomic library screens because they do not cleave the analogs described below (M. Howard, unpublished observations).

Chitin analogs have been well documented as a valuable tool for initial identification of chitinolytic-enzyme-producing strains and clones [10, 34, 39, 55, 56]. 4-Methylumbelliferyl β-D-N,N′-diacetylchitobioside (MUF-diNAG) (Sigma, M 9763) and 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside (MUF-triNAG) (Sigma, M 5639) are prepared by linking a 4-methylumbelliferyl moiety to the reducing end of chitobiose or chitiotriose, respectively [45]. When cleaved by a chitin-degrading enzyme, methylumbelliferone is released, which can be detected by its bright blue fluorescence under UV light [45]. Quantification of this activity can be performed using a fluorometer (355 nm excitation, 465 nm emission) and a standard curve prepared with 4-methylumbelliferone (Sigma, M 1381) [45]. A variety of enzymes can be detected with these analogs, including some chitin depolymerases, chitodextrinases, and N-acetylglucosaminidases (chitobiases). Chitinolytic enzymes can be tentatively classified (i.e., endo- vs exo-acting) based on their activity toward analogs, but results should be confirmed by testing activity against unmodified chitooligosaccharides. MUF analog solutions should be prepared in 100 mM sodium phosphate buffer and stored at −20°C until use. These analogs can be used to screen outgrown colonies on agar by using a 0.7% agarose solution supplemented to a final concentration of 5 μM MUF-diNAG or MUF-triNAG [50] as a top-agar overlay. After incubation of overlay plates at the desired temperature, colonies or transformants producing chitinolytic enzymes will be surrounded by a blue halo when visualized with UV light.

While the above plate screen is effective, diffusion of liberated 4-methylumbelliferone through the agarose may be problematic. An assay utilizing individual broth cultures of genomic clones is an alternative to agar media. Individual *E. coli* transformants are grown in 96-well microtiter plates at 25°C overnight and 1.5 nmol of a MUF analog is then added to each well and further incubated for 24–48 h with gentle shaking [24]. UV light can be used to visualize fluorescing cultures.

Several derivatives of MUF analogs have been prepared that can be used to specifically screen for chitodextrinase production. 4-Methylumbelliferyl-N-N′-diacetyl-4-thio-β-chitobioside (Mu-TCB) and 4-methylumbelliferyl-N-N′-N″-triacetyl-4-thio-β-chitotrioside (Mu-TCT) contain thio-glycosidic linkages between GlcNAc residues [63]. This alternative linkage prevents

N-acetyl-glucosaminidases from degrading the non-reducing end of these substrates. Mu-TCT has been demonstrated to be the optimal analog for chitodextrinase identification in enzymatic screens [62]. To identify chitin depolymerase producing clones, which may also cleave Mu-TCB and Mu-TCT, all clones producing a positive reaction should be subsequently screened for chitin depolymerase activity using one of the methods described above.

The paranitrophenol (PNP) chitin analogs 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma, N 9376), 4-nitrophenyl N,N′-diacetyl-β-D-chitobioside (Sigma, N 6133), and 4-nitrophenyl N,N′,N″-triacetylchitotrioside (Sigma, N 8638) are well-established compounds for the analysis of chitinolytic enzymes, particularly N-acetyl-β-D-glucosaminidases [36, 51]. PNP-analogs can be used much like the MUF analogs described above. When PNP is liberated from the carbohydrate polymer, it can be detected spectrophotometrically by monitoring the absorbance at 410 nm (A_{410}). Svital et al. [55] describe a method for analyzing crude soluble protein extracts from *E. coli* expressing chitinolytic enzymes cloned from *Vibrio harveyii*. Reaction buffer (10 mM Tris-Cl, 666 µM PNP-chitobiose, pH 7.5) is combined with an enzyme preparation and incubated at the desired temperature. Stival et al. report success with this procedure after a 30 min incubation. Three volumes of a 1 M Tris base solution are added to stop the reaction. The amount of liberated PNP can then be quantified using a standard curve prepared with 4-nitrophenol (Sigma, 104-8).

Analogs can also be used to screen for the presence of chitosanases. Honda et al. [23] utilized a chitin deacetlyase purified from *Colletotrichum lindemuthianum* to deaceylate the GlcNAc residues of MUF-triNAG. MUF-triNAG was dissolved in 800 µl reaction buffer (10 mM sodium tetraborate-HCl, pH 8.4) and combined with 0.46 U chitin deacetylase. The reaction was incubated at 37°C for 24 h and the enzyme was then separated from the analog using a centrifugal filter. Deacylated MUF-triNAG (MUF-triGluN) can then be used to assay for chitosanase activity as described above. Though chitosanases have been shown to cleave all of the bonds in MUF-triGluN, cleavage of the methylumbelliferyl moiety is sufficient for detection of activity using assays similar to those described for MUF-triNAG/chitinase assay [23].

Detection of chitin modifying enzymes in culture supernatants and whole cell lysates using zymograms

Chitin depolymerases produced by an organism or a genomic clone can be identified and enumerated by using zymograms. Native or denaturing polyacrylamide gel electrophoresis (PAGE) [31] can be employed in this type of zymography. Native gels do not require an overnight refolding step to restore enzymatic activity, but estimating the molecular weight of separated proteins requires additional steps (for method, see [20, 44]). Denaturing gels provide better separation of proteins and permit an estimation of molecular weight to be made directly, but are dependent upon successful refolding of the enzymes. A number of methods for detection of chitin depolymerase activity have been described that are amenable to both native and denaturing gels. Zymograms can be performed with whole cell lysates, membrane fractions, or concentrated culture supernatants. To ensure that supernatants are free of cells, they should be sterilized with a 0.22 µm filter, and concentrated using a centrifugal filter device prior to analysis. A 50–100 fold concentration is recommended.

Because of its solubility, EGC should be used as a substrate in zymograms when evaluating chitin depolymerase activity [58]. EGC (0.01%) can be incorporated into an otherwise standard SDS-PAGE separating gel. Protein samples can then be fractionated by denaturing PAGE and incubated in refolding buffer (50 mM Tris base, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.5) [39] for 16 h at 4°C. Gels containing fractionated proteins should be washed in 10 mM Na phosphate buffer (pH 7.0) for 1 h with several changes of wash buffer to remove trace amounts of EDTA and β-mercaptoethanol. Gels are then incubated in 10 mM sodium phosphate buffer (pH 7.0) for 12–36 h at the desired temperature to allow refolded, active enzymes to degrade the EGC incorporated into the separating gel. After incubation, zymograms are washed in 10 mM Tris-HCl (pH 7.4) containing 0.01% Calcofluor (Sigma, F 3543) for 5 min, taking care to fully dissolve the Calcofluor. The gel is then rinsed with dH_2O and periodically evaluated under UV light. Calcofluor will bind the glycol chitin and fluoresce brightly under UV light, while zones of depolymerase activity will appear dark.

An alternative method for producing zymograms has been described that detects not only the presence of some chitin depolymerases, but also chitodextrinase and N-acetylglucosaminidase activity [32]. Proteins are fractionated by standard denaturing SDS-PAGE and incubated in refolding buffer as described in the previous screen. Gels are then placed in activity buffer (10 mM Na phosphate, 5 µM MUF analog of choice, pH 7.0) for 30 s. After incubation at the desired temperature, gels are observed under UV light for activity. It is important that gels not be submerged during incubation in order to minimize diffusion of the released methylumbelliferone. Li et al. [32] report detection of chitinase activity with this method after a 5 min incubation.

Chitin deacetlyase activity can also be detected in zymograms by incorporating EGC into the separating gel as in the above chitin depolymerase screen [59]. Gels should be run as described above, stained with Calcofluor, and rinsed. Calcofluor has a higher binding affinity for chitosan than chitin [59], thus zones of chitin deacetylase activity will appear as bands of bright blue fluorescence against a blue fluorescent background.

Detection of chitinases by the release of reducing sugars

Chitooligomers, chitobiose, and GlcNAc, all of which have a reducing end, are released as chitin is degraded. This increase in reducing activity can be detected and quantified using a variety of methods. Commercially available kits for monitoring reducing sugar levels can be used, but an easy and inexpensive method described by Garcia et al. [19] makes use of bicinchoninic acid (Sigma, D 8284). Culture supernatants obtained from minimal medium/chitin grown cultures can be tested for chitinolytic activity with this method. Additionally, mixtures containing purified chitinolytic enzymes and a solution of chitin or chitooligosaccharides can be analyzed to determine reaction rates (e.g., micromoles reducing sugar produced per minute per milligram enzyme) or substrate specificities.

To perform the Garcia protocol, prepare Solution A [per liter: 54.28 g Na_2CO_3, 24.0 g $NaHCO_3$, 1.942 g 4,4′-dicarboxy-2–2′-biquinoline (bicinchoninic acid, disodium salt)] and Solution B (per liter: 1.248 g $CuSO_4 \cdot 5H_2O$, 1.262 g L-serine). Equal amounts of Solutions A and B are mixed immediately before use to prepare the reducing sugar assay reagent. Reactions should be prepared by mixing equal volumes of reducing sugar reagent and substrate (supernatant, enzyme reaction, or standard) and incubated at 80°C for 30 min. The samples are cooled to room temperature and the A_{560} measured. Reducing sugars are quantified by comparison to a standard curve prepared with GlcNAc. Note that the presence of protein will also produce a positive reaction, and therefore appropriate controls must be included.

An alternative to the Garcia protocol is the dinitrosalicylic acid reducing sugar test optimized by Miller [37]. This assay makes use of a reagent that reacts only with reducing sugars and is not affected by the presence of protein or amino acids. After an enzyme/substrate reaction has been incubated for a desired time, add an equal volume of DNSA reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide) and boil for 5 min. The A_{575} is measured after boiling and levels of reducing sugars estimated by comparison to a standard curve prepared with the reducing sugar of interest (e.g., GlcNAc).

A method for the detection of reducing sugars by fluorescent labeling and subsequent high performance liquid chromatography (HPLC) of the products has been described [30]. A fluorescent compound, 1-amino-1,3-naphthalene disulfonic acid, reacts with the reducing end of a sugar. The products of the reaction can then be separated by HPLC and the amount of labeled sugars quantified. Though more costly, this method has the advantage of allowing the researcher to determine the abundance of specific chitooligosaccharides produced by a specific enzyme.

Degenerate PCR primers and probes for environmental DNA samples, culturable, and nonculturable microorganisms

Many microorganisms are not culturable on known laboratory media [14]. For example, DNA can be isolated from marine sediments and terrestrial soil that appears to be from organisms not identified in outgrown cultures of environmental samples. The conserved nature of certain domains found within bacterial chitin-modifying proteins makes it possible to identify the cognate genes for chitin-processing enzymes from environmental samples by using molecular techniques. Many known microbial chitinases contain a GH18 domain and/or chitin-binding domains; GH18 domains are the predominant catalytic domain in microbial chitin depolymerases, and chitin-binding domains are common among bacterial chitinolytic enzymes [22]. The nucleotide sequence corresponding to these conserved domains can be amplified from a known species, radiolabeled or labeled with a colorimetric detection kit, and used to probe a set of environmental isolates or a genomic library. Probes and degenerate primers can be used to identify chitinase genes from naked DNA in the environment or from a collection of microbes in an environmental sample that cannot be cultured.

Ramaiah et al. [49] prepared a probe for chitinase genes derived from the *chiA* gene of *V. harveyii*. The primers 5′-GATATCGACTGGGAGTTCCC-3′ (forward) and 5′-CATAGAAGTCGTAGGTCATC-3′ (reverse) were designed using the *chiA* sequences of *Serratia marcescens* (X03657, Z36294), *Alteromonas* sp. (D13762), *Bacillus circulans* (M57601), and *Aeromonas caviae* (U09139). These primers were then used to amplify a 225 bp fragment of *chiA* from *V. harveyii* (ATCC 14216) genomic DNA. This amplified fragment was radiolabeled and used to probe environmental isolates from the Chesapeake Bay using standard hybridization techniques. In some cases, a chitinase gene fragment could be directly amplified from colonies that hybridized the probe using the above primers.

Cottrell et al. [10] described another set of primers that amplify chitinase genes, predominantly from α- and γ-proteobacteria isolated from environmental samples of marine origin. These primers are derived from a highly conserved region found within the *chiA* genes of *Alteromonas* sp. strain O-7, *A. caviae*, *S. marcescens*, and *Enterobacter agglomerans* and compensate for the variation in the third nucleotide position of each codon. The forward primer sequence is 5′-WSIGTIGGIGGITGGCANYT-3′ and the reverse primer sequence is 5′-ATRTCICCRTTRTCIGCRTC-3′ where W = A or T, S = C or G, Y = C or T, R = A or G, N = any nucleotide, and I = deoxyinosine. PCR conditions used to screen environmental isolates with these primers employed 35 cycles of: 1 min denaturation at 94°C, 1 min annealing at 50°C, and 3 min extension. The expected product size

of a positive result is 900 bp. The amplified fragment can then be sequenced or ligated into an AT vector (Invitrogen) for further analysis. The nucleotide sequence of any amplified fragment must be determined to confirm the presence of a GH18-like sequence.

Detection of specific chitooligosaccharides generated by the activity of chitin depolymerases or chitodextrinases

Biochemical assays are necessary to further classify chitinolytic enzymes. True rates of reaction and native substrate specificities are difficult to extrapolate from enzyme/analog reactions. Therefore, it is important that methods for detection and quantification of native chitooligosaccharides be presented here. Chitooligosaccharides are commercially available with degrees of polymerization ranging from 2 (chitobiose) to 8 (chitooctose) (Sigma). Degradative activity can be evaluated using chitin or various chitooligosaccharides and purified enzyme preparations followed by analysis to determine the products of each reaction.

To separate the reaction products for qualitative analysis, a relatively simple thin-layer chromatography (TLC) procedure can be performed. Samples of reaction mixtures can be spotted on a silica gel plate and dried. Chromatograms are then developed using 2-propanol:ethanol:water at a ratio of 5:2:1 (v/v) [61]. Plates should be thoroughly air-dried and then sprayed with 10% sulfuric acid in ethanol. The plates are again air-dried and baked at 120°C for 5–20 min. Chitooligosaccharides will appear as dark spots against a white background. Standards containing a mixture of chitooligosaccharides of various lengths are commercially available, or can be prepared from individual solutions. Chromatograms should be photographed soon after developing because spots will fade over time and are easily smeared.

An alternative method for fractionating chitin degradation products by TLC has been described [32]. The chromatogram is developed with *n*-butanol/methanol/28% ammonia/water (5/4/2/1) (v/v). After drying, plates are sprayed with diphenylamine-aniline-phosphate reagent (0.4 g diphenylamine, 0.4 ml aniline, 3 ml 85% phosphoric acid, 20 ml acetone) and incubated at 80°C for 20 min. Chitooligosaccharides will appear as dark spots on a white background.

While TLC analysis allows for a qualitative assessment of product formation, it is relatively insensitive and not quantitative. More advanced and much more sensitive analysis can be performed to better characterize the products released by chitin-degrading enzymes using analytical instrumentation. Several methods that employ HPLC have been described for the separation and quantification of chitooligosaccharides. Difficulties involving limits of detection are reduced as compared to TLC, and quantification of end-products is possible. HPLC has been used to determine the products formed by the action of chitodextrinases and N-acetylglucosaminidases [29], and also to determine the length of chitooligosaccharides released by the degradation of chitin [6].

Table 1 Retention time of chitooligosaccharides in a LiChrospher 100 NH2 column with 5 μm packing chromatographed with 75/25 (v/v) acetonitrile/water at 1 ml/min. *GlcNAc* N-acetyl-D-glucosamine

Chitooligosaccharide	Retention time (min)
GlcNAc	5.73
$(GlcNAc)_2$	8.40
$(GlcNAc)_3$	12.00
$(GlcNAc)_4$	17.87
$(GlcNAc)_5$	26.67
$(GlcNAc)_6$	40.07

Chang et al. [6] found that a LiChrospher 100 NH2 column (Fisher Scientific) with 5 μm packing coupled with an injection volume of 20 μl and a mobile phase composed of 75/25 (v/v) acetonitrile/water at a flow rate of 1 ml/min resulted in optimum separation of chitooligosaccharides. Detection of chitooligosaccharides was performed by monitoring A_{205} and peaks corresponding to specific products eluted as in Table 1. Other protocols that make use of different column packing and/or mobile phase components can also be explored [3, 15, 46].

Mass spectroscopy and NMR can also be utilized to determine the size of chitooligosaccharides produced by the activity of chitinases or chemical depolymerization of chitin. These methods can be modified for use with most mass spectrometers [35, 43, 48, 57, 66]. Lopatin et al. reported a method that allows identification of specific chitooligosaccharides within a mixture containing chitooligosaccharides of varying lengths [35]. A standard mass spectroscopic protocol involves electrospray injection of a sample to determine the molecular weight of reaction products, though care should be taken to limit the salt and buffer content of the reaction.

Finally, oligosaccharide gel electrophoresis can be performed to determine chitooligosaccharide production or degradation in a reaction using a 2-aminobenzoic acid tag. A modified version of the Huang et al. [25] protocol has been used in our laboratory to label and separate chitooligosaccharides between two and seven residues in length (Fig. 2). To perform this procedure, chitooligosaccharide degradation products are dried under vacuum. Each reaction is then mixed with 100 μl labeling solution (1.0 M sodium cyanoborohydride, 0.2 M 2-aminobenzoic acid, prewarmed to 65°C). Standards are prepared by adding 10 μl of a 1 mM solution of known chitooligosaccharides to 100 μl labeling solution. Each labeling reaction is incubated at 65°C for 2 h with periodic mixing. After cooling, six volumes of acetonitrile are added and samples are mixed briefly by vortexing. Products are then collected by centrifugation at 16,000 *g* for 2 min. Most of the supernatant (at least 500 μl) should be discarded, taking care not to disturb or remove the gelatinous clusters at the bottom of the tube. Water (300 μl) is then added to each tube, thor-

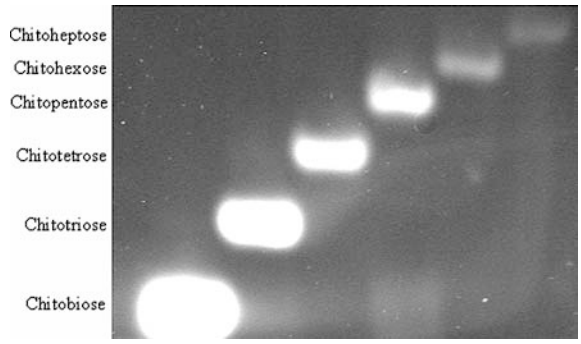

Fig. 2 2-Aminobenzoic-acid-labeled chitooligosaccharides The reducing end of chitooligosaccharides can be labeled with 2-aminobenzoic acid and separated on a standard 15% SDS-PAGE gel. Labeled chitooligosaccharides can be visualized under UV light

oughly mixed, and dried under vacuum. Dried, labeled chitooligosaccharides should then be resuspended in 25 μl SDS-PAGE loading buffer.

To separate the products of each labeling reaction, prepare a standard 1.5 mm, 10 cm ×7 cm SDS-PAGE gel (15% acrylamide, Tris-HCl buffer) using a standard Tris-glycine buffer system. No stacking gel is necessary. Samples (2–10 μl) are separated using constant current (45 mA) for 45 min. Labeled chitooligosaccharides can be visualized under UV light. The integrating function of gel visualization software may allow detection of 2-aminobenzoic acid-labeled saccharides that cannot be observed with the naked eye [25].

Chitin-binding assays and isolation of chitin-binding proteins and enzymes

A significant number of proteins associated with chitin degradation contain chitin-binding domains [22, 40]. These binding domains could conceivably be used to purify chitinolytic enzymes from protein preparations. In fact, a chitin-binding domain from a chitinase of *B. circulans* is employed as a purification tag in the Impact protein expression systems (New England Biolabs, Beverly, Mass.).

Secreted chitin-binding proteins or enzymes can be easily isolated from bacterial cultures by their affinity for chitin. This is especially useful in the event that promoters from an organism of interest are not active in *E. coli*, or if screening of genomic libraries is not possible due to gene toxicity. Chitin incorporated into liquid cultures can be isolated and washed to remove non-specifically adsorbed proteins. Many chitin-binding domains appear to bind the chitin polymer so tightly that conventional methods for eluting proteins from a matrix are ineffective. New England Biolabs reports that the *B. circulans* chitin-binding domain is not eluted from chitin when treated with N-acetyl-glucosamine, chitooligosaccharides, or high concentrations of sodium chloride. SDS-PAGE loading dye can be used to elute bound proteins for use in zymograms. Alternatively, addition of 1% SDS or 6 M guanidinium hydrochloride followed by incubation at 50–80°C can be used to denature and remove bound proteins (http://www.neb.com).

To determine if a purified protein of interest binds chitin, combine 1 mg binding substrate [61] (α- or β-chitin, chitosan, etc.) with binding buffer (50 mM Tris-HCl, 0.1 M NaCl, pH 7.0). After addition of a known amount of enzyme, reactions are incubated at a desired temperature for 1 h with agitation. The mixture is then centrifuged at 25,000 *g* for 10 min and the supernatant collected. The amount of protein in the supernatant is determined using a standard protein quantification protocol (e.g., a bovine serum albumin protein quantification kit). The amount of bound protein is calculated by subtracting the amount of protein detected in the supernatant from the initial amount of protein added to the reaction.

Conclusions

The methods presented here can be used to identify and characterize new chitin-degrading organisms and enzymes, and therefore increase our ability to modify chitin for applied uses. The non-toxic nature and absorbent properties of chitin make it environmentally safe for bioremediation and an excellent pharmaceutical tool for drug discovery and delivery. In addition, chitin is abundant and inexpensive, making it an attractive material for industrial processes and large-scale applications.

The conserved nature of several domains commonly found within chitin-modifying enzymes allows conserved and semi-conserved chitinases to be detected from raw nucleotide sequence. Extensive sequence analysis of the expanding list of microbial genome sequences is facilitated by the large number of web-based analysis tools, many of which are available at no cost to the researcher. Analysis of genomic sequence can lead to promising discoveries that must be subsequently demonstrated and characterized biochemically. Many of the methods presented here can be combined with bioinformatics to discover new and valuable chitin-modifying enzymes.

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