Cloning and Expression of a *Streptomyces plicatus* Chitinase (Chitinase-63) in *Escherichia coli*

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4-Methylumbelliferyl (4-MU) glycosides of N-acetylglucosamine oligosaccharides were used as substrates to detect expression of a *Streptomyces* chitinase in *Escherichia coli*. Low levels of enzyme were detected when *S. plicatus* DNA was cloned into a bacteriophage λ vector (EMBL-4). Subcloning into *E. coli* plasmids also gave low but detectable levels of enzyme expression. High level expression was achieved by resection of the cloned *S. plicatus* DNA with Bal31 followed by in-frame fusion to the amino-terminal peptide sequence of β-galactosidase found in the pUC vectors. The *Streptomyces* chitinase was secreted into the periplasmic space of *E. coli*, and its signal sequence was removed.

We characterized the activity of the cloned enzyme and compared it to three other purified *Streptomyces plicatus* chitinases with respect to hydrolysis of the 4-MU oligosaccharides. We found that two of the enzymes form 4-methylumbelliflorone much more rapidly from the 4-MU disaccharide than from the trisaccharide. These same enzymes convert the 4-MU trisaccharide primarily to diacetylchitobiose and the 4-MU monosaccharide, a nonfluorescent product. The latter compound is not hydrolyzed appreciably by any of the enzymes. On the basis of these results, we suggest a new definition of "exo" and "endo" chitinase that differs from that found in the literature. We propose that exochitinase activity be defined as processive action starting at the nonreducing ends of chitin chains with release of successive diacetylchitobiose units, and that endochitinase activity be defined as random cleavage at internal points in chitin chains.

Bacteria of the genus *Streptomyces* actively secrete enzymes that digest protein and complex carbohydrates. Chitin, the β-1,4-linked polymer of N-acetylglucosamine, is an excellent carbon and nitrogen source for many *Streptomyces* strains, and the *Streptomyces* chitinases have been the subject of several publications (1-3). There is a consensus that growth on chitin induces the synthesis and secretion of several enzymes that have endo- and/or exochitinase activities, but little is known about the number of enzymes involved, the mechanism of their activities, or the control systems involved in their induction. We have initiated studies on this interesting group of enzymes by cloning one of the enzymes of the *S. plicatus* chitinase complex.

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Papers in the older literature have defined "exochitinases" as enzymes that release acetylgalactosamine from the nonreducing ends of chitin chains (4, 14). Although enzymes of this type may exist, we feel that the formation of acetylgalactosamine may in many cases be the result of hydrolysis of small oligosaccharides by contaminating hexosaminidases. We propose that the term exochitinase be used for enzymes that catalyze processive release of diacetylchitobiose units from the nonreducing ends of chitin chains, and that enzymes which release acetylgalactosamine from either chitin or from oligosaccharides be called β-N-acetylglucosaminidases.

**MATERIALS AND METHODS**

The bacteriophage λ cloning vector EMBL-4 was purchased from Promega Biotech. The recommended Promega procedures were used for restriction enzyme digestion of the vector and ligation of DNA with phage arms. *S. plicatus* DNA (5) was digested with Sau3A, size-selected on a sucrose gradient (6), and the 10-20-kb fraction was treated with calf intestinal alkaline phosphatase before ligation with phage that had been digested with BamHI and EcoRI. Ligation of 1 μg of phage arms with 0.6 μg of *S. plicatus* DNA gave a total yield of about 6 X 10⁶ recombinant plaques following packaging and infection of *Escherichia coli* NM538. Plaques were purified, and phage DNA was isolated by standard procedures (6). The plasmid cloning vectors pKK-322, pUC-18, and pUC-19 were purchased from Pharmacia LKB Biotechnology Inc. and 4-methylumbelliferyl substrates were obtained from Sigma and from Carbohydrates International, Arlo, Sweden. Comparison of the trisaccharide derivative from the two sources by paper chromatography in n-butyl alcohol:ethanol:water (4:1:1) showed that the material from Carbohydrates International was pure, but that the Sigma preparation was about one-third 4-MU trisaccharide and two thirds a second 4-MU oligosaccharide derivative. This second derivative has a slightly greater mobility than the 4-MU trisaccharide on paper and is refractory to hydrolysis by chitinase-61. It is hydrolyzed, however, by enzymes in the culture supernatant of *S. plicatus* grown on chitin.

Periplasmic extracts of *E. coli* were prepared by the method of Koshland and Botstein (7). Procedures described in the *Streptomyces* methods manual (8) were used for growth of *Streptomyces* and plasmid transformation procedures. For enzyme assay, culture supernatants and purified enzyme preparations were diluted in solution A (0.8% NaCl, 0.02% KCl, 0.12% Na₂HPO₄, 0.02% KH₂PO₄).

Chitinases-61 and -47 were isolated from protein fractions obtained during endoglycosidase H isolation from *S. plicatus* culture supernatants. Culture of the organism and fractionation was carried out as described by Tarentino et al. (9). A chitinase with apparent molecular weight of 47,000, chitinase-47, was found to represent over 80% of the protein in the SP-Sephadex C-25 peak (step 4) that precedes endo H during column elution. The enzyme was kept frozen at -20 °C. Chitinase-61 was isolated from the protein fraction that precedes endo H during DEAE-column elution. This fraction was lyophilized, dissolved in a small volume of H₂O, and applied to Sephadex G-75. The first protein peak from the G-75 column was fractionated on SP-
**RESULTS**

Cloning in EMBL-4—The primary EMBL-4 library was plated on 150-mm LB plates with 4-methylumbelliferyl substrates in the soft agar overlay (12.5 µg/ml of the trisaccharide or 25 µg/ml disaccharide). Plates were incubated at 30 °C and were observed under long wavelength ultraviolet light as soon as plaques began to form. Although the signal was extremely weak, faint fluorescent halos were observed around six of 5- to 10⁵ plaques. Most positive plaques were detected after overnight incubation at 30 °C.

Although some positive clones were detected with the disaccharide substrate and others with the trisaccharide derivative, restriction mapping of the inserted DNA suggested that the same enzyme was probably being expressed in all cases. As can be seen from Fig. 1, all six inserts were in perfect register at one end. This suggested that cloning at only a single Sau3A site in the genomic DNA was compatible with detectable expression in EMBL-4.

Plasmid Subcloning—The most logical explanation for all of the λ clones being in register at one end was that the 5′-terminus of the gene in question was located near the common junction site with the vector DNA (see “Discussion”). Subcloning proved that this was indeed the case. Ligation of one end of the X362 insert into the EcoRV site of pBR-322 gave low but detectable levels of enzyme (Fig. 2). Bal31 resection followed by a second subcloning into pUC-18 gave the following results.

(a) Incomplete removal of the λ-DNA gave moderate levels of enzyme expression. (b) Removal of 150–200 base pairs from the junction site with the vector DNA (see “Discussion”) gave very high levels of expression (see below). (c) Removal of more than 200 base pairs from the insert led to complete inactivation.

The Streptomyces insert gave rise in some cases to very high levels of expression (see below). (c) Removal of more than 200 base pairs from the insert led to complete inactivation.

These results suggest that high level expression was the result of fusion of the chitinase gene with the short β-galactosidase sequence of the pUC vector. One of these high level expression plasmids, pCT-F1, was chosen for detailed analysis.

**Sequence Analysis of pCT-F1**—Fig. 3 shows the DNA sequence of the junction region between pUC-18 and the chitinase gene of pCT-F1. The sequence clearly suggests that nucleotides 7–9 code for the ATG start codon of the chitinase gene which in this case is fused in-frame to the pUC-1β-
galactosidase sequence. The first 30 amino acids of the chitinase are typical of the signal sequences found in secreted proteins of Gram-positive bacteria (10). Amino-terminal amino acid analysis of the protein purified from periplasmic extracts of *E. coli* carrying plasmid pCT-F1 gave a sequence of 15 amino acids corresponding exactly to the deduced sequence that starts at amino acid 31. This result confirms the general assumptions concerning the location and orientation of the gene and shows that the *E. coli* signal peptidase cleaves the protein between the 2 alanine residues, amino acids 30 and 31.

**Enzyme Purification**—Large scale periplasmic lysates were prepared by scaling up the Koshland and Botstein procedure (5) 1000-fold. In one case, 40 ml of lysate from cells carrying pCT-F1 was adsorbed to a 1-×15-cm DE52 column in 0.01 M phosphate buffer, pH 8.4. Following washing with 100 ml of 0.32 M phosphate, the enzyme was eluted with 10 ml of 3 M sodium acetate, pH 4.6. This preparation was dialyzed against 0.01 M Tris, pH 8, lyophilized, taken up in 1 ml of H2O, and applied to Superose 12. The column was eluted with solution A on the Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography apparatus. The enzyme was eluted as a sharp peak near the inclusion volume. Since this result suggested that the enzyme was being adsorbed to Superose 12, a second purification was devised involving sequential application to Sephadex G-50, where the enzyme is well included, followed by Superose 12 chromatography. In this procedure, 1 ml of a lyophilized, redissolved periplasmic extract was placed on a 1-×27-cm Sephadex G-50 column. Material eluting between 8 and 11 ml was lyophilized and taken up in 0.3 ml of H2O. The sample was placed on Superose 12 and eluted as above. The enzyme was again found near the inclusion volume clearly separated from partially included protein fractions. As shown by the SDS-acrylamide gel in Fig. 4, the purified enzyme gives a single protein band with an apparent molecular weight of 63,000. Specific activity measurements show that chitinase-63 is present as 5–10% of the secreted protein in periplasmic extracts of cells carrying pCT-F1.

**Enzymatic Properties**—Chitinase-63 hydrolyzes radioactive chitin most rapidly at pH 6 and has approximately half-maximal rates for this reaction at pH 4.5 and 7.5. By contrast, the maximum rate of hydrolysis of the 4-methylumbelliferyl trisaccharide occurs at pH 4 in 0.1 M citrate buffer. The enzyme catalyzes hydrolysis of the trisaccharide derivative over a wide pH range with appreciable activity even in 0.1 M glycine buffer, pH 10.4 (approximately 10% of the rate at pH 4).

Chitinase-63 has a high affinity for the oligosaccharide substrates; *Km* for the 4-MU trisaccharide is about 0.5 μM and for the 4-MU disaccharide is about 5 μM. Higher concentrations produce severe substrate inhibition. By contrast, chitinase-61 has a *Km* for the disaccharide derivative in the millimolar range.

Chitinase-63 is stable when heated in solution A at 63 °C, but is largely inactivated when heated at 80 °C for 1 min. Following brief boiling in solution A, the enzyme can be renatured. For example, in one experiment, the boiled enzyme was incubated at 4 °C for 15 h, after which 60% of the enzyme activity had returned.

The rates of hydrolysis of various 4-methylumbelliferyl substrates by the four *S. pilicus* chitinases were compared. The results are summarized in Table I. It is striking that two of the enzymes, chitinases-47 and -61, produce 4-methylumbelliferone about 10 times faster from the 4-MU disaccharide than from the trisaccharide derivative. If chitinases-47 and -61 are exo enzymes as defined above, they should react primarily at the nonreducing ends of chitin chains, releasing successive diacetychitobiose units. When the 4-MU trisaccharide is hydrolyzed by these enzymes, one would predict the products to be diacetychitobiose and 4-MU-GlcNAc, a non-fluorescent compound that is not hydrolyzed by the enzymes. Hydrolysis of the tetrascaraccharide derivative, on the other hand, should give primarily 4-MU and little if any 4-MU-GlcNAc. Table I shows that these predictions are largely borne out, although the specificity is not absolute.

Chitinase-49 and -63 present a different picture. These enzymes produce 4-methylumbelliferone more rapidly from the trisaccharide than from the disaccharide derivative, and both enzymes form more 4-methylumbelliferone than 4-MU-GlcNAc from both the 4-MU trisaccharide and tetrasaccharide. Thus, both chitinase-49 and -63 might be classified as endochitinases.

The lack of complete endo or exo specificity may be a result of the artificial nature of the substrates. It should be noted that, in general, the aglycone may have either a positive or negative impact on the rate and extent of 4-MU release. The 4-MU residue is a good leaving group and may interact strongly with residues at the active site of the enzyme. This could lead, for example, to the unexpected release of 4-MU from the trisaccharide derivative by exo enzymes or to hydrolysis of the disaccharide substrate by endo enzymes. On the other hand, the fact that the 4-MU is not acetylglicosamine might have negative effects. For example, the appreciable formation of 4-MU-GlcNAc from the trisaccharide derivative by chitinase-63 might reflect its preference for cleavage of...
reaction is diffusible. Therefore, if the rate of hydrolysis in the region of a phage plaque is less than the diffusion rate of the product, a localized signal will not be seen although the plates may gradually become fluorescent. It was observed, in cloning of chitinase-63 are extremely sensitive reagents, they expression is unknown, but is presumably associated with ratios of products when two plant chitinases were used to suffer from the limitation that the fluorescent product of the use of “signatures” for enzyme identification. It should be DNA permits expression in cloning at only one or possibly two Sau3A sites in the genomic transcription in E. coli. fact, that some plates did become fluorescent after the gene was fused to the pUC vector 8-galactosidase stream DNA is inactive or even inhibitory with respect to level expression of chitinase-63 in E. coli. Cloning at the next “upstream” Sau3A site does not allow “downstream” Sau3A site would destroy the gene. The reason that cloning at the next “upstream” Sau3A site does not allow expression is unknown, but is presumably associated with inhibition of transcription or translation in E. coli or with toxicity problems. Severe restrictions on cloning sites compatible with expression obviously decreases the frequency of recovery of positive clones. The requisite number of transforms can be monitored with λ, but with few other available vectors.

GlcNAc-GlcNAc linkages as opposed to the GlcNAc-4-MU linkage. In any case, the ratio of rates of reaction with various substrates and ratios of end products formed should serve as useful “signatures” for enzyme identification. It should be noted that Powxing and Izykiewicz (11) found different ratios of products when two plant chitinases were used to hydrolyze chitin oligosaccharides. They also found that one of their enzymes was more active than the other with large oligosaccharides. It would obviously be of interest to compare the plant and Streptomyces enzymes with respect to mode of action on chitin and the 4-MU oligosaccharides.

**DISCUSSION**

Although the 4-methylumbelliferyl substrates used in the cloning of chitinase-63 are extremely sensitive reagents, they suffer from the limitation that the fluorescent product of the reaction is diffusible. Therefore, if the rate of hydrolysis in the region of a phage plaque is less than the diffusion rate of the product, a localized signal will not be seen although the plates may gradually become fluorescent. It was observed, in fact, that some plates did become fluorescent after 2 or 3 days while others remained dark. By carrying out serial transfers from appropriate dilutions of positive plates, it should be possible to isolate plaques that produce even lower levels of signal than those detected directly in the present study. High level expression of chitinase-63 in E. coli was achieved only after the gene was fused to the pUC vector β-galactosidase sequence. This demonstrates clearly that Streptomyces upstream DNA is inactive or even inhibitory with respect to transcription in E. coli. However, poor codon usage predicted from the DNA sequence does not impede expression unduly (12).

The DNA inserts of six independent clones isolated in AEMBL-4 varied in size from 12 to 17 kilobase pairs, but had identical termination points at one end. This suggests that cloning at only one or possibly two Sau3A sites in the genomic DNA permits expression in λ. Cloning at the next “downstream” Sau3A site would destroy the gene. The reason that cloning at the next “upstream” Sau3A site does not allow expression is unknown, but is presumably associated with inhibition of transcription or translation in E. coli or with toxicity problems. Severe restrictions on cloning sites compatible with expression obviously decreases the frequency of recovery of positive clones. The requisite number of transforms can be monitored with λ, but with few other available vectors.

Only preliminary studies have been carried out on the expression of chitinase-63 in Streptomyces. However, even though only 200 base pairs of upstream DNA are present in available clones, it is clear that expression of Streptomyces chitinase-63 expression from the cloned gene is strongly stimulated by growth on chitin. We intend to use this gene as well as fusions of the gene to other secreted proteins to study the mechanism of induction and the role played by catabolite repression in synthesis of the chitinase complex in Streptomyces.

The lack of absolute specificity during hydrolysis of the 4-MU substrates may accurately reflect the properties of the enzymes with chitin as substrate, i.e. the enzymes may each have characteristic ratios of both endo and exo activities. On the other hand, the enzymes may show absolute or nearly absolute endo or exo activity with chitin. In this case, the lack of absolute specificity found here reflects differences between chitin as substrate and the small 4-MU oligosaccharides. Other enzymes contaminating the chitinase-47 and chitinase-61 preparations might also contribute to apparent lack of specificity.

Our definition of exo and endo chitinase will require confirmation and extension with chitin itself as substrate. These experiments are difficult, however, since oligosaccharide products tend to remain hydrogen-bonded to the insoluble, incompletely hydrolyzed substrate. However, since solvents do exist that dissolve and dissociate chitin chains (13), it should be possible to determine whether an enzyme is acting processively or is catalyzing random chain cleavage. A different approach to classification of endo and exo chitinases based on relative activities toward fibrous and collapsed chitin has been proposed by Bade et al. (15).

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