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A Modular Family 19 Chitinase Found in the Prokaryotic Organism *Streptomyces griseus* HUT 6037

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The specificity of chitinase C-1 of *Streptomyces griseus* HUT 6037 for the hydrolysis of the β-1,4-glycosidic linkages in partially acetylated chitosan is different from that of other microbial chitinases. In order to study the primary structure of this unique chitinase, the *chiC* gene specifying chitinase C-1 was cloned and its nucleotide sequence was determined. The gene encodes a polypeptide of 294 amino acids with a calculated size of 31.4 kDa. Comparison of the amino acid sequence of the deduced polypeptide with that of other proteins revealed a C-terminal catalytic domain displaying considerable sequence similarity to the catalytic domain of plant class I, II, and IV chitinases which form glycosyl hydrolase family 19. The N-terminal domain of the deduced polypeptide exhibits sequence similarity to substrate-binding domains of several microbial chitinases and cellulases but not to the chitin-binding domains of plant chitinases. The previously purified chitinase C-1 from *S. griseus* is suggested to be generated by proteolytic removal of the N-terminal chitin-binding domain and corresponds to the catalytic domain of the chitinase encoded by the *chiC* gene. High-performance liquid chromatography analysis of the hydrolysis products from *N*-acetyl chitotetraose revealed that chitinase C-1 catalyzes hydrolysis of the glycosidic bond with inversion of the anomeric configuration, in agreement with the previously reported inverting mechanism of plant class I chitinases. This is the first report of a family 19 chitinase found in an organism other than higher plants.

Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine. Recently, chitinases have been receiving renewed attention because of their possible application for the biological control of chitin-containing organisms and also for the exploitation of natural chitinous materials. They are present in a wide range of organisms including bacteria, insects, viruses, plants, and animals and play important physiological and ecological roles. Chitinases so far sequenced are classified in two different families (namely, families 18 and 19) of the classification of glycosyl hydrolases based on amino acid sequence similarities (13, 14). Family 18 contains chitinases from bacteria, fungi, viruses, and animals and class III and V chitinases from plants. On the other hand, class I, II, and IV plant chitinases belong to family 19, and this family solely comprises chitinases of plant origin. The two different families of chitinases display no sequence similarities with each other and have different three-dimensional structures (5).

Oligosaccharides produced from partially acetylated chitosan by microbial chitinases have been previously studied in an attempt to clarify their specificity for β-1,4-*N*-acetylglucosaminic versus β-1,4-glucosaminic linkages (26, 27, 30). Except for chitinase C-1 from *Streptomyces griseus* HUT 6037, all of the examined microbial chitinases hydrolyzed GlcNAc-GlcNAc and GlcNAc-GlcN linkages in partially acetylated chitosan but did not hydrolyze GlcN-GlcNAc or GlcN-GlcN linkages. In other words, for these enzymes, the residue at the nonreducing side of the linkages must be GlcNAc to be hydrolyzed. On the other hand, chitinase C-1 produced by *S. griseus* HUT 6037 hydrolyzed GlcN-GlcNAc and GlcNAc-GlcNAc linkages but not GlcNAc-GlcN or GlcN-GlcN linkages (25). *S. griseus* chitinase C-1 can therefore hydrolyze β-1,4-*N*-acetylglucosaminic and β-1,4-glucosaminic bonds as long as the residue at the reducing end side of the linkage is a GlcNAc. These observations led us to suspect that chitinase C-1 was unique among microbial chitinases in terms of structure of catalytic site, three-dimensional folding, and evolutionary origin. In order to verify this, the gene specifying chitinase C-1 was cloned into *Escherichia coli* and its nucleotide sequence was determined. Comparison of the encoded amino acid sequence with those of other proteins revealed high sequence similarity to the catalytic domain of class I, II, and IV plant chitinases, indicating that this chitinase belongs to family 19 of the glycosyl hydrolases. This is the first report of the finding of family 19 chitinase in an organism other than higher plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *S. griseus* HUT 6037 was used as a source of chitinase C-1 and of chromosomal DNA for cloning of the gene specifying chitinase C-1 (25). *E. coli* JM109 *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD* (*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*] was used as a host organism, and pUC119 was used as a vector for chitinase gene cloning.

S. griseus HUT 6037 was grown at 30°C in a medium containing 0.2% colloidal chitin, 0.05% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0), and 0.001% $FeSO_4$ for enzyme production and 1.0% mannitol, 0.2% peptone, 0.1% meat extract, and 0.1% yeast extract (pH 7.0) for chromosomal DNA preparation. *E. coli* JM109 carrying pUC119 and its derivatives were grown at 37°C in Luria-Bertani medium containing 100 μg of ampicillin per ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE in 12.5% slabs was conducted as described by Ames (1), with the buffer system of Laemmli (21). After electrophoresis was completed, renaturation of the enzymes in the gel and detection of chitinase activity were performed when necessary as described previously (43).

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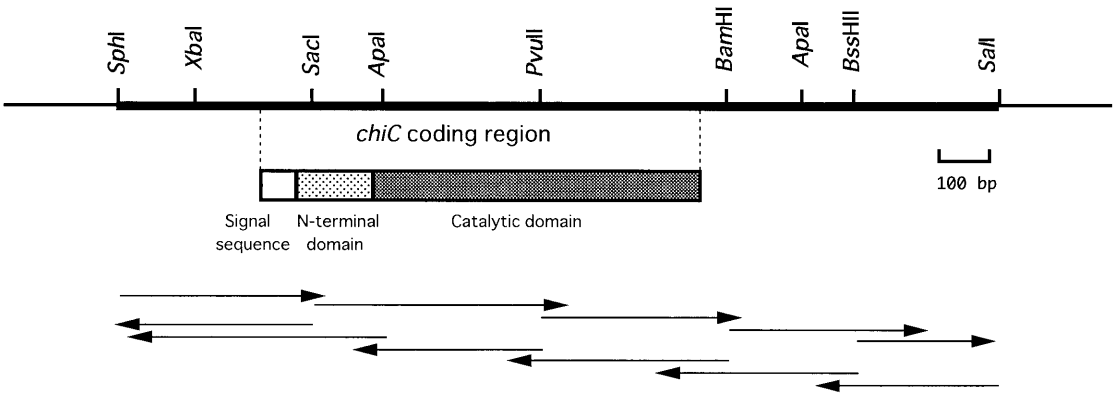

FIG. 1. Restriction map of the DNA fragment carrying the chitinase gene on the plasmid pGC01. The thick line indicates the inserted DNA fragment derived from *S. griseus* HUT 6037. The thin line represents the DNA of vector plasmid pUC119. The direction and extent of sequence determinations are shown by horizontal arrows.

Purification of chitinases of *S. griseus* HUT 6037 and N-terminal amino acid sequence analysis. Chitinases C-1 and C-2 were purified by combination of ammonium sulfate precipitation, Butyl-Toyopearl chromatography, and chromatofocusing as described previously (25). For N-terminal amino acid sequencing, purified chitinases C-1 and C-2 were run on SDS-PAGE, and the proteins on the gel were electroblotted onto a polyvinylidene difluoride membrane, as described by Matsudaira (24). The membrane was briefly stained with Coomassie brilliant blue R-250 for protein band visualization, and the excised chitinase bands were sequenced on an Applied Biosystems 473 gas phase sequencer (Foster City, Calif.).

Genomic DNA extraction, library construction, and screening. Chromosomal DNA of *S. griseus* HUT 6037 was extracted from the mycelia according to the method described by Hopwood et al. (15) with minor modifications. For Southern hybridization, restriction enzyme-digested DNA was fractionated in a 0.7% agarose gel, transferred onto a nylon membrane (Hybond N; Amersham) by the capillary method, and hybridized with a ^{32}P-labeled synthetic oligonucleotide probe, 5′-GAGGCICAGTTCAACCAGATGTTCCC(CI)AAC(CA)G(CI)AAC GCITTCTACAC-3′.

Chromosomal DNA of *S. griseus* HUT 6037 was doubly digested with *Sal*I and *Sph*I and separated on a 0.7% agarose gel. The gel segment corresponding to the size between 1.5 and 2.3 kb was cut out, and DNA fragments in the gel were recovered from the gel by using GENECLEAN II (Bio 101, Inc., Vista, Calif.) according to the supplier's instructions. The DNA was ligated to *Sal*I- and *Sph*I-digested pUC119 and used to transform *E. coli* JM109 cells. The library was screened by colony hybridization with the ^{32}P-labeled synthetic oligonucleotide probe described above at 37°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% SDS, 20% formamide, and 50 mM sodium phosphate. Washing of the membrane was done at 37°C with a solution containing 2× SSC and 0.1% SDS.

Nucleotide sequence determination. Various restriction fragments of pGC01 were subcloned into pUC119, and the resulting plasmids were sequenced with an automated laser fluorescence sequencer (Pharmacia). Sequencing reactions were done with the Autoread sequencing kit (Pharmacia) according to the supplier's instructions with double-stranded template.

Nucleotide sequence data were analyzed with the SDC Genetyx system (Software Kaihatsu Co., Tokyo, Japan). The amino acid sequence was compared with those available in the National Biomedical Research Foundation protein data bank by using the Lipman-Pearson algorithm (22).

Enzyme and protein assay. Chitinase activity was determined with glycol chitin as the substrate unless otherwise stated. Activity was determined by measuring the reducing sugar, according to the procedure described by Imoto and Yagishita (16). One unit of chitinase was defined as the amount of enzyme that liberated reducing sugar corresponding to 1 μmol of GlcNAc per min under the stated conditions. Soluble protein concentration was measured according to the method of Lowry et al. with bovine serum albumin as a standard (23).

Analysis of hydrolysis products of colloidal chitin. The reaction mixture (final volume, 150 μl) contained 0.2 mg of colloidal chitin and 0.003 U of enzyme and was incubated at 37°C for various times. The mixture was filtered through a cellulose acetate membrane filter and analyzed by high-performance liquid chromatography (HPLC). HPLC analysis was carried out on an Ultron-NH_2 column (0.45 by 25 cm) in a liquid chromatograph (LC-6A system; Shimadzu Corp., Kyoto, Japan) with water-acetonitrile as the eluent. Elution was monitored by measuring UV A_{200} with a Shimadzu SPD-6A spectrophotometer.

Analysis of anomeric configuration of hydrolysis products. Five microliters of chitinase C-1 of *S. griseus* HUT 6037 (1 U/ml in water) was added to 40 μl of a solution of chitotetraose in water (1 mg/ml in water). The mixture was incubated for 5 min at 40°C. The mixture was then microdialyzed for 10 min at 4°C and analyzed by reverse-phase HPLC with a C_{18} Nucleosyl column (5 μm, Interchim) equipped with a refractometric detector and eluted with water.

Chemicals. Glycol chitin and colloidal chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo, Japan) by the method described by Jeuniaux (18) and Yamada and Imoto (44), respectively. Regenerated chitin was prepared from chitosan 8B (80% deacetylated chitin) purchased from Funakoshi Chemical Co. as described by Molano et al. (28).

RESULTS

Cloning of an *S. griseus* chitinase gene. Chitinases C-1 and C-2 are major chitinases detected in the culture supernatant of *S. griseus* HUT 6037 grown in a medium containing colloidal chitin as a sole carbon source (25). The two chitinases are very similar to each other in terms of biochemical properties such as molecular weight, optimum pH, pH stability, thermal stability, and substrate specificity and were therefore suspected to be derived from a single gene. The only difference was with their isoelectric point of 7.7 for C-1 and 7.3 for C-2.

In order to design a hybridization probe for cloning of the gene specifying chitinases C-1 and C-2 of *S. griseus* HUT 6037, the N-terminal amino acid sequences of both purified chitinases C-1 and C-2 were first determined. The N-terminal amino acid sequence of chitinase C-1 was GEGPGGNNG FVVSEAQFNQMFPNRNAFYTYKGLTDALS-, and Asn-8 in the sequence was replaced by an Asp residue in chitinase C-2. The resemblance of the biochemical properties between the two chitinases and their N-terminal amino acid sequences suggest that chitinase C-2 is a derivative of chitinase C-1 generated by a deamidation of Asn-8. A synthetic oligonucleotide probe based on the sequence from residue Glu-14 to Thr-29 of the determined N-terminal amino acid sequence was designed, radiolabeled, and used for Southern hybridization experiments. One strong signal at a position around 2 kb was observed when chromosomal DNA was doubly digested with *Sal*I and *Sph*I. Thus, for library construction, the chromosomal DNA was digested with *Sal*I and *Sph*I, and the fragments with sizes between 1.5 and 2.3 kb were collected after electrophoresis on an agarose gel. The resulting DNA fragments were ligated with *Sal*I- and *Sph*I-cut pUC119 and transformed into *E. coli* JM109 cells. Colony hybridization was carried out for the transformant colonies with the same radiolabeled probe, and four positive clones were detected among 1,000 transformant colonies. The four positive clones contained an identical plasmid with 1.7 kb of inserted DNA, and this plasmid was designated pGC01. The physical map of the inserted DNA of pGC01 is shown in Fig. 1. Restricted fragments of appropriate

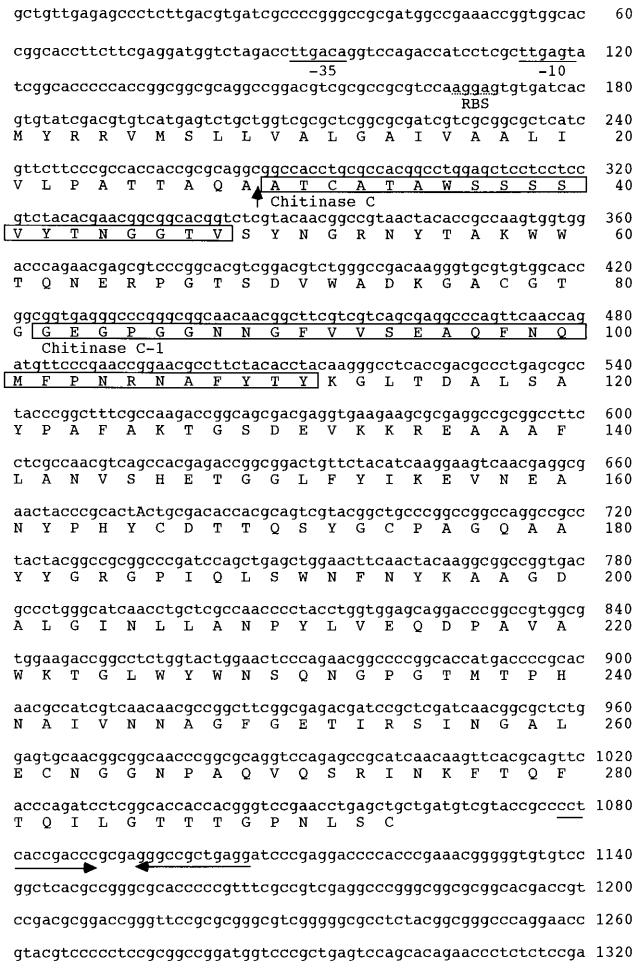

FIG. 2. Nucleotide sequence of the *chiC* gene of *S. griseus* HUT 6037 and deduced amino acid sequence of the gene product. A possible ribosome-binding sequence (RBS) and −10/−35 hexamers of the possible promoter are dashed-underlined and underlined, respectively. Amino acid sequence regions matched to the determined N-terminal amino acid sequences of chitinase C and C-1 are boxed. The vertical arrow indicates the cleavage site of the signal sequence. The horizontal arrows indicate the inverted repeat.

sizes were subcloned, and nucleotide sequences were determined.

Nucleotide sequence. The nucleotide sequence of the 1.7-kb fragment is shown in Fig. 2. One open reading frame of 885 nucleotides starting from a GTG initiation codon was identified in the sequenced region. The overall G+C content of the reading frame was 66.4% with 55.1% in the first, 49.3% in the second, and 94.9% in the third position of the codons. The translated polypeptide is 294 amino acids long with a calculated size of 31.4 kDa, significantly larger than the size of purified chitinase C-1 (27 kDa) estimated by SDS-PAGE. The N-terminal region of the deduced polypeptide exhibited the typical features of a signal sequence, i.e., a hydrophilic segment containing a positively charged amino acid(s) followed by a stretch of hydrophobic amino acids. The cleavage site of the signal sequence could not be deduced since the N-terminal amino acid sequence of chitinase C-1 did not match any region near the N terminus of the deduced polypeptide. Instead, the experimentally determined N terminus of both chitinases C-1 and C-2 matched the sequence starting from Gly-82 of the deduced polypeptide, suggesting that both chitinases C-1 and C-2 are truncated forms of the initial product (termed chitinase C) of this gene.

A probable ribosome-binding sequence, AAGGAG, complementary to the 3′ end of 16S rRNA of *Streptomyces lividans* (36), was found upstream from the proposed initiation codon. The −10 and −35 hexamers with a perfect match to the vegetative consensus of streptomycete promoters (36) were observed upstream from the probable ribosome-binding sequence. The gene cloned and sequenced in the present study was designated *chiC*.

Comparison of deduced amino acid sequence of the *chiC* gene with that of other proteins. The deduced amino acid sequence of the *chiC* gene was compared with that of other chitinases and related proteins and also with the sequences in the National Biomedical Research Foundation protein data bank. Surprisingly, a large C-terminal region of the deduced sequence showed significant similarity to the catalytic domains of plant chitinases of classes I, II, and IV forming family 19 of the glycosyl hydrolases. The region which showed similarity to plant chitinases corresponds to the region starting from the N-terminal amino acid sequence of chitinase C-1 (Gly-82), which is therefore considered to be the catalytic domain of this chitinase. The region did not show significant similarity to the catalytic domain of any other bacterial chitinases of family 18. The highest similarity was observed with class I chitinases from Japanese yam (NBRF A44039) followed by maize chitinase A (A42424), kidney bean chitinases (S16579), and other family 19 chitinases. The sequence alignment of the deduced amino acid sequence of the *chiC* gene with the catalytic domain of the Japanese yam chitinase is shown in Fig. 3. Until now, family 19 contained chitinases exclusively of plant origin (13, 14). Chitinase C is thus the first family 19 chitinase identified in an organism other than higher plants.

On the other hand, the N-terminal region of the deduced polypeptide was found to have sequence similarity with certain domains of bacterial polymer-degrading enzymes, i.e., chitinases, cellulases, and protease as shown in Fig. 4. In many of these enzymes, this domain has been shown to lack catalytic activity and to serve as an insoluble substrate-binding domain (9). For example, the related regions of chitinase A1 and D of *Bacillus circulans* WL-12 (Fig. 4) were experimentally demonstrated to be chitin-binding domains and essential for efficient hydrolysis of insoluble chitin (40). Therefore, although the catalytic domain of chitinase C is clearly related to family 19 plant chitinases, its N-terminal domain, which probably serves as a chitin-binding domain, appears related to microbial chitin- and cellulose-binding domains and not to plant chitin-binding domains.

Identification of the initial product of the *chiC* gene. Comparison between the N-terminal amino acid sequence of chitinase C-1 and the deduced amino acid sequence of the cloned

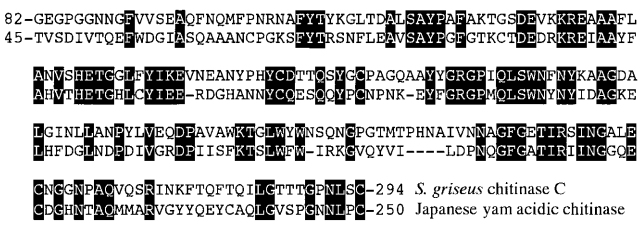

FIG. 3. Alignment of chitinase C with the catalytic domain of acidic class I chitinase from Japanese yam (NBRF A44039) as an example of family 19 chitinases. Identical amino acids between the two chitinases are shown on a black background.

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  S.griseus ChiC          30 ATCATAWSSSSVYTNGGTVSYNGRNYTA-KWWT----QNERPG-TSDVWADKGACGTG 82

CHITINASE
  Aeromonas sp.ChiII     491 PGGCAAWAEGNTYTAGTCASYGGKDYVAQVTHTAYVGANWNEAATPTLWKLK 542
  A.cavie ChiA            768 ANHPAWSAGTVYNTNDKVSHKQLVWQA-KYWT----QGNEPSRTADQWKLVS  814
                          815 QVQLGWDAGVVYNGGDVTSHNGRKWKA-QYWT----KGDEPG-KAAVWVDQGAASCN 865
  Alteromonas sp.Chi85   773 GAEYPTWDRSTVYVGGDRVIHNSNVFEA-KWWT----QGEEPG-TADVWKAVTN 820
  B.circulans ChiA1      650 NPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHT--SLAGWEPSNVPALWQLQ 699
  B.circulans ChiD1       31 AAQWQAGTAVKQGDLVTYLNKDYECIQPHT--ALTGWEPSNVPALWKYV 77
  J.lividum CHI69         50 VVACVPWQEGGTYNAGTVVTYLGGNYTALVTQTDHVGSGWNPVSTPSLWSAGGTCDGG 106
                         148 GTCALAWAAGTAYSAGATVSYAGTNYRA-NYWT----QGDNPSTSSGGAGTGKPWTSQ 200
  S.marcescens ChiB      450 IMTAPAYVPGTTYAQGALVSYQGYVWQT-KW----GYITSAPG-SDSAWLKVGRLA 499
CELLULASE
  Bacillus sp. CelA      354 PGEYPAWDPTQIYTN-EIVYHNGQLWQA-KWWT----QNQEPGYPYGPWEPLN 488
  Bacillus sp. CelB      363 PGDYPAWDPNTIYTD-EIVYHNGQLWQA-KWWT----QNQEPGDPYGPWEPLN 409
PROTEASE
  S.griseus ProC         408 DSPGGTWAVGTAYAAGATVTYGGATYRCLQAHT--AQPGWTPADVPALWQRV 457
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FIG. 4. Sequence similarity of the N-terminal domain of chitinase C with other enzymes. Amino acid residues identical to those of chitinase C are indicated by a black background. Amino acid sequences shown here are from *Aeromonas cavie* chitinase A (4), *Alteromonas* sp. strain O-7 chitinase 85 (NBRF A40633), *S. marcescens* chitinase B (NBRF S04856), *B. circulans* WL-12 chitinase A1 (NBRF A38368) and chitinase D1 (NBRF A41961), *Janthinobacterium lividum* chitinase (10), *Aeromonas* sp. strain 10S-24 chitinase II (GenBank AEOCII), *Bacillus* sp. strain N-4 cellulase A (NBRF A251560) and cellulase B (NBRF B25156), and *S. griseus* protease C (34). Numbers represent positions in each protein sequence.

gene led us to assume that chitinase C-1 was a proteolytically processed form of chitinase C, the initial product of the *chiC* gene. Sequence comparisons of the deduced protein with other proteins supported this assumption and suggest that the initial product of the gene is the complete form which consists of the catalytic domain and the putative N-terminal chitin-binding domain.

Chitinases C-1 and C-2 are the major chitinases detected in the culture supernatant of *S. griseus* HUT 6037 when cultivated under previously described conditions (25). In order to detect the initial product of the gene, cultivation of *S. griseus* was started with a smaller inoculum size and terminated earlier than the culture for chitinase C-1 purification. As shown in Fig. 5, in these conditions, a large amount of chitinase was detected with an estimated size of 33 kDa, a size larger than the activity band of chitinases C-1 and C-2. The protein band corresponding to the putative chitinase C was electrophoretically blotted onto a polyvinylidene difluoride membrane, and its N-terminal amino acid sequence was determined. The determined sequence, ATCATAWSSSSVYTNGGTVN, perfectly matched the deduced amino acid sequence from Ala-30. The signal sequence is therefore suggested to be Met-1 to Ala-29. These results indicate that the gene product is initially secreted in the culture medium as chitinase C, the mature form of the chitinase which carries the N-terminal putative chitin-binding domain. Chitinases C-1 and C-2 appear to be truncated forms of this initial chitinase, presumably generated by the action of protease(s) present in the culture supernatant of *S. griseus.*

In addition to the activity bands of chitinases C-1, C-2, and C, an activity band a little smaller than 45 kDa was detected as seen in Fig. 5B, indicating the presence of another chitinase gene than *chiC* in this organism.

Hydrolysis products of colloidal chitin. Most bacterial chitinases reported so far produce diacetylchitobiose as a major hydrolysis product from colloidal chitin. Other products, Glc NAc and chitooligosaccharides larger than $(GlcNAc)_3$, are either produced in a small amount or not observed at all (3, 33, 37, 43). This has led to the proposal that these enzymes would be exochitinases liberating chitobiose units from the nonreducing end of chitin. On the other hand, plant chitinases have been reported to be of the endo type and to produce mixtures of oligosaccharides of various sizes (19, 29, 32). Since the catalytic domain of chitinase C of *S. griseus* HUT 6037 is significantly related to plant class I, II, and IV chitinases but not to bacterial chitinases so far reported, it was important to determine if this chitinase indeed hydrolyzes chitin and produces chitobiose as a predominant product.

Chitinase C-1 was incubated with colloidal chitin at 37°C for 5 min, and hydrolysis products generated during the reaction were analyzed by HPLC. The enzyme produced $(GlcNAc)_2$ and $(GlcNAc)_3$ as major products of colloidal chitin hydrolysis, indicating endo-type action of this chitinase.

Stereochemical course of hydrolysis reaction catalyzed by chitinase C-1. We have recently demonstrated that chitinases A1 and D of *B. circulans* WL-12 both produce β-chitobiose from chitotetraitol and that these chitinases are therefore retaining enzymes (2). On the other hand, Fukamizo et al. reported that class I chitinase from Japanese yam produced only the α-anomer as an initial product from chitopentaitol and that this chitinase is therefore an inverting enzyme (7). The former two chitinases belong to glycosyl hydrolase family 18 while the latter belongs to family 19. A correspondence between the stereochemical outcome of the hydrolysis reaction and the classification in families based on amino acid sequence similarities has been reported for several cellulases and xylanases (8). If this was true also for chitinases, chitinase C-1 of *S. griseus* should be an inverting enzyme, since this chitinase is a new member of family 19. In order to verify this hypothesis,

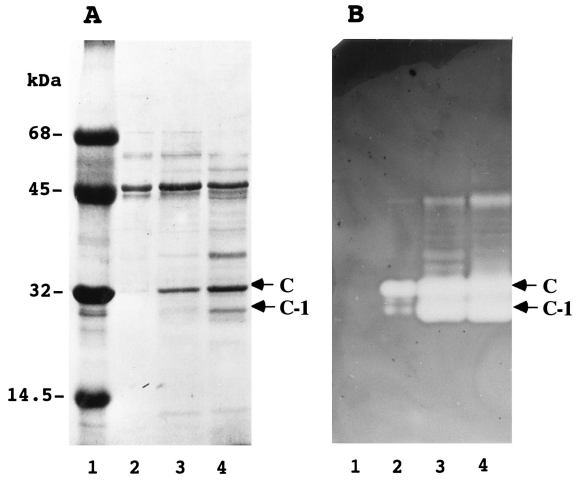

FIG. 5. Detection of chitinase C, mature form of the initial product of the *chiC* gene in the culture supernatant of *S. griseus* HUT 6037. (A) Protein staining of the polyacrylamide gel with Coomassie brilliant blue R-250; (B) chitinase activity detected on an agar replica of the polyacrylamide gel. Lanes 1, size marker; lanes 2, day 1; lanes 3, day 2; lanes 4, day 3 of cultivation. Positions of chitinases C and C-1 are indicated.

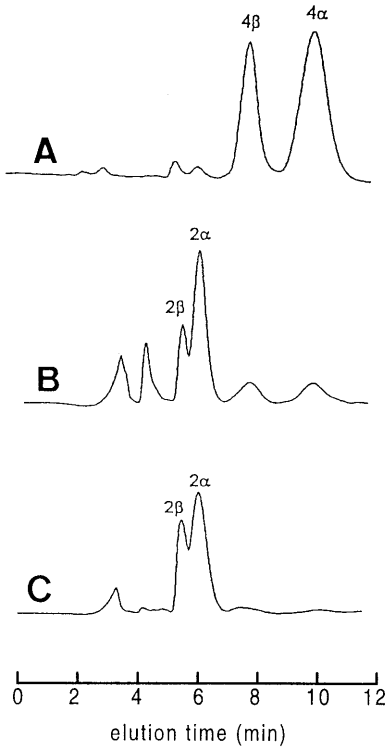

FIG. 6. HPLC determination of the stereochemistry of hydrolysis of chitotetraose by *S. griseus* chitinase C-1. (A) Chitotetraose; the β- and α-anomers are labeled 4β and 4α, respectively. (B) Chitobiose produced after 5 min of hydrolysis of chitotetraose by chitinase C-1; the β- and α-anomers are labeled 2β and 2α, respectively. (C) Chitobiose produced after 24 h of hydrolysis of chitotetraose by chitinase C-1; the β- and α-anomers are labeled 2β and 2α, respectively.

chitotetraose was hydrolyzed with chitinase C-1 and the hydrolysis products were analyzed by HPLC under conditions which can separate the α- and β-anomers of chitooligosaccharides (2). The chromatogram recorded after 5 min of hydrolysis shows an almost complete hydrolysis of chitotetraose into chitobiose with approximately 75% of the α-anomer and 25% of the β-anomer (Fig. 6B). It is likely that most of the β-anomer present in the reaction mixture originates from the initial proportion of the β-anomer in chitotetraose (Fig. 6A). The chromatogram recorded after a 24-h reaction shows that, after mutarotation, the α/β ratio has evolved to its normal equilibrium value of about 2 (Fig. 6C). This set of experiments demonstrates the accumulation of α-chitobiose in the early stages of the reaction, and this allows the conclusion that the enzyme indeed operates, as expected, by inversion of the anomeric configuration. The obtained results are in agreement with the working hypothesis that the mechanism of inversion versus retention of anomeric configuration of hydrolysis products is characteristic of each of the two chitinase families.

DISCUSSION

We have studied chitinase C-1 of *S. griseus* HUT 6037 because the specificity of this enzyme for the various linkages in partially acetylated chitosan is different from that of other microbial chitinases. Chitinase C-1 hydrolyzes GlcN-GlcNAc and GlcNAc-GlcNAc linkages whereas the other microbial chitinases hydrolyze only GlcNAc-GlcNAc and GlcNAc-GlcN linkages in partially acetylated chitosan. All chitinases absolutely require at least one GlcNAc residue at one side of the linkages undergoing hydrolysis. However, the position of the GlcNAc residue required for hydrolysis is different between chitinase C-1 and other microbial chitinases. This observation suggested that the structure of the catalytic site of chitinase C-1 could be different from that of the other microbial chitinases. It is well known that chitinases of family 18, which include all microbial chitinases so far sequenced, display short conserved regions of amino acid sequence (12, 20, 42). The importance of these conserved regions, and especially those containing a perfectly conserved glutamic acid residue, in catalytic activity has been shown by site-directed mutagenesis of *B. circulans* chitinase A1 (41) and later confirmed by the three-dimensional structures of chitinases from *Serratia marcescens* (31) and *Hevea brasiliensis* (39). There is no region in the amino acid sequence of chitinase C-1 of *S. griseus* that contains the above-mentioned conserved segments, and instead, the sequence was found to be clearly related to family 19 plant chitinases, whose three-dimensional structure (11) is clearly different from that of family 18 enzymes (5).

In addition to the linkage specificity in the hydrolysis of partially acetylated chitosan, we also examined the stereochemical course of the hydrolysis reaction catalyzed by chitinase C-1. Bacterial chitinases, e.g., chitinases A1 and D of *B. circulans* WL-12, have been shown to operate by a mechanism leading to overall retention of the anomeric configuration (2). On the other hand, a chitinase from Japanese yam has been shown to be an inverting enzyme as reported by Fukamizo et al. (7). Chitinase C-1 was also found to be an inverting enzyme, substantiating the idea that the classification of chitinases into two families (family 18 and family 19 of glycosyl hydrolases) correlates with the stereochemistry of the reaction and the linkage specificity in the hydrolysis of partially acetylated chitosan. Family 18 chitinases have been shown to act via a retaining mechanism (2, 38) likely to involve some anchimeric assistance by the acetamido group at C-2 of the substrate (38). A likely consequence of such a mechanism is that deacetylation of the sugar moiety undergoing hydrolysis should considerably slow down, or even prevent, hydrolysis. This mechanism is supported by the fact that microbial family 18 chitinases are unable to cleave the glycosidic bonds of GlcN in partially acetylated chitosan. On the other hand, family 19 chitinases operate by an inverting mechanism (6, 17) probably involving the direct attack of the sugar anomeric carbon by a nucleophilic water molecule (35). This mechanism, which has recently been shown to also prevail in a chitosanase (6), does not involve participation of the acetamido group at C-2 and, consequently, allows the hydrolysis of β-linked GlcNAc as well as GlcN. This is compatible with the observation that *S. griseus* chitinase C is able to cleave GlcNAc-GlcNAc as well as GlcN-GlcNAc bonds in partially acetylated chitosan. The results obtained in the present study lead us to speculate that plant family 19 chitinases, because of their inverting mechanism, would also be able to hydrolyze GlcNAc-GlcNAc and GlcN-GlcNAc linkages in partially acetylated chitosan. Experiments aimed at elucidating the hydrolysis pattern of plant class I (family 19) and class III (family 18) chitinases on partially acetylated chitosan are now under way.

The features of *S. griseus* chitinase C-1 raise the question of the evolution of this enzyme: why was a family 19 chitinase found only in a particular strain of *S. griseus*, although the primary structures of a large number of microbial chitinases have been determined? Considering the high sequence similarity between chitinase C-1 and several plant chitinases, the most probable explanation is that family 19 chitinases have evolved along with plant evolution and that chitinase C-1 was

acquired by *S. griseus* from plants through horizontal gene transfer. However, other questions remain unanswered, such as why family 19 chitinases are rare in prokaryotic organisms, how *S. griseus* HUT 6037 acquired such a chitinase, and by which mechanism was a chitin-binding domain fused to the N terminus of the *S. griseus* chitinase C-1? It is possible that the number of determined sequences is not sufficient and that family 19 chitinase might be more widespread in prokaryotic organisms than it appears at the present time. Accumulation of primary sequences of chitinases not only from bacteria but also from animals and fungi will perhaps help to answer these questions.

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