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Chitinases of *Streptomyces olivaceoviridis* and Significance of Processing for Multiplicity

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Five extracellular chitinases of 20.5, 30, 47, 70, and 92 kDa purified from the culture filtrate of *Streptomyces olivaceoviridis* ATCC 11238 differed in their sequences at the amino termini of the protein chains. In the native state, the chitinases were found to be resistant to proteolysis by trypsin, papain, and *Staphylococcus aureus* V8 protease. The latter produced several fragments of identical molecular mass from chitinases denaturated with sodium dodecyl sulfate. Five proteases were detected in the protein concentrate from the culture filtrate, and two of them showing ability to cleave chitinases in the native state were purified. One, a protease of 42 kDa, released a 30-kDa protein from the 70-kDa chitinase that reacts with anti-30 kDa chitinase antibodies; the other, a protease of 29 kDa, split the 30-kDa chitinase into 20.5-, 18-, and 16-kDa fragments. From these results, it was deduced that the 70-kDa chitinase is the precursor protein of the 30- and 20.5-kDa chitinases.

After cellulose, chitin is the most abundant biopolymer found in nature (31). Chitin, a β-(1,4) polymer of *N*-acetylglucosamine, is a structural component of the arthropod exoskeleton and is a common constituent of fungal cell walls (24). Chitin-degrading enzymes have been found to be secreted by many bacteria, e.g., *Aeromonas* (20), *Bacillus* (29, 34), *Clostridium* (25), *Nocardia* (23), and especially *Streptomyces* (2, 10, 27, 30) spp. Some of these microorganisms produce several chitinases with different molecular masses (6, 29, 30, 34).

Recently the assumption has been made that the multiplicity of chitinases in *Bacillus circulans* (34) is due to proteolytic modification of one or two precursor protein(s), and two amylases from *Streptomyces griseus* were shown to be derived from the same gene product by an intracellular processing mechanism (9). Posttranslational splitting of a precursor protein led to an α- and a β-amylase in *Bacillus polymyxa* (33).

We had previously reported on the purification of several chitinases from the extracellular fluid of *Streptomyces olivaceoviridis* (6). We now describe N-terminal amino acid sequences, proteolytic cleavage, and substrate specificities of the chitinases. The results, including antibody typing, substantiate the hypothesis of sequence homologies between different chitinases and posttranslational proteolytic cleavage of a larger primary gene product.

MATERIALS AND METHODS

Microorganisms and culture conditions. *Streptomyces* sp. ATCC 11238, identified as *S. olivaceoviridis* (16), was maintained on yeast extract agar and grown in fermentors (Biostat V; Braun Diessel Biotech, Melsungen, Germany) as described previously (1), except that a modified mineral medium (13) containing 7.5 g of chitin was used. After 9 to 10 days at 27°C, 200 rpm, and 2 liters of air per min, the fermentation was stopped by centrifugation.

Concentration of extracellular protein and purification of chitinases. The supernatant obtained after centrifugation was subjected to microfiltration (Microdyn MD 020 TP 2 N cartridge; ENKA AG, Wuppertal, Germany) and ultrafiltration (hollow-fiber cartridge SPS4002-6; Fresenius AG, St. Wendel, Germany). A 200-ml sample of the concentrate containing 280 to 300 mg of protein in 20 mM Tris-HCl buffer (pH 9.0) was subjected to fast-protein liquid chromatography (FPLC) in Pharmacia equipment (6) on a Sepharose Q fast-flow column (HR 16/10; 16-mm inner diameter, 100 mm long), and three chitinase pools (A, C, and D) were collected. After concentration of pools A, C, and D in an Amicon pressure cell with a Diaflo PM10 membrane (Amicon, Witten, Germany), ammonium sulfate was added to make a final concentration of 1.0 M (pH 7.0). Each of the pools was applied separately to a phenyl-Sepharose column equilibrated with 1.2 M ammonium sulfate in 20 mM Tris-HCl buffer (pH 7.0). Protein from pool D was eluted by a descending linear gradient of ammonium sulfate from 1.2 to 0 M (170 ml) at a flow rate of 2 ml/min. Proteins from pools A and C were eluted with the 20 mM Tris buffer (pH 7.0) (1 to 5 bed volumes). Fractions of 2 ml were collected.

Enzyme assays. Chitin from crab shell (Sigma Chemie, Deisenhofen, Germany) was ball milled for 7 days and washed (26). Carboxymethyl chitin (CM-chitin) was obtained from Katakura Chikkarin Co., Tokyo, Japan. Colloidal chitin was prepared as described by Jeuniaux (14).

Chitinases were tested as follows. To 0.5 ml of chitin suspension (10 mg of chitin per ml in 0.2 M citrate–0.4 M phosphate buffer [pH 6.5]), the enzyme sample and water were added to a final volume of 1 ml. The test tube was placed in a water bath at 37°C and stirred magnetically for 30 min. The reaction was stopped by boiling for 5 min. The amounts of released reducing sugars were determined as described by Dygert et al. (7). One unit of chitinase activity was defined as the amount of enzyme which releases 1 μmol of reducing sugar per min in the standard test. Protein was determined by the method of Lowry et al. (18).

To determine protease activity, the reaction mixture contained 0.55 ml of 1% casein solution and 0.2 ml of enzyme. The substrate was dissolved in 50 mM sodium citrate–citric acid buffer (pH 7.0). After 1 h at 37°C, the reaction was stopped with 5 ml of 10% trichloroacetic acid. Hydrolysis products were measured by the method of Lowry et al. (18).

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One unit of the enzyme was defined as the amount of enzyme which yields an ΔE_{540} of 1.0/h.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-slab gel electrophoresis was performed according to Laemmli (17), using an electrophoresis system from Biometra (Göttingen, Germany). Calibration proteins were obtained from Pharmacia LKB (Freiburg, Germany). Staining was done with Coomassie brilliant blue R-250 or the silver stain of Blum et al. (3).

Determination of the N-terminal amino acid sequence. Gas-phase sequencing (8, 12) was carried out by using a 470A sequencer and on-line analysis of phenylthiohydantoin-derived amino acids with 120A high-pressure liquid chromatography equipment, both from Applied Biosystems Inc., Weiterstadt, Germany.

C-terminal amino acids. The proteins (20 nmol in 20 μl of 50 mM sodium citrate buffer [pH 6.0] containing 1.0% SDS) were denaturated for 30 min at 60°C. The SDS concentration was then reduced to 0.01% by dilution, and the carboxyl-terminal amino acids were cleaved with 0.2 nmol of carboxypeptidase Y (sequencing grade; Boehringer, Mannheim, Germany) at 25°C. Aliquots were withdrawn at intervals from 30 s to 30 min, the reaction was stopped by the addition of 5 μl of glacial acetic acid, and the samples were lyophilized. Free amino acids were analyzed in a model LC5001 amino acid analyzer (Biotronik, Maintal, Germany).

Proteolytic cleavage of chitinases. Cleavage with protease from *Staphylococcus aureus* V8 (SAP; Sigma Chemie, Deisenhofen, Germany) was done as described by Cleveland et al. (5). Samples (30 μg) of the chitinases were diluted in buffer (0.375 M Tris-HCl [pH 6.8], 1.5% SDS), boiled for 2 min, and after cooling to room temperature, incubated with 5 U of SAP for 1 h at 37°C. The reaction was stopped by boiling for 2 min, and the mixture was then separated by SDS-polyacrylamide gel electrophoresis (PAGE).

For digestion with papain, 10-μg samples of the native chitinases were incubated with 66 μg of papain in 50 mM citrate buffer (pH 7.0) containing 5 mM L-cysteine and 2 mM EDTA. For digestion of native chitinases by trypsin or SAP, 30 μg of chitinase, diluted in 25 μl of 0.15 M Tris-HCl buffer (pH 6.8), was incubated with 15 μg of trypsin or 0.8 μg of SAP and incubated for 1 h at 37°C.

Cleavage with *S. olivaceoviridis* proteases was done with the native chitinases. Samples (6 μg) of the enzymes were incubated with 10 μl (2 μg of protein) of the protease. After 15, 30, and 60 min, samples were taken and analyzed by SDS-PAGE.

Preparation of antisera. Polyclonal antisera against 20- and 30-kDa chitinases were obtained from rabbits after 7 weeks. A 100-μg dose of antigen was applied for initial immunization, and 400 μg was used for a booster injection 4 weeks later.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed on microtiter plates (Nunc-Immuno-Plates MaxisorbF96; Nunc GmbH, Wiesbaden, Germany), using biotinylated goat anti-rabbit antibodies, streptavidin-peroxidase conjugates (both diluted 1:20,000), and 2,2′-azino-di-(3-ethylbenzthiazolin-6-sulfonic acid) as the substrate.

Immunoblotting. In general, the method of Harlow and Lane (11) was followed. The proteins separated by SDS-PAGE were blotted onto a PVDF-Immobilon membrane (Millipore, Eschborn, Germany), using a Fast-Blot apparatus (Biometra). Visualization of immunoreactive bands was done by using goat anti-rabbit immunoglobulin G, streptavidin-alkaline-phosphatase conjugates, and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium staining.

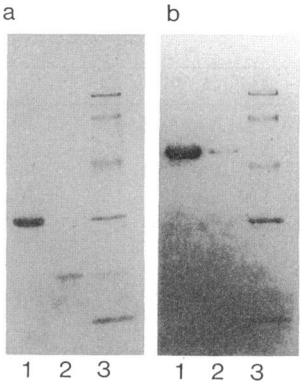

FIG. 1. SDS-PAGE of purified chitinases after hydrophobic interaction chromatography on phenyl-Sepharose. (a) Lanes: 1, 12 μg of 30-kDa chitinase; 2, 4 μg of 20.5-kDa chitinase; 3, molecular mass standards. (b) Lanes: 1, 8 μg of 47-kDa chitinase; 2, 2 μg of 47-kDa chitinase; 3, molecular mass standards.

RESULTS

Multiplicity of chitinases. The previously published method for concentration of the culture filtrate and purification by FPLC (6) was refined. After a primary separation on Q-Sepharose, pure enzymes were prepared in high yields by hydrophobic-interaction chromatography on high-capacity phenyl-Sepharose columns and subsequent gel filtration on Superose-12 (three chitinases from pool A, one from pool C, and another from pool D). The details of this purification procedure will be described elsewhere.

From SDS-PAGE, the molecular masses of the purified enzymes could be deduced (Fig. 1). The chitinase in pool D had a molecular mass of 20.5 kDa, and the three chitinases from pool A had molecular masses of 92, 70, and 30 kDa, respectively. Pool C contained a chitinase with a molecular mass of 47 kDa.

After separation and identification of the different chitinases by FPLC, it was possible to monitor the appearance of the different chitinases in the time course of fermentation. It was shown that 47-kDa chitinase is already present in the chitin-free inoculum, while 92- and 70-kDa chitinases were detected after 72 h, and 30- and 20.5-kDa chitinases were detected after 115 and 145 h in the chitin-containing medium, respectively.

Purification of proteases. By hydrophobic interaction chromatography of pool A, four proteases could be separated in the descending gradient. One of these eluted at 0.1 M $(NH_4)_2SO_4$ as a single band, showed proteolytic activity against 30-kDa chitinase, and had a molecular mass of 29 kDa in SDS-PAGE. Another protease with a molecular mass of 42 kDa was purified to homogeneity from pool C by using the same chromatographic method and conditions. This protease was eluted at 0.3 M $(NH_4)_2SO_4$. The specific activity of 29-kDa protease was 1.9 U/mg, and that of 42-kDa protease was 1.0 U/mg; only faint protein bands of both proteases were seen upon SDS-PAGE of the crude concentrated protein before chromatography. The activity of both purified proteases is inhibited by phenylmethylsulfonyl fluoride and by trypsin inhibitor, indicating that the enzymes belong to the group of serine proteases.

N- and C-terminal amino acids. The sequences at the amino terminus are shown in Table 1. No apparent homology could be observed.

The first amino acid released from 20.5- and 30-kDa

TABLE 1. N-terminal amino acid sequences of five chitinases from *S. olivaceoviridis*

Size (kDa)	Position																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
20.5	X[a]	Gly	Tyr	Thr	Asp	Leu	Pro	Val	Ser	Arg	Gln	Lys	Met	Cys	Gln	Asn	Gly	Met	Val	Thr	Asn	Cys					
30	Ala	Ala	Cys	Ser	Ser	Tyr	Pro	Ser	Trp	Val	Ala	Gly	Arg	Ser	Tyr	Ala	Ala	Gly	Asp	Ile	Val	Tyr	Tyr	Thr	Asp		
47	Ala	Gly	Ser	Lys	Val	Val	Gly	Tyr	Phe	Thr	Glu	Trp	Gly	Thr	Tyr	Asp	Arg	Lys	Tyr	Tyr	Val	Lys	Asn	Ile	Glu	X	Ser
70	X	Thr	Ser	Ala	Thr	Ala	Thr	Tyr	Ala	Lys	Thr	Gln	Asp	Trp	Gly	Ser	Cys	Phe	Glu	Gly	Lys	Trp	Thr	Ile	Lys	Asn	Thr
92	X	X	Glu	Glu	Ser	Ala	Arg	Pro	Asp	Gly	Leu	Tyr	Arg	Thr	Pro	Gly	Val	Asp	Val	Pro	Tyr	Asp	Ser	Val	Tyr		

[a] X, not determined.

chitinases by carboxypeptidase Y was threonine, and the first released from 47-kDa chitinase was asparagine. Threonine and alanine were released concomitantly from 70-kDa chitinase. The yield of released amino acids in 60 min ranged from 0.14 to 0.91 nmol in the presence of 0.01% SDS. In the presence of 1% SDS, the amount of free amino acids was too low to be determined with certainty.

Proteolytic digestion. The chitinases were found to be stable in the native state against proteolytic digestion by SAP, trypsin, or papain but could be cleaved by SAP after SDS treatment and heat denaturation. From the 47-kDa chitinase, fragments of 32, 24, 17.8, 14.5, and 13.7 kDa appeared in the enzymatic digests (Fig. 2). The bands at 17.8, 14.5, and 13.7 kDa were also apparent after digestion of 70-, 30-, and 20.5-kDa chitinases. The most abundant product of the cleavage of 30-kDa chitinase was a fragment with a molecular mass of 23.4 kDa. The amino-terminal sequence of this peptide was AlaGlnPheAsnGlnMetPheProAsnArg AsnSerPheTyrSerTyr.

After incubation of native 70-kDa chitinase with the homologous (from *S. olivaceoviridis*) 42-kDa protease, fragments of 46, 30, 17.5, and 17.3 kDa were detected after SDS-PAGE and silver staining. The cleavage of native 70- and 30-kDa chitinases with the homologous 29-kDa protease led to fragments of 20, 18, and 16 kDa. The 16-kDa fragment was isolated by gel filtration on Superose-12 and had no chitinase activity.

Immunological assays. Antibodies were prepared against 20.5- and 30-kDa chitinases. The titers rose considerably following the booster injection. After a period of 46 days, the titers were found to be high enough (Table 2). Higher titers were obtained with 30-kDa chitinase than with 20.5-kDa chitinase.

Anti-30-kDa chitinase sera showed no cross-reaction with the other chitinases in the ELISA, while anti-20.5-kDa chitinase sera reacted with 30-kDa chitinase and to a lesser extent with 47-kDa chitinase.

Immunoblotting of the fragments generated from 70-kDa chitinase by proteolytic digestion with 42-kDa protease revealed that the 30-kDa fragment reacted with anti-30-kDa chitinase antibodies. No cross-reaction was observed with the smaller fragments (Fig. 3).

Properties of the chitinolytic enzymes. The substrate specificities of the purified chitinases were tested with different substrates. There were only slight differences in recognition and activity against chitin and derivatives (Table 3). All of the purified chitinases produced low-molecular-weight oligomers from colloidal as well as from native chitin. The main product is a always dimer, but trimers and tetramers could be observed for 20- and 30-kDa chitinases in smaller amounts. In general, all chitinases are able to cleave oligomers from $n = 3$ to 6 (data not shown).

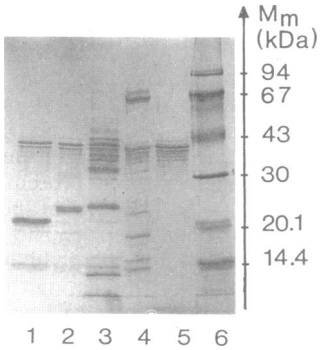

FIG. 2. SDS-PAGE of proteolytic cleavage of chitinases by SAP. Lanes: 1 to 4, digests of 20.5-, 30-, 47-, and 70-kDa chitinases, respectively; 5, SAP; 6, molecular mass (M_m) standards.

DISCUSSION

The five chitinases purified from the extracellular culture fluid of *S. olivaceoviridis* differ in their activities against crystalline and colloidal chitin and CM-chitin but have similar catalytic properties. The product spectrum is in accordance with the finding of Ueno et al. (32) that two immunologically distinct chitinases from the same *Streptomyces* strain form mainly chitobiose. While 47-kDa chitinase is present even in the inductor-free inoculum, the other four enzymes appear in fermentations with chitin as a substrate initially at 92 and 70 kDa and then consecutively at 30 and 20.5 kDa. The amino acid sequences at the amino-terminal end showed no homology. We determined the carboxy-terminal amino acids by cleavage by the method of Klemm (15), with the modification that SDS was diluted to 0.01%. Threonine was the C-terminal amino acid in the two inducible chitinases tested, but no sequence could be determined

TABLE 2. Relative titers of antisera and extents of cross-reactions

Rabbit no.	Antibodies against chitinase	Relative titer[a]	Cross-reactions (%)[b] against chitinase		
			20.5 kDa	30 kDa	47 kDa
1	20.5 kDa	21,833	100	17	3
2	20.5 kDa	67,900	100	4	0
3	30.5 kDa	218,000	0	100	0
4	30.5 kDa	310,000	0	100	0

[a] Dilution at which $A_{414} = 1$ in ELISA.
[b] Antisera were used at the same dilution as given for relative titers.

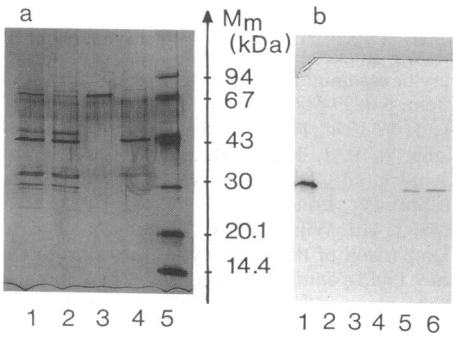

FIG. 3. Fragments of proteolytic digestion of 70-kDa chitinase with *S. olivaceoviridis* 42-kDa protease. (a) Silver stain. Lanes: 1 and 2, digests after incubation for 15 and 60 min, respectively; 3, 70-kDa chitinase; 4, 42-kDa protease; 5, molecular mass (M_m) standards. (b) Immunoblot. Lanes: 1, 30-kDa chitinase; 2, molecular mass standards; 3, 42-kDa protease; 4, 70-kDa chitinase; 5 and 6, digests after incubation for 15 and 60 min, respectively.

because the activity of carboxypeptidase Y against the chitinases was very low.

On proteolytic digestion of the 70-, 47-, 30-, and 20.5-kDa chitinases with SAP, which had earlier been shown to cleave proteins reproducibly (5), fragments of the same molecular masses (17.8, 14.5, and 13.7 kDa) appeared. Because of the very low concentration of 92-kDa chitinase in the extracellular protein, no proteolytic experiments have yet been performed.

A homologous protease of 29 kDa yields fragments of the same molecular masses (20.5, 18.0, and 16.0 kDa) from 30- and 70-kDa chitinases but cleaves the protein chains at other positions as well. Moreover, an immunologically active 30-kDa fragment was produced by a homologous 42-kDa protease when 70-kDa chitinase was the substrate. The identities of 30-kDa chitinase and the 30-kDa fragment were proven (4) by two-dimensional peptide mapping (19) after iodination.

The apparent homology between 20.5-kDa chitinase and 30-kDa chitinase was substantiated by the immunological relationships. Antibodies against 20.5-kDa chitinase could recognize 30-kDa chitinase at least in part. If one assumes that antibodies against 30-kDa chitinase are caused by antigenic determinants on the 10-kDa fragment at the NH_2 end, no cross-reaction with 20.5-kDa chitinase should be observed, as shown in Table 2.

It is thus reasonable to assume that 30-kDa chitinase is derived from 70-kDa chitinase and that 20.5-kDa chitinase is derived from 30-kDa chitinase by proteolytic removal of the amino terminus. While the active site of the chitinases is retained in the 20.5-kDa protein, there is a substantial drop in specific activity compared with that of the chitinases of higher molecular masses which might be due to changes in the binding site.

TABLE 3. Substrate specificity of the purified chitinases

Substrate	Chitinase sp act (mU/mg)				
	20.5 kDa	30 kDa	47 kDa	70 kDa	92 kDa
Chitin	84	472	512	291	150
Colloidal chitin	146	6,344	1,967	1,353	775
CM-chitin	612	8,507	2,942	1,618	1,375

The extent of cross-reactions of 47-kDa chitinase with anti-20.5-kDa chitinase serum is definitely lower than that of 30-kDa chitinase (Table 2). Differences in amino acid sequence between 47- and 30-kDa chitinases are visualized by the fact that SAP cleaves a 32-kDa fragment from the former and a 23.4-kDa fragment from the latter. This finding indicates that there are major changes in the amino acid sequence in the 20- to 30-kDa region from the COOH terminus of 47-kDa chitinase.

From the evidence presented, it may be concluded that two chitinase genes exist in *S. olivaceoviridis*: one for 47-kDa chitinase and another for 20.5-, 30-, 70-, and probably also 92-kDa chitinases, which are derived from a common precursor protein. The active site in all chitinases is located at the 20-kDa region on the COOH terminus, while the NH_2-terminal region may contain sequences involved in a protease recognition or excretion mechanism.

Recently the chitinase genes from *Streptomyces lividans* 66 have been cloned (21). From four clones, four chitinases of different molecular mass were expressed. Two of these chitinases showing strong similarities in enzymatic activity were localized on a common DNA fragment. The assumption was made that the smaller was derived from the larger by proteolytic modification. One of the genes was not expressed in the wild-type strain.

Few examples for the proteolytic modification of polysaccharide-degrading enzymes have been described. Cleavage from the amino terminus was observed with chitinases from *B. circulans* WL-12 (34) and *Bacillus licheniformis* (29) and with amylase from *S. griseus* (9). Processing by cleavage from the carboxyl terminus was only suggested for glucamylase from *Rhizopus* sp. (28) and demonstrated for amylase from *Pseudomonas stutzeri* (22).

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