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Isolation of Extracellular 28- and 42-Kilodalton β-1,3-Glucanases and Comparison of Three β-1,3-Glucanases Produced by *Bacillus circulans* IAM1165

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***Bacillus circulans* IAM1165 produces three major extracellular β-1,3-glucanases (molecular masses, 28, 42, and 91 kDa) during the stationary phase of growth. The 28- and 42-kDa enzymes were purified to homogeneity from the culture supernatant in this study. The properties of these two enzymes were examined, together with those of the 91-kDa enzyme previously isolated. The enzymatic properties of the 28- and 42-kDa β-1,3-glucanases closely resemble each other. The enzymes belong to a category of endo type 1,3-β-D-glucan glucanohydrolases. The enzymes were active at pH 4.0 to 7.0. The optimum temperature of the reactions was 60°C when laminarin (a soluble β-1,3-glucan) was used as the substrate at pH 7.0. The enzymes hydrolyzed barley glucan and lichenan (β-1,3-1,4-glucans) more effectively than laminarin. Of the three enzymes, the 42-kDa enzyme lysed fungal cell walls the most effectively.**

Bacillus circulans IAM1165 was previously isolated because of its ability to lyse fungal mycelia (12–16). This organism is the first microbial isolate producing enzymes that lyse fungal cells. Fungal cell walls are generally constructed of several polysaccharides. It is likely that fungal mycelia are degraded by concerted actions of several enzymes that hydrolyze a variety of polysaccharides. One of the major lytic enzymes excreted by strain IAM1165 has been characterized as an endo type of β-1,3-glucanase (1,3-β-D-glucan glucanohydrolase; EC 3.2.1.39). The molecular mass of this enzyme was estimated as 28 kDa by Sephadex gel chromatography (12). An analysis of the cell walls of *Aspergillus oryzae* with the enzyme revealed that a basal structure in the cell walls is β-1,3-D-glucan (15).

Fungal cell wall-lytic enzymes can provide a means for degrading fungal mycelia and yeast cells. These enzymes might be applied for preparation of protoplasts from fungi or yeasts for studies involving gene manipulation, utilization as biomass resources of yeast cell walls wasted by brewing industries, or control of pathogenic fungi infecting plants or rotting foods during storage.

Many microorganisms are known to produce simultaneously two or more enzymes that hydrolyze a particular insoluble polysaccharide. Multiple β-1,3-glucanase systems are known in many microorganisms (8, 10, 17, 21, 26, 27). The multiple systems may contain not only genetically different isozymes but also enzymatically active small products resulting from one of the enzymes by some proteolytic processing. The lytic enzyme complement of *B. circulans* IAM1165 might contain several enzymes under similar circumstances.

We have reexamined the extracellular β-1,3-glucanases produced by *B. circulans* IAM1165 and isolated an extracellular 91-kDa β-1,3-glucanase (1). The molecular mass of this enzyme was inadequately estimated to be 87 kDa, as described in our previous reports (1, 2, 20, 30). Here, we report that the correct molecular mass is 91 kDa. On the previous isolation of the 91-kDa enzyme, we noticed that the organism produces some extracellular β-1,3-glucanases with distinctive chromatographic behavior (1). The organism has a multiple-β-1,3-glucanase system consisting of at least three major extracellular enzymes with distinct molecular masses (28, 42, and 91 kDa). This diversity of β-1,3-glucanases was supported by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the culture supernatant followed by activity staining (unpublished results). These enzymes are respectively designated β-1,3-glucanases L (low molecular mass [28 kDa]), M (medium molecular mass [42 kDa]), and H (high molecular mass [91 kDa]) in this report to emphasize their different molecular sizes.

Extracellular β-1,3-glucanases would provide a good model of how production of proteins is regulated by environmental factors. We purified β-1,3-glucanases L and M from the culture supernatant. This is the first successful purification of all β-1,3-glucanase species extracellularly produced by a certain strain belonging to the genus *Bacillus*.

In this report, we describe the purification and characterization of β-1,3-glucanases L and M and a comparison of the three enzymes (L, M, and H) produced by the organism.

MATERIALS AND METHODS

Organism and cultivation medium. *B. circulans* IAM1165 was grown in a medium containing 10 g of yeast extract, 5 g of pachyman (an insoluble β-1,3-glucan), and 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter of 0.1 M Na_2HPO_4-KOH buffer (pH 8.0).

Purification of β-1,3-glucanases. (i) Step 1. The organism was grown aerobically in the medium described above at 37°C for 48 h. The culture (3 liters) was centrifuged at 6,000 × *g* for 10 min at 4°C.

(ii) Step 2. Proteins in the supernatant fluid (2,860 ml) were precipitated with 40% saturated $(NH_4)_2SO_4$ at 4°C overnight. The precipitate was recovered by centrifugation (6,000 × *g*, 30 min, 4°C). β-1,3-Glucanase M was obtained mainly in this precipitate. Enzyme L was retained mainly in the supernatant. Proteins that remained in the supernatant solution were precipitated by further addition of $(NH_4)_2SO_4$ to give a final concentration of 80% saturation. The precipitate containing β-1,3-glucanase L was recovered by centrifugation.

(iii) Step 3A. The precipitate [0 to 40% saturated $(NH_4)_2SO_4$] was suspended in 300 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.0). The suspension was stirred at 4°C overnight. Insoluble matter was recovered by centrifugation (10,000 × *g*, 10 min, 4°C). The precipitate was suspended in 100 ml of 1 M HEPES-NaOH (pH 7.0) to dissolve β-1,3-glucanase M. This suspension was centrifuged at 10,000 × *g* for 10 min at

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TABLE 1. Purification of β-1,3-glucanases L and M

Step No.	Treatment	Vol (ml)	Activity (U)	Protein (mg)	Sp act (U/mg)	Recovery (%)[a]
1	Culture fluid	2,860	11,700	358	33	100
2	Precipitation with 0 to 40% saturated $(NH_4)_2SO_4$	300	5,850	226[b]	26	50 (100)
	Precipitation with 40 to 80% saturated $(NH_4)_2SO_4$	181	3,290	167	20	28 (100)
3A	Precipitate washing with low-strength buffer	50	225	0.64[b]	352	2 (4)
3B	1st DEAE chromatography	187	2,950	45.4	65	25 (90)
4	Hydrophobic chromatography	177	1,790	6.0	298	15 (54)
5	2nd DEAE chromatography	40	626	1.2	522	5 (19)

[a] The values in parentheses are recoveries of the 28- or 42-kDa enzyme, shown as percentages of activity after $(NH_4)_2SO_4$ fractionation, which were defined as 100%.
[b] Proteins were measured by the method of Lowry et al. (19). Proteins in the other samples were measured by the Bradford method (4).

4°C. The supernatant was recovered and dialyzed against 2 liters of 10 mM HEPES-NaOH (pH 7.0) overnight.

The β-1,3-glucanase that had been precipitated during the dialysis was recovered by the centrifugation. This precipitate was suspended in 0.01% NaN_3–10 mM HEPES-NaOH (pH 7.0) and kept at 4°C. This suspension was used as a purified preparation of β-1,3-glucanase M. When a high concentration of the enzyme solution was needed, the precipitate was dissolved in 1 M HEPES-NaOH.

(iv) Step 3B. The precipitate [40 to 80% saturated $(NH_4)_2SO_4$] obtained at step 2 was dissolved in 100 ml of 10 mM HEPES-NaOH (pH 7.0) and dialyzed against the same buffer. The nondiffusible matter (181 ml) was loaded onto a column (2.5 by 18 cm) of DEAE-Toyopearl 650M (Seikagaku Kogyo Co., Tokyo, Japan) which had been equilibrated with HEPES-NaOH buffer. The column was washed with 150 ml of the buffer at a flow rate of 15 ml/h. The effluent and wash containing β-1,3-glucanase L were recovered and combined.

(v) Step 4. $(NH_4)_2SO_4$ was added to the effluent plus wash to a final concentration of 25% saturation. The solution was centrifuged at 7,000 × *g* for 15 min at 4°C. The supernatant solution (187 ml) was loaded onto a column (2.5 by 14.5 cm) of Butyl-Toyopearl 650M (Seikagaku Kogyo Co.) equilibrated with the HEPES-NaOH buffer containing 25% saturated $(NH_4)_2SO_4$. The column was washed with 150 ml of the salt buffer at 4°C and then eluted with a decreasing linear gradient of 25 to 0% saturated $(NH_4)_2SO_4$ in 1 liter of HEPES-NaOH buffer at a flow rate of 40 ml/h. Fractions (10 ml) were collected, and the β-1,3-glucanase-positive fractions that eluted at 20% saturated $(NH_4)_2SO_4$ were pooled.

(vi) Step 5. The solution (100 ml) was dialyzed against HEPES-NaOH buffer. Nondiffusible matter was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant solution (177 ml) was loaded onto a column (2.5 by 14 cm) of DEAE-Toyopearl 650M equilibrated with HEPES-NaOH buffer, and the column was washed with 150 ml of the buffer at 4°C. The column was eluted with a linear gradient of 0 to 0.3 M NaCl in 1 liter of HEPES-NaOH buffer at a flow rate of 54 ml/h. Fractions (10 ml) were collected. The β-1,3-glucanase-positive fractions that eluted at 30 mM NaCl were pooled, and the enzyme was salted out with $(NH_4)_2SO_4$ (80% saturation). The precipitate was dissolved in HEPES-NaOH buffer (pH 7.0) containing 0.01% NaN_3. The solution was dialyzed against the same buffer, kept at 4°C, and used as a purified preparation of β-1,3-glucanase L.

Assay for β-1,3-glucanase activity. A 20-μl portion of the enzyme solution was mixed with 80 μl of 5% (wt/vol) laminarin (a soluble β-1,3-glucan) and 100 μl of 0.2 M HEPES-NaOH (pH 7.0) prewarmed to 40°C, unless otherwise stated. The mixture was incubated at 40°C for 10 min. The enzyme was inactivated by adding 1 ml of a dinitrosalicylic acid solution and heating the preparation in a boiling water bath for 5 min (24). Reducing sugars liberated from the laminarin were colorimetrically determined by measurement of A_{510} after addition of 3 ml of water to the mixture. One unit of β-1,3-glucanase activity was defined as the amount of the enzyme that released reducing saccharides equivalent to 1 μmol of glucose per min under these conditions (1).

Assay for fungal cell wall-lytic activity of β-1,3-glucanases. Cell walls (20 mg) prepared from *A. oryzae* were suspended in 10 ml of 0.2 M citric acid-NaOH buffer (pH 6.0) by brief sonication. The cell walls were washed once with the buffer. Each β-1,3-glucanase preparation (1 U/ml) was prepared with the buffer. The enzyme solution (400 μl) was added to 400 μl of the cell wall suspension. This reaction mixture was shaken at 37°C for 2 h and then centrifuged at 20,000 × *g* for 10 min. The amount of glucans solubilized in the supernatant was determined by the phenol-sulfuric acid reagent (3) before and after incubation.

Determination of protein concentration. Protein concentration was determined by the method of Bradford (4), unless otherwise stated. A few samples were assayed by the method of Lowry et al. (19). Bovine serum albumin was used as the standard.

SDS-PAGE of protein. Samples were dissolved in a solution containing 2% (wt/vol) SDS, 2.5% (vol/vol) β-mercaptoethanol, 20% (vol/vol) glycerol, and 16 mM Tris-HCl (pH 6.8) and heated in a boiling water bath for 5 min. The samples were run on an SDS-polyacrylamide gel as described by Laemmli (18). The molecular mass markers used were rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), egg white ovalbumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa), obtained from Bio-Rad Laboratories, Richmond, Calif.

Isoelectric focusing gel electrophoresis of protein. Samples were dissolved in a solution containing 4.8 M urea, 1% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 1% Ampholine (pH 3.5 to 10; Pharmacia LKB, Uppsala, Sweden), and 2.5% (vol/vol) β-mercaptoethanol at room temperature (23). The samples were run on a 4% polyacrylamide gel containing 8 M urea, 2% (vol/vol) Nonidet P-40, and 5% (vol/vol) Ampholine (pH 3.5 to 10). A kit of isoelectric focusing markers were obtained from Pharmacia LKB. After electrophoresis, proteins were fixed with 10% (vol/vol) trichloroacetic acid and 5% (vol/vol) sulfosalicylic acid for 1 h. The gels were washed with 30% (vol/vol) methanol and 10% (vol/vol) acetic acid overnight and stained with Coomassie brilliant blue R-250.

Sequencing of N-terminal amino acids. N-terminal amino acids of the native enzymes were sequenced by automated Edman degradation of the samples with an Applied Biosystems 477A protein-peptide sequencer and a 120A on-line phenylthiohydantoin analyzer.

Materials. Several types of glucan preparations were purchased as follows: laminarin produced by *Laminaria digitata* (Sigma, St. Louis, Mo.), curdlan produced by *Agrobacterium* sp. (Wako Pure Chemicals, Osaka, Japan), paramylon produced by *Euglena gracilis* (Wako Pure Chemicals), lichenan produced by *Getraria islandica* (Sigma), pstulan prepared from lichens (Calbiochem, San Diego, Calif.), and barley glucan (Sigma). Pachyman was prepared from commercial fruiting bodies of the basidiomycete *Poria cocos*. A portion of this pachyman preparation was milled in a mortar and suspended in deionized water to give a concentration of 5% (wt/vol). The suspension was sonicated (40 W, 2 min) in an ice-water bath and then centrifuged at 1,000 × *g* for 1 h at room temperature. The supernatant fluid was recovered and used as a partially degraded pachyman preparation.

The cell wall preparation of *A. oryzae* used was a kind gift from K. Horikoshi (14). Extracellular β-1,3-glucanases A and N of alkaliphilic *Bacillus* sp. strain AG-430 (21, 22) and rabbit antisera against these enzymes were obtained from Y. Nogi.

RESULTS

Purification of β-1,3-glucanases L and M. Production of enzyme H was remarkably suppressed under the culture conditions used. The predominant β-1,3-glucanase species produced in the culture supernatant were enzymes L and M. β-1,3-Glucanase L was soluble in a 40% saturated $(NH_4)_2SO_4$ solution, while enzyme M was insoluble. These two β-1,3-glucanases were roughly separated from each other by fractional precipitation with $(NH_4)_2SO_4$ (Table 1).

Enzyme M salted out at 40% saturated $(NH_4)_2SO_4$ was scarcely soluble in low-concentration buffers. The solubility of the enzyme was below 0.5 U/ml in 10 mM HEPES-NaOH buffer. However, the enzyme was soluble in high-concentration buffers. The solubility was above 100 U/ml in 1 M HEPES-NaOH buffer. Most of the proteins (including small amounts of enzymes L and H) precipitated together with enzyme M were soluble in low-ionic-strength buffers. There were other concomitant impurities (proteins and polysaccharides) that were not soluble even in 1 M HEPES-NaOH buffer. Enzyme

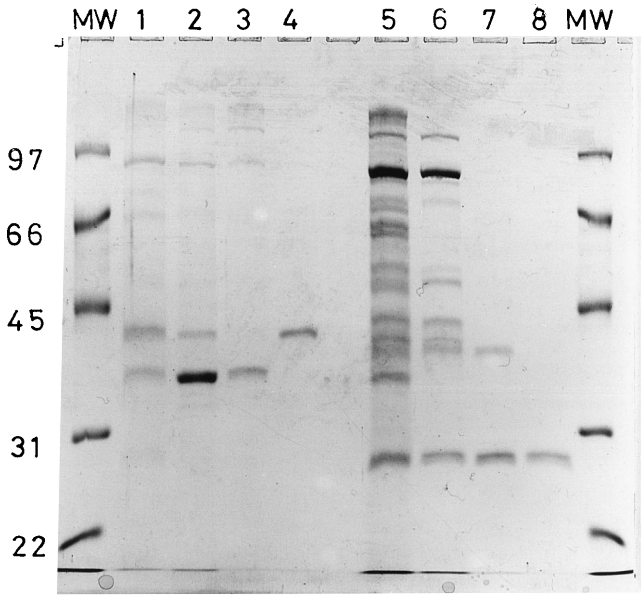

FIG. 1. SDS-PAGE of β-1,3-glucanase samples during purification steps. Samples were electrophoresed on an SDS–10% polyacrylamide gel. Proteins in the gel were stained with Coomassie brilliant blue R-250. Lanes: 1, culture supernatant (0.04 U); 2, proteins precipitated from the culture supernatant with 40% saturated $(NH_4)_2SO_4$; 3, first wash of the 40% $(NH_4)_2SO_4$ precipitate; 4, purified enzyme M precipitated during dialysis against 10 mM buffer (0.03 U); 5, proteins precipitated with 40 to 80% saturated $(NH_4)_2SO_4$ (0.08 U); 6, fraction from the first DEAE-Toyopearl 650M column (0.08 U); 7, fraction from a Butyl-Toyopearl 650M column (0.08 U); 8, purified enzyme L preparation purified by the second DEAE-Toyopearl column (0.08 U). MW, molecular weight markers (10^3).

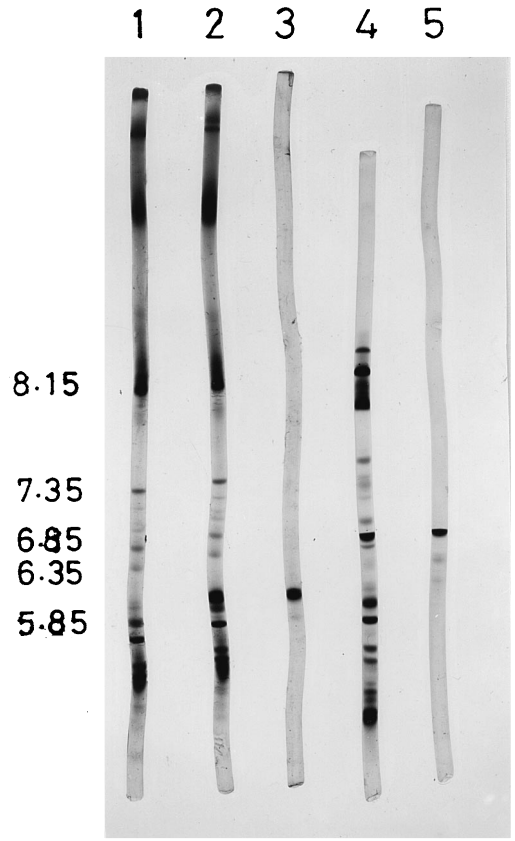

FIG. 2. Isoelectric focusing of purified β-1,3-glucanases L and M. A purified enzyme preparation (0.05 μg) was electrophoresed on 4% polyacrylamide gels containing 5% Ampholine (pH 3.5 to 10), 2% Nonidet P-40, and 8 M urea. Proteins in the gels were stained with Coomassie brilliant blue R-250. Lanes: 1, pI markers (Pharmacia); 2, pI markers plus β-1,3-glucanase L; 3, β-1,3-glucanase L; 4, pI markers plus β-1,3-glucanase M; 5, β-1,3-glucanase M. Lanes 1 to 3 were electrophoresed at 400 V for 12 h and then at 800 V for 1 h. Lanes 4 and 5 were electrophoresed at 400 V for 18 h and then at 800 V for 2 h. The pI markers used were bovine carbonic anhydrase *b* (pI 5.85), human carbonic anhydrase *b* (pI 6.55), horse myoglobin (pIs 6.85 and 7.35), and lentil lectin (pI 8.15).

M was purified as a protein that was highly soluble in 1 M HEPES-NaOH buffer but not in 10 mM HEPES-NaOH buffer (Fig. 1).

Enzyme L precipitated with 80% saturated $(NH_4)_2SO_4$ was soluble in 10 mM HEPES buffer. Enzyme L passed through the column of DEAE-Toyopearl 650M under the conditions used for the first anion-exchange chromatography step. Most of the enzymatic activity was recovered in the effluent and wash. The recovery was 90% of the total activity applied to the column. This step gave 3.3-fold purification of β-1,3-glucanase, as calculated simply on the basis of specific activity measurements (Table 1). Enzyme L itself was probably purified at above 3.3-fold.

Enzyme L adsorbed to Butyl-Toyopearl 650M only at a high concentration of $(NH_4)_2SO_4$ and eluted from the column at 20% saturated $(NH_4)_2SO_4$. This chromatographic behavior of the enzyme indicated that the enzyme was highly hydrophilic. This step gave 4.5-fold purification of the enzyme, although the recovery was relatively low at 60% of the amount that had been applied onto the column. On the second anion-exchange chromatography step, enzyme L was adsorbed with DEAE-Toyopearl 650M, probably because of a decrease in the impurities in the fraction, and eluted at a low concentration of NaCl (30 mM). This step gave a purified preparation of enzyme L (Fig. 1).

The purification procedures are summarized in Fig. 1 and Table 1. During the procedures, it was shown that solubility and chromatographic behavior differed significantly among the three β-1,3-glucanases. The preparations purified in this study (enzymes L and M) and the enzyme H previously purified from the culture supernatant of the organism (1) were used in the experiments described below.

Physicochemical properties of β-1,3-glucanases. Each of the purified enzyme preparations produced a single band on an SDS-polyacrylamide gel, and its molecular mass was estimated to be approximately 28 or 42 kDa (Fig. 1). The pI of enzyme L was about 5.7 as measured by isoelectric focusing on an Ampholine-PAGE plate (pH 3.5 to 9.5; Pharmacia LKB) (results not shown), while the pI of β-1,3-glucanase H is 4.3 (1). Enzyme M was not electrophoresed because of its low solubility at low ionic strength.

The pIs of enzymes L and M, measured in the presence of 8 M urea and 2% Nonidet P-40 (Fig. 2), were 5.7 and 6.6, respectively. The pI of enzyme L agreed with that measured without any detergents as described above. It has been reported that the pI of the 40-kDa β-1,3-glucanase produced by *Escherichia coli* carrying the corresponding gene cloned from *B. circulans* WL-12 is 6.5 (10). The enzyme produced by *E. coli* seems to be soluble even in low-ionic-strength buffer.

Mode of action of β-1,3-glucanases on a soluble β-1,3-glucan. Laminarin was hydrolyzed with each β-1,3-glucanase at 40°C. The enzymic reactions were periodically interrupted by thermal inactivation of the enzymes in the reaction mixtures at 100°C for 10 min. The products were analyzed periodically by silica gel thin-layer chromatography (Fig. 3). All of the en-

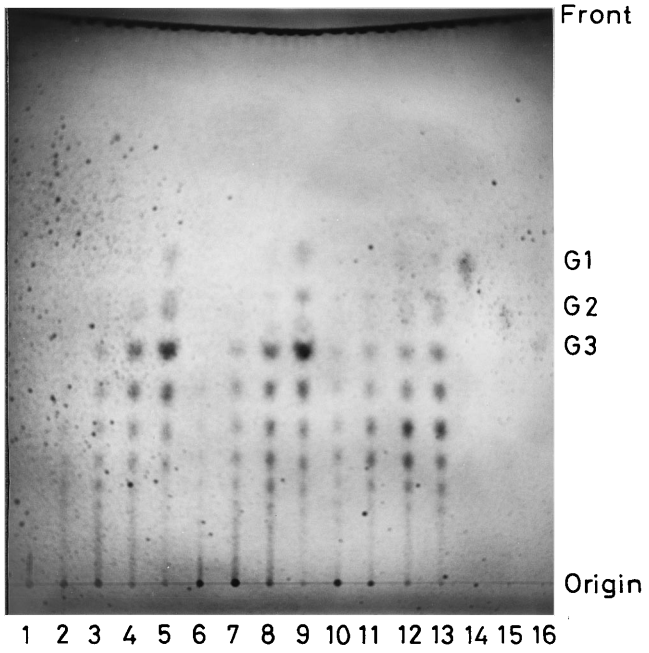

FIG. 3. Thin-layer chromatogram of products obtained from laminarin with β-1,3-glucanases. A solution of each glucanase preparation (0.12 U/40 μl) was mixed with 0.2 ml of 1% laminarin–0.1 M citric acid-NaOH (pH 5.0). After incubation at 40°C, each enzyme was denatured by heating in a boiling water bath for 10 min. Products in the reaction mixtures (0.5 μl) were developed on a thin-layer silica gel in ethyl acetate-acetic acid-water (2:2:1, vol/vol/vol) at room temperature. The sugar compounds liberated from laminarin by the enzymatic hydrolysis were colored by spraying with orcinol-sulfuric acid reagent (5% [wt/vol] orcinol in 50% [wt/vol] sulfuric acid) and then heating with hot air (5). Lanes: 1, laminarin before digestion; 2 to 5, laminarin digestions with enzyme L; 6 to 9, digestions with enzyme M; 10 to 13, digestions with enzyme H. Hydrolysis was carried out for 15 (lanes 2, 6, and 10), 30 (lanes 3, 7, and 11), 60 (lanes 4, 8, and 12), or 120 (lanes 5, 9, and 13) min. The standards used were glucose (lane 14, G1), laminaribiose (lane 15, G2), and laminaritriose (lane 16, G3).

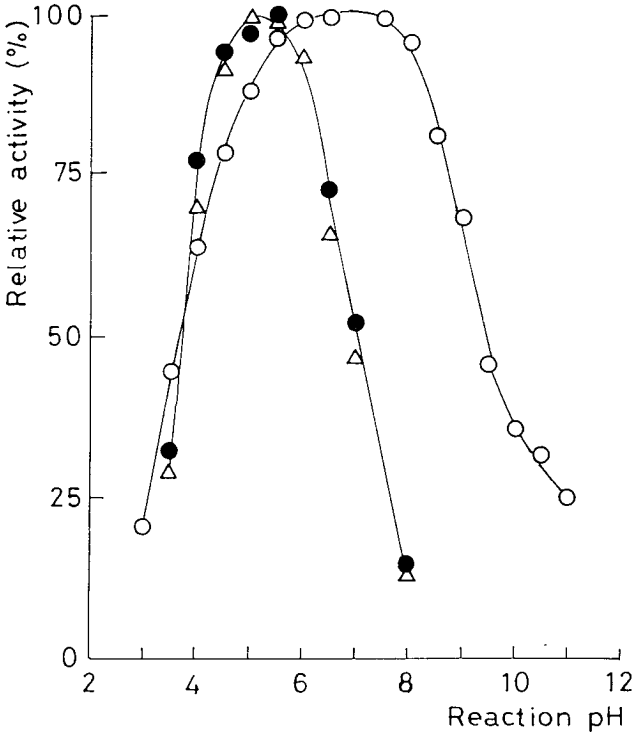

FIG. 4. Effect of reaction pH on the activities of β-1,3-glucanases. pHs were adjusted with the following buffer systems: 0.1 M citric acid-NaH_2PO_4 (pHs 3.0 to 3.5), 0.1 M citric acid-NaOH (pHs 3.5 to 6.0), 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH (pHs 6.0 to 7.0), 0.1 M HEPES-NaOH (pHs 6.5 to 8.2), 0.1 M *n*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)–NaOH (pHs 7.7 to 9.5), and 0.1 M monoethanolamine-HCl (pHs 9.5 to 11.0). Activity was measured at 40°C for 10 min. The level of activity of each enzyme at its optimum pH was defined as 100%. Symbols: ●, enzyme L; ▲, enzyme M; ○, enzyme H.

zymes hydrolyzed laminarin at random, and the products were glucose, laminaribiose, laminaritriose, laminaritetraose, and higher laminarioligosaccharides. Enzymes L and M gave the same hydrolytic products from laminarin. A major saccharide produced with the enzymes was laminaritriose. A major product obtained with enzyme H under the conditions used was laminaripentaose. Oligosaccharides higher than the pentasaccharide were major products at the beginning of all of the reactions. Lower oligosaccharides were released from laminarin or laminarioligosaccharides after the prolonged hydrolytic reaction processes. These results indicated that both of the glucanases were endo-β-1,3-glucanases that randomly cleaved laminarin as a substrate.

Each of the enzymes (0.12 U) was reacted on 600 μg of laminarioligosaccharides (di- to pentasaccharides). All of the enzymes hydrolyzed laminaritetraose and laminaripentaose (results not shown). The enzymes did not show glucosyl transference. Previously, the 28-kDa enzyme was reported to hydrolyze laminaritriose (16). However, laminaritriose was hardly hydrolyzed by any of the enzymes. Small amounts of laminaribiose and glucose were produced after prolonged incubation with large amounts of the enzymes. Laminaribiose was not hydrolyzed by any of the enzymes.

Effect of pH on β-1,3-glucanase activity. The β-1,3-glucanase activities of the enzyme preparations were measured at various pHs with laminarin as the substrate (Fig. 4). The reaction pHs were adjusted to 3.0 to 11.0 with a variety of buffers. The other experimental conditions were the same as those described for the standard assay.

Enzymes L and M were active over a relatively broad pH range. The pH-enzyme activity profiles of these enzymes were similar. The pH optima for these enzyme reactions were 5.0 to 5.5. The enzyme activity levels found between pHs 4.0 and 7.0 were more than half of the activity level at the optimum pH. On the other hand, β-1,3-glucanase H exhibits activity at pH 4.0 to 9.0 and its optimum pH range is 6.0 to 7.5 (1).

Optimum temperature of the enzyme reaction and thermal stability of the enzymes. The optimum temperature of the enzyme reaction was determined by varying the incubation temperature at pH 7.0 (Fig. 5). The temperature-enzyme activity profiles of enzymes L and M were similar. The two enzymes were most active at 60°C. The levels of activity at 60°C were approximately 2.1-fold (enzyme L) and 2.6-fold (enzyme M) higher than those at 40°C. The standard assay conditions used in this study included a temperature of 40°C. The optimum temperature of β-1,3-glucanase H is 70°C (1).

The enzyme preparations were mixed in 0.2 M citrate buffer (pH 6.0) containing 10 mM $CaCl_2$ or EDTA. After the enzymes were heated at various temperatures for 10 min, the residual activity was measured. Enzyme L was inactivated at temperatures above 50°C in the absence of the substrate and Ca^{2+} (Fig. 6A). The enzyme was relatively stable at 55°C in the presence of 10 mM $CaCl_2$. β-1,3-Glucanase M showed similar Ca^{2+}-dependent stability (results not shown). Laminarin or Ca^{2+} stabilized enzymes L and M. Enzyme H is unstable at

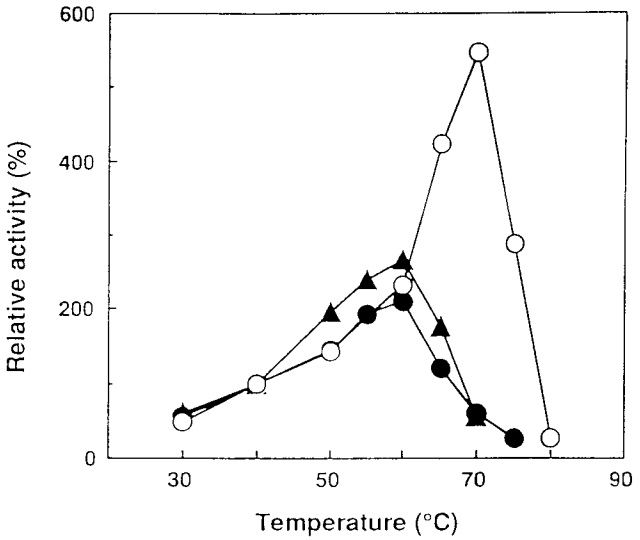

FIG. 5. Effect of reaction temperature on the activities of β-1,3-glucanases. The enzyme reaction was carried out in 0.1 M phosphate buffer (pH 7.0) for 10 min at a concentration of 0.05 to 0.30 U of enzyme L (●), M (▲), or H (○) per ml. The level of activity of each enzyme at 40°C (under standard assay conditions) was defined as 100%.

temperatures above 60°C in the absence of the substrate (1). The thermal stability of this enzyme was improved with laminarin but not influenced by addition of 10 mM $CaCl_2$ or EDTA (Fig. 6B).

Substrate specificities of β-1,3-glucanases on various glucans. The substrate specificities of the three enzymes were examined by using several β-glucans (Table 2). Enzymes L and M hydrolyzed β-1,3-1,4-glucans (barley glucan and lichenan) more effectively than laminarin. Enzymes L and M were β-1,3-1,4-glucan-degrading enzymes rather than β-1,3-glucan-degrading enzymes, although a glucosyl linkage cleaved with the enzymes has not been examined. Enzyme H was poorly reactive toward the β-1,3-1,4-glucans.

The reactivity of the three enzymes toward β-1,3-glucans was dependent on the polysaccharide preparations. The three enzymes reacted preferably on laminarin. Enzyme H had a low level of activity on the other insoluble β-1,3-glucans. Enzymes L and M showed relatively broad substrate specificity for β-1,3-glucans. These two enzymes hydrolyzed curdlan considerably. Enzyme L was active toward glucan with a low degree of polymerization prepared from pachyman by partial degradation but had a low level of activity toward pachyman with a high degree of polymerization. This pachyman preparation showed that the ratio of glucose residues, determined with the phenol-sulfuric acid reagent (3), to the reducing terminal, determined with the dinitrosalicylic acid reagent (24), was 250:1, whereas that of degraded pachyman was 36:1. A number average degree of polymerization of pachyman of 690 has been reported (11). Paramylon, a reserve β-1,3-glucan of *Euglena* sp., was not hydrolyzed by any of the β-1,3-glucanases. These results indicated that the reactivity of the three enzymes was dependent on not only a local linkage between glucose residues but also the overall conformation of the polysaccharide preparations.

None of the enzymes hydrolyzed other types of β-glucans, such as soluble carboxymethyl cellulose (β-1,4-glucan) and pstulan (β-1,6-glucan), or α-glucans, such as soluble starch (α-1,4-glucan) and dextran (α-1,6-glucan) (results not shown).

Fungal cell walls derived from *A. oryzae* were degraded by each β-1,3-glucanase. β-1,3-Glucanase M was the most active

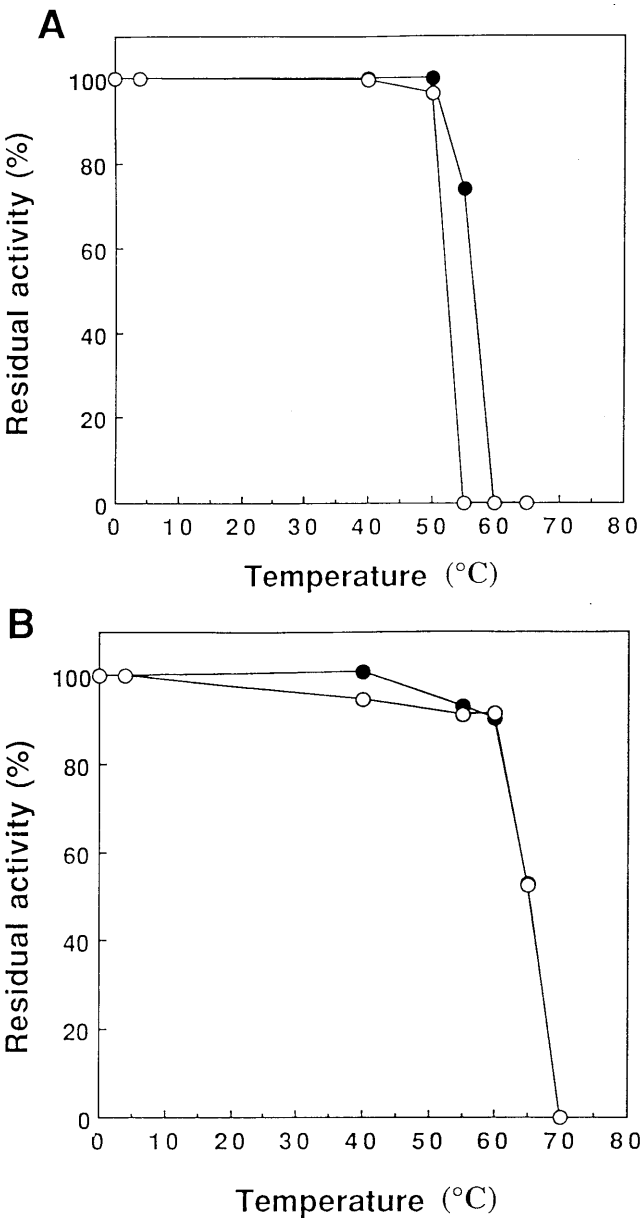

FIG. 6. Thermal stability of β-1,3-glucanases. Enzymes L (A) and H (B) were dissolved in 0.1 M citric acid-NaOH buffer (pH 6.0) containing 10 mM EDTA (○) or $CaCl_2$ (●). The enzyme solutions were heated at the temperatures indicated for 10 min. Residual activity was assayed at 40°C. The residual activity of each enzyme stored in the buffer containing 10 mM $CaCl_2$ at 0°C was defined as 100%.

lytic enzyme of the three. About 15% of the cell walls was solubilized under the conditions used. Enzymes L and M solubilized 6 and 9% of the cell walls, respectively. The fungal cell wall-lytic activity of enzyme M was much higher than that of enzyme L, which was previously purified as the lytic enzyme (12, 16). Enzyme L showed the lowest level of lytic activity of the three.

Influence of various enzyme-inhibitory reagents on the activity of β-1,3-glucanases. β-1,3-Glucanase activity was measured under standard assay conditions in the presence of various 1 mM metal ions (Table 3). Among the ions tested, only Hg^{2+} partially inhibited the enzymic activity. β-1,3-Glucanase

TABLE 2. Relative activities of β-1,3-glucanases on a variety of β-glucans

Substrate[a]	Main linkage	Relative activity of β-1,3-glucanase[b]:		
		L	M	H
Laminarin	β-1,3	100	100	100
Curdlan	β-1,3	95	79	36
Degraded pachyman[c]	β-1,3	70	28	15
Pachyman	β-1,3	32	20	14
Paramylon	β-1,3	<5	<5	<5
Barley glucan	β-1,3-1,4	173	160	5
Lichenan	β-1,3-1,4	147	123	10
Carboxymethyl cellulose	β-1,4	<5	<5	<5
Pstulan	β-1,6	<5	<5	<5

[a] Glucans were hydrolyzed with each enzyme (about 0.2 U/ml) in 0.1 M citric acid-NaOH buffer at 37°C. Soluble glucans (laminarin and carboxymethyl cellulose) were reacted for 10 min, and insoluble glucans were reacted with continuous shaking of the reaction mixtures for 30 min. Other experimental conditions were the same as those described in Materials and Methods.

[b] After the reactions, reducing saccharides released by enzymic hydrolysis were determined. The amounts of reducing saccharides released per minute are shown as percentages of that measured for laminarin, which was defined as 100%.

[c] Pachyman prepared from fruiting bodies of *P. cocos* was partially degraded by sonication.

L was the most sensitive to Hg^{2+}. Enzyme H was relatively tolerant to Hg^{2+}.

N-Bromosuccinimide completely inhibited the activity of every enzyme, indicating that tryptophan residues are involved in the catalytic sites or glucan recognition sites. A tryptophan residue has been found near the catalytic site of enzyme H (30). Monoiodoacetic acid or EDTA did not inhibit the activity, suggesting that a cysteine residue or metal ion is not involved in the activity of these three enzymes. In fact, enzyme H possesses no cysteine residue (30).

N-terminal amino acid sequences of β-1,3-glucanases. The N-terminal amino acids of enzyme H have been sequenced (30), as shown in Fig. 7. The N-terminal amino acid sequences of enzymes L and M were sequenced in this study (Fig. 7). These two sequences strongly resemble each other but not that

TABLE 3. Influence of various reagents on the activity of β-1,3-glucanases

Reagent[a]	Relative activity of β-1,3-glucanase[b]:		
	L	M	H
$PbCl_2$	98	93	107
$MnCl_2$	119	112	117
$MgSO_4$	103	89	100
$ZnSO_4$	104	102	108
$HgCl_2$	43	62	90
$CaCl_2$	100	94	103
$FeSO_4$	100	89	91
$FeCl_3$	111	96	97
$CuSO_4$	85	88	95
$CuCl_3$	84	80	95
$CoSO_4$	93	94	80
NBS[c]	0	0	0
MIA[d]	104	97	107
EDTA	99	88	92

[a] All reagents were used at 1 mM.

[b] Activity of the three β-1,3-glucanase was assayed under standard conditions in the presence of the reagents shown. Activity is shown as a percentage of that measured without any reagent, which was defined as 100%.

[c] NBS, *N*-bromosuccinimide.

[d] MIA, monoiodoacetic acid.

Origin	Molecular mass		
IAM1165	28	1	***W****X*W****X**X*N---
IAM1165	42	1	APNXNLVWSDEFNGTXLNNA---
IAM1165	91	1	*ETAGTTITSMSYFSTADGFG---
		385	IEGM**I*Q******A*DQS---
WL-12	40	1	*T*W**********S---
WL-12	68	1	XXPEVTSMEYFCPAD---
WL-12	72	1	*GTTVTSMEYFSPADGPVI---
AG-430	26	1	***WS*********NS**PANWT---
WL-12 (β-1,3-1,4)	40	1	***KPFPQHTTYTSGSIKPNHVT---

FIG. 7. N-terminal amino acid sequences of mature β-1,3-glucanases. The sequences of β-1,3-glucanases L, M, and H (30) from *B. circulans* IAM1165, *B. circulans* WL-12 (10, 29), and alkaliphilic *Bacillus* sp. strain AG-430 (21) and that of β-1,3-1,4-glucanase from *B. circulans* WL-12 (6) are compared. The sequence of enzyme H (positions 385 to 404) is also shown. Amino acid residues identical to those of β-1,3-glucanase M are represented by asterisks in each sequence.

of enzyme H. The sequences of enzymes L and M were highly similar to those of the 40-kDa β-1,3-glucanase of *B. circulans* WL-12 (10) and the 26-kDa enzyme of alkaliphilic *Bacillus* sp. strain AG-430 (22). A gene encoding the 40-kDa β-1,3-1,4-glucanase has been cloned from strain WL-12 (6). The N-terminal amino acid sequence of β-1,3-glucanase L or M was not homologous to that of the 40-kDa β-1,3-1,4-glucanase.

A gene (*bglH*) encoding β-1,3-glucanase H has already been cloned in *E. coli* (2). The amino acid sequence of the enzyme has been deduced from the nucleotide sequence of the cloned gene (30). The amino acid sequences (positions 5 to 17) of enzymes L and M are highly conservative with respect to that of enzyme H (positions 389 to 401) (Fig. 7). However, the overall N-terminal amino acid sequence of enzyme L and M did not correspond to any portion of the amino acid sequence of enzyme H (30). This indicates that neither enzyme L nor M was generated by proteolytic digestion of enzyme H. We concluded that enzymes L and M are encoded by a gene(s) other than *bglH*.

Immunological differentiation of β-1,3-glucanases. The β-1,3-glucanases of *B. circulans* IAM1165 were made to cross-react by the dot method with antisera against two β-1,3-glucanase preparations derived from *Bacillus* sp. strain AG-430 (Fig. 8). The antiserum against enzyme A reacted with enzyme

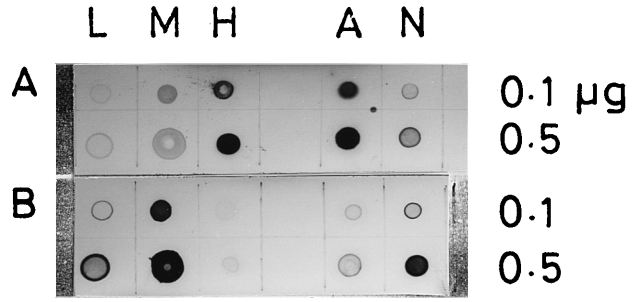

FIG. 8. Immunological differentiation of β-1,3-glucanases. Enzyme preparations (0.1 or 0.5 μg) from *B. circulans* IAM1165 (L, M, and H) and *Bacillus* sp. strain AG-430 (A and N) were spotted on two nitrocellulose filter sheets. One of the sheets was reacted with antiserum against the β-1,3-glucanase A of strain AG-430 (A), and the other was reacted with antiserum against the enzyme N derived from strain AG-430 (B). The proteins which cross-reacted with the β-1,3-glucanases of strain AG-430 were detected with goat anti-rabbit horseradish peroxidase conjugate obtained from a commercial source.

H. However, this antiserum did not react with enzyme L or M of strain IAM1165 or enzyme N of strain AG-430. Therefore, enzymes L and M are immunologically different from enzyme H. Meanwhile, the antiserum prepared against enzyme N of strain AG-430 reacted with enzyme M but not enzyme L or H. This result supports the conclusion that enzymes M and H do not immunologically cross-react. Thus, enzyme M was homologous to enzyme N of strain AG-430. Furthermore, the findings indicated that enzymes L and M are immunologically distinguishable from each other.

DISCUSSION

Relationships among molecular species of multiple diverse β-1,3-glucanases produced by *Bacillus* spp. are implicated mainly because the available information on the enzymes is poor and incomplete. *B. circulans* WL-12, which belongs to the same species as strain IAM1165, has been reported to produce up to six molecular species of extracellular β-1,3-glucanases with different sizes (9, 10, 25). Induction patterns of the enzymes are variable in response to the structures of the glucans used as growth substrates (25). Some of the enzymes are probably proteolytic products processed from certain authentic mature enzymes. Therefore, the organism may have β-1,3-glucanases with similar characteristics.

To understand the diversity of the multiple enzymes, it is important to obtain systematic information on each enzyme as follows: (i) enzymatic and biochemical properties of the individual extracellular β-1,3-glucanase purified from a culture of the producer microorganism, (ii) the N-terminal amino acid sequence of the enzyme, (iii) the nucleotide sequence of the gene encoding the enzyme, and (iv) coincident properties of the original enzyme purified from the organism used as a DNA donor and the cloned enzyme purified from cells harboring the gene.

It is likely that several β-1,3-glucanase genes have been cloned to circumvent the difficulties of purifying individual β-1,3-glucanases from a culture supernatant containing multiple enzymes. There are few β-1,3-glucanases that satisfy the ideal criteria described above. Such incomplete information has historically resulted from attempts to clone the genes. This strategy has led to a dilemma since the biological nature of the cloned enzyme in the DNA donor was not always clear.

Two genes coding for β-1,3-glucanases of *B. circulans* WL-12 have been cloned in *E. coli* (10). These genes encode enzymes immunologically unrelated to each other. Therefore, it is now known that this organism has at least two genes coding for β-1,3-glucanases. These two genes likely give 40 (βGl B1)- and 72 (βGl A1)-kDa extracellular enzymes as authentic mature products. The other minor β-1,3-glucanases of strain WL-12 might be enzymatically active fragments derived from the two authentic enzymes by proteolytic processing. It has been suggested that limited proteolysis of βGl A1 yields three enzymatically active fragments with a variety of molecular sizes (28, 29). From strain WL-12, another gene (*bgc*) encoding β-1,3-1,4-glucanase has been cloned (6, 7). The *bgc* product hydrolyzes β-1,3-1,4-glucan but not β-1,3- or β-1,4-glucan (7). This substrate specificity indicates that the *bgc*-encoded enzyme differs from enzyme L or M. The N-terminal sequence of putative the mature *bgc*-encoded enzyme is not homologous to those of the 40-, 68-, and 72-kDa β-1,3-glucanases of *B. circulans* WL-12.

Strain IAM1165 contains different-size enzymes, and the amounts of the enzymes vary in response to the cultivation conditions. We have purified all species of the major enzymes produced by the organism. The physicochemical properties of these three enzymes were distinctive (Fig. 1 and 2 and Table 1). They can be divided into two clearly different groups on the basis of enzymatic properties (optimum pH, optimum temperature, and Ca^{2+}-dependent thermal stability) (Fig. 4 to 6). One group consists of enzymes L and M, and the other contains only enzyme H. Of the β-1,3-glucanases isolated from strain IAM1165, enzyme M was the most reactive on fungal cell walls.

Enzymes L and M have highly similar enzymatic properties. Additionally, the 20 N-terminal amino acids of enzymes L and M are almost the same (Fig. 7). These sequences are not identical to the N-terminal sequence of enzyme H or βG1 A1. However, the sequences are somewhat homologous to a portion of the enzyme H sequence (Fig. 7), although the biological significance of the homologous sequences is unclear. Enzymes L, M, and H are immunologically unrelated one another (Fig. 8). Enzymes M and H cross-reacted with enzymes N and A of strain AG-430, respectively. Enzyme L did not cross-react with enzyme A or N. This indicates that enzyme L differs structurally from enzyme M. Two possible differences between enzymes L and M can be considered. (i) One possibility is that enzymes L and M are isozymes encoded separately on individual genes. The homologous N termini of the enzymes might be a conservative domain possessing some function essential for the activity of both of the enzymes. The presence of a homologous sequence in enzyme H (Fig. 7) supports this possibility. (ii) The other is that enzyme L is a product generated from enzyme M by removal of its 14-kDa C-terminal region. The product (enzyme L) might not have strong immunological reactivity for the antiserum to enzyme M.

The results described above indicate that *B. circulans* IAM1165 produces at least three molecular species of extracellular β-1,3-glucanases. Enzyme H is an isozyme distinguishable from enzymes L and M. However, it is still hard to reach a conclusion about the relationship between enzymes L and M. The characteristics of the enzyme L purified in this study are almost the same as those of the 28-kDa enzyme previously described by Horikoshi (12). Further examination of the amino acid sequences of these enzymes is needed to conclude whether these enzymes originated from one gene or two distinct genes. Additional work to determine the sequences of the genes involved in β-1,3-glucanase production is under way.

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