APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 177–184
0099-2240/89/010177-08$02.00/0

Vol. 55, No. 1

Purification and Characterization of an Endo-(1,3)-β-D-Glucanase from *Trichoderma longibrachiatum*

B. TANGARONE,† J. C. ROYER,‡ AND J. P. NAKAS*

Department of Environmental and Forest Biology, College of Environmental Science and Forestry, State University of New York, Syracuse, New York 13210

Received 10 August 1988/Accepted 26 October 1988

A laminarinase [endo-(1,3)-β-D-glucanase] has been purified from *Trichoderma longibrachiatum* cultivated with D-glucose as the growth substrate. The enzyme was found to hydrolyze laminarin to oligosaccharides varying in size from glucose to pentaose and to lesser amounts of larger oligosaccharides. The enzyme was unable to cleave laminaribiose but hydrolyzed triose to laminaribiose and glucose. The enzyme cleaved laminaritetraose, yielding laminaritriose, laminaribiose, and glucose, and similarly cleaved laminaripentaose, yielding laminaritetraose, laminaritriose, laminaribiose, and glucose. The enzyme cleaved only glucans containing β-1,3 linkages. The pH and temperature optima were 4.8 and 55°C, respectively. Stability in the absence of a substrate was observed at temperatures up to 50°C and at pH values between 4.9 and 9.3. The molecular mass was determined to be 70 kilodaltons by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis, and the pI was 7.2. Enzyme activity was significantly inhibited in the presence of $HgCl_2$, $MnCl_2$, $KMnO_4$, and *N*-bromosuccinimide. The K_m of the enzyme on laminarin was 0.0016%, and the V_{max} on laminarin was 3,170 μmol of glucose equivalents per mg of the pure enzyme per min.

Laminarinase (EC 3.2.1.6) are enzymes capable of hydrolyzing β-1,3-glucans. They have been reported in a great variety of organisms, including bacteria (29), actinomycetes (14), yeasts (9), filamentous fungi (40), mollusks (32), and higher plants (2, 26, 35). Laminarinases are divided into two hydrolytic classes on the basis of their mode of hydrolysis. Endo-type enzymes (EC 3.2.1.6) generally cleave internally and yield laminarin oligosaccharides, while exo-type enzymes (EC 3.2.1.56) generally release glucose.

β-1,3-Glucanases have been utilized for elucidation of fungal cell wall structure (17, 36) and β-1,3-glucan structure (17, 30) and have been useful in discerning taxonomic and evolutionary relationships among yeasts (15). They have been used indirectly as biocontrol agents against plant-pathogenic fungi through the use of soil amendments that lead to enhancement of β-1,3-glucanase- and chitinase-producing microorganisms (21, 22) and are important in the brewing industry (19).

In nature, β-1,3-glucanases play a role in fungal cell lysis (28, 31, 36), parasitism of fungi by other fungi (10), degradation of marine algae (3), seed germination (33), and various yeast life cycle processes requiring cell wall hydrolysis (4, 6).

In previous research (34), the crude enzyme generated by *Trichoderma longibrachiatum* cultured on *Agaricus bisporus* fruiting bodies was characterized. In the present study, *T. longibrachiatum* was grown on D-glucose in order to limit the production of repressible extracellular enzymes. A specific, endo-type β-1,3 glucanase was purified and characterized with respect to several physiochemical and enzymatic properties.

* Corresponding author.
† Present address: Genetics Institute, Andover, MA 01810.
‡ Present address: Center for Plant Biotechnology and Department of Botany, University of Toronto, Erindale Campus, Mississauga, Ontario, Canada L5L 1C6.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin, soluble laminarin (from *Laminaria saccharina*), xylan (from oat spelts), lichenan (obtained from *Usnea barbata*), dextran and pullulan (obtained from *Aureobasidium pullulans*), and laminaribiose were obtained from Sigma Chemical Co., St. Louis, Mo. β-1,3-Xylan (from *Penicillus dumetosus*) was obtained from J. P. Utille, Center de Recherches sur les Macromolecules Vegetales, Cedex, France. Pustulan (from *Umbilicaria papullosa*) and pachyman (from *Wolfiporia cocos*) were obtained from Calbiochem-Behring, La Jolla, Calif., soluble starch was from Difco Laboratories, Detroit, Mich., and carboxymethyl cellulose (degree of substitution, 0.4 to 0.5) was from Hercules Inc., Wilmington, Del. Laminarin oligosaccharides were supplied by Elwin Reese of the U.S. Army Troop Support Command, Natick Research, Development and Engineering Center, Natick, Mass. All other chemicals used were of reagent grade.

Culture. *T. longibrachiatum* Rifai, an unnumbered wild-type strain, was supplied by C. J. K. Wang of the State University of New York College of Environmental Science and Forestry, Syracuse, N.Y. The culture was maintained on petri plates containing a medium supplemented with basal salts as described previously (34) and 2% lactose and was transferred monthly.

Medium and culture conditions. The basal medium contained 2% glucose and salts (34) and was adjusted to pH 5.0. All inoculations were performed with spores at a final concentration of 5×10^4/ml of culture medium (J. R. Royer and J. P. Nakas, Enzyme Microb. Technol., in press). The fungus was grown in a 2-liter Multigen fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) at 28°C for 4 days; it was agitated at 200 rpm at a working volume of 1.5 liters. The pH was maintained at 6.5 by using a pH controller (pH monitor, controller, and recorder, model 5650; Horizon Ecology Co., Chicago, Ill.), antifoam was added as required, and the fermentor was sparged with filter-sterilized air (1.5 liters/min).

Enzyme assay. Enzyme was incubated with laminarin (250

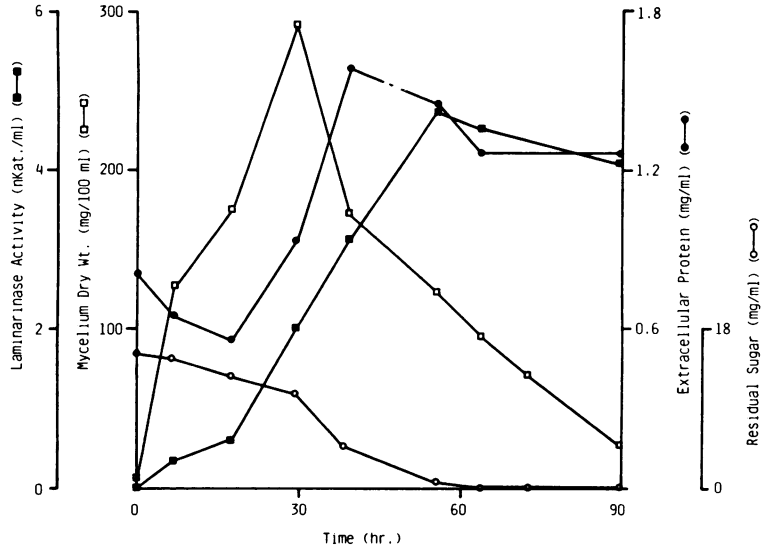

FIG. 1. Time course of laminarinase production by *T. longibrachiatum*. A spore-inoculated culture was grown on 2.0% glucose in a 2-liter fermentor as described in Materials and Methods.

μg) in 1 ml of sodium acetate (50 mM, pH 5.0) for 10 min at 50°C. The quantity of enzyme was adjusted to generate between 20 and 120 μg of reducing sugar in the assay. The reaction was terminated by boiling for 2 min, and reducing sugar liberated was detected by the Somogyi procedure (37), with glucose as a standard.

Activity was expressed in nanokatals (nanomoles of glucose released per second). Protein was estimated by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Isolation of laminarin oligosaccharides. Mixtures of laminarin oligosaccharides were resolved by gel filtration on a column (5.0 by 100 cm) of Bio-Gel P-2 (Bio-Rad). Water was used as the eluate, and the column was maintained at 50°C by using a Lauda K-2/R circulating water bath (Brinkmann Instruments, Inc., Westbury, N.Y.). The column flow rate was 30 ml/h; 3-ml fractions were collected.

Oligosaccharides were identified by high-performance liquid chromatography (HPLC) by using a Bio-Rad HPX42A column maintained at 85°C and a Series 10 liquid chromatograph and Series 10 LC controller (The Perkin-Elmer Corp., Norwalk, Conn.). Detection was by A_{192} by using a Perkin-Elmer LC spectrophotometric detector. Distilled water was used as the eluate at a flow rate of 0.5 ml/min.

Enzyme purification and ethanol precipitation. The crude broth used in the ethanol precipitation step was the supernatant obtained following centrifugation (8,000 × *g*, 15 min) of the culture fluid. Ethanol fractionation of 900 ml of the culture supernatant was performed at −25°C by the method of Kaufman (11). An equal volume of cold ethanol (−20°C) was added, and the mixture was centrifuged at 8,000 × *g* for 15 min. An additional 900 ml of ethanol (−20°C) was then added to the supernatant, mixed for 15 min, and centrifuged (8,000 × *g*, 15 min). The resulting pellet was dissolved in sodium acetate buffer (50 mM, pH 5.0).

Gel filtration. A portion of the redissolved ethanol precipitate was applied to a Bio-Rad Econocolumn (100 by 1.5 cm) packed with Bio-Gel P-200 (Bio-Rad) and was eluted with

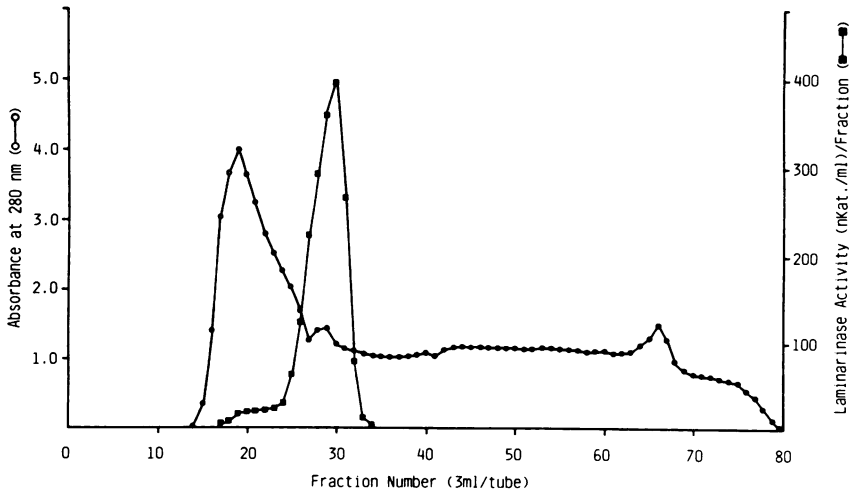

FIG. 2. Gel filtration of ethanol-precipitated laminarinase on Bio-Gel P-200. Fractions (3 ml each) were assayed for protein (A_{280}) and laminarinase activity.

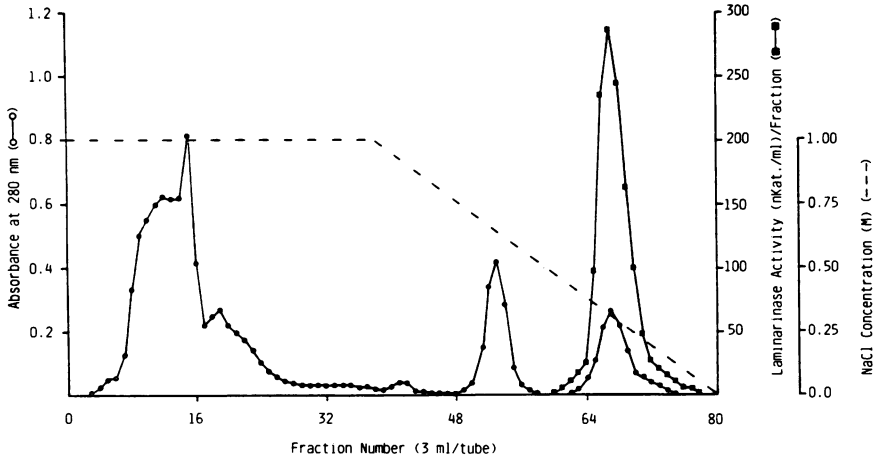

FIG. 3. Hydrophobic-interaction chromatography of laminarinase on phenyl-Sepharose after gel-filtration column chromatography. Fractions (3 ml each) were assayed for protein (A_{280}) and laminarinase activity.

sodium acetate (100 mM, pH 5.5) at a rate of 21 ml/h. Fractions (3 ml each) were collected and analyzed for laminarinase activity as described previously, and protein was determined by A_{280}. Active fractions were pooled for a total volume of 24 ml.

Hydrophobic-interaction chromatography. A portion of the pooled gel-filtration fraction was adjusted to 1 M NaCl and applied to an Econocolumn (10 by 2.5 cm) packed with phenyl-Sepharose (Pharmacia, Uppsala, Sweden). The column was eluted with 1 M NaCl in Tris (50 mM, pH 7.0) followed by elution with a linear gradient of 1.0 to 0 M NaCl in Tris buffer. The flow rate was 24 ml/h, and 3-ml fractions were collected. Active fractions were pooled for a total volume of 27 ml.

Ion-exchange chromatography. A portion of the pooled fractions from hydrophobic-interaction chromatography was dialyzed in sodium acetate (15 mM, pH 5.0) and applied to an Econocolumn (2.5 by 10 cm) packed with carboxymethyl Bio-Gel (Bio-Rad). After elution with start buffer (15 mM sodium acetate, pH 5.0), a linear gradient of NaCl (0 to 300 mM) was applied. The flow rate was 24 ml/h, and 3-ml fractions were collected and analyzed for laminarinase activity and protein content.

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run in an SE 600 vertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) by the method of Laemmli (16) by using 12.5% polyacrylamide. Bio-Rad low-molecular-mass gel electrophoresis protein standards were used for molecular mass determination. Protein was visualized by silver staining (23).

Michaelis-Menten kinetics. Laminarinase assays were performed by using a range of 0.002 to 0.1% (wt/vol) laminarin in a 10-ml volume of sodium acetate (50 mM, pH 5.0). Assays were performed at 50°C by using an enzyme concentration of 80 pg/ml of the assay mixture. One-milliliter portions were removed at 2-min intervals for a total of 10 min. The rate of product formation (micromoles of reducing sugar per minute) was determined by linear regression. Michaelis-Menten constants were determined by using a Lineweaver-Burk plot.

pH optimum and stability. The effect of pH on laminarinase activity was measured over a range of 3.0 to 7.8 by using a Na_2PO_4-citric acid buffer system (20) under standard enzyme assay conditions.

The effect of pH on laminarinase stability was determined by using the McIlvaine buffer system (20) in the pH range of 2.5 to 9.0 and by using the glycerine-sodium hydroxide buffer system (38) in the pH range of 9.0 to 10.2. After incubation of the enzyme (80 pg in 200 μl of the various

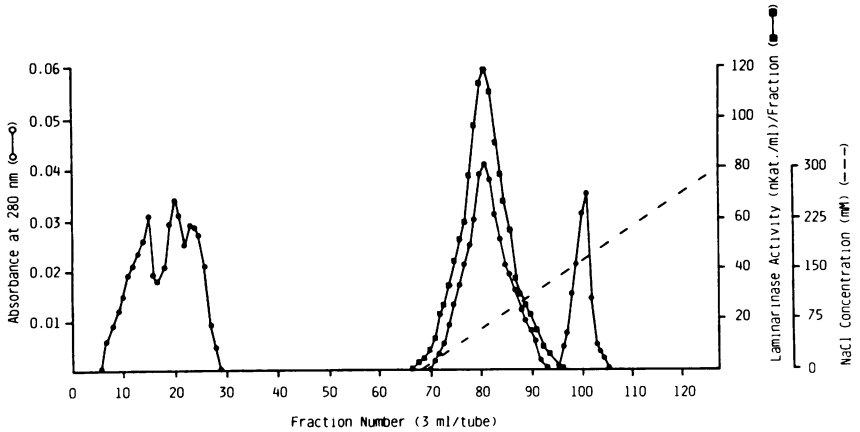

FIG. 4. Ion-exchange chromatography of laminarinase with Bio-Rad carboxymethyl Bio-Gel after hydrophobic-interaction chromatography. Fractions (3 ml each) were assayed for protein (A_{280}) and laminarinase activity.

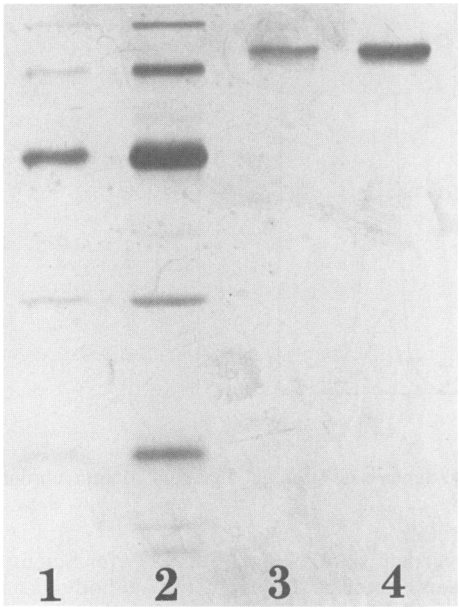

FIG. 5. SDS-PAGE of purified laminarinase. A polyacrylamide concentration of 12.5% was used, and gels were silver stained as described in Materials and Methods. Lanes: 1 and 2, molecular mass protein standards, with 1 and 5 μg of each protein, respectively; 3 and 4, 1 and 5 μg of purified protein, respectively.

buffers) for 12 h, the pH was adjusted to 5.0 with 800 μl of laminarin substrate (0.025%) in sodium acetate buffer (100 mM, pH 5.0).

Temperature optimum and stability. The temperature optimum was determined by performing the standard assay within the temperature range of 30 to 70°C.

Thermal stability was determined by assaying for residual laminarinase activity after incubation of laminarinase (80 pg/ml in sodium acetate buffer [50 mM, pH 5.0]) at various temperatures for 30 min. Following the incubation, the enzyme was cooled and the residual activity was determined under standard enzyme assay conditions.

Effect of various compounds and inhibitors. Laminarinase standard reaction mixtures were incubated with various compounds and known enzyme inhibitors at 1 mM for 1 h at 20°C. Chloride salts of all pertinent metals were used. Following the preincubation, the remaining activity was determined by using the standard enzyme assay conditions. The metals used were as follows: $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $HgCl_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, RbCl, $SnCl_2$, and $ZnCl_2$. The enzyme inhibitors used were as follows: urea, $KMnO_4$, EDTA, acetamide, *N*-bromosuccinimide, iodoacetic acid, *N*-ethylmaleimide, and L-cysteine.

Substrate specificity of laminarinase. Purified laminarinase (0.8 μg) was incubated with a variety of glucans and xylans of various linkage types. The enzyme assay was conducted in the standard manner by using substrates at 1% (wt/vol). Somogyi-Nelson standard curves were prepared for both glucose and xylose.

Identification of reaction products of laminarin and oligosaccharide hydrolysis. A range of 5.5 to 55 units of purified enzyme was incubated with laminarin (0.5%) or laminarin oligosaccharides (0.5%) in 1 ml of ammonium acetate buffer (50 mM, pH 5.0) for 1 h. Following hydrolysis, the samples were lyophilized and redissolved in 1/10 volume of deionized water. Products of the hydrolysis were determined through HPLC analysis as previously described.

Isoelectric focusing of laminarinase. Isoelectric focusing was performed by using a Hoefer SE 600 vertical slab gel apparatus by using a modification of the method of Giulian et al. (7). Gels contained 3/10.5 ampholyte (Pharmacia-LKB, Uppsala, Sweden) and were poured on Gel Bond (FMC Corp., Rockland, Maine). Gels were prefocused for 15 min and focused for 2 h at 15 W (2,500-V maximum) at 15°C. Following isoelectric focusing, the pH gradient was measured with a surface electrode.

RESULTS

Enzyme production. Production of laminarinase using D-glucose as the carbon source in the Multigen fermentor resulted in approximately one-fourth the laminarinase units per milliliter of crude broth as compared with the amount produced by *T. longibrachiatum* cultivated on *A. bisporus* (5.2 versus 22.91 units).

The time course of laminarinase production on D-glucose is illustrated in Fig. 1. Under the conditions used, fungal biomass increased for approximately 30 h, at which point there was a decrease in the filterable biomass. Residual glucose declined at a nearly linear rate for the first 63 h. Laminarinase production followed the peak in biomass, probably as a result of autolytic release of the enzyme. Extracellular protein exhibited a similar increase following the biomass peak.

Enzyme purification. Gel-filtration chromatography of ethanol-precipitated enzyme is illustrated in Fig. 2. The single laminarinase activity peak corresponded to a small peak in protein in fractions 28 and 29. Active fractions pooled from the gel-filtration step were further purified on phenyl-Sepharose (Fig. 3). Contaminating protein eluted through the column at a high salt concentration. Enzyme activity was desorbed from the column at a decreasing NaCl concentration. Active fractions were subsequently chromatographed on carboxymethyl Bio-Gel (Fig. 4). Contaminating protein passed through the column under the initial conditions, and laminarinase activity was eluted from the column at the onset of the NaCl gradient. Silver staining of 5 μg of purified enzyme on polyacrylamide 12.5% SDS-PAGE revealed a

TABLE 1. Summary of purification of laminarinase from *T. longibrachiatum*

Purification step	Vol (ml)	Protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture filtration	900	391.50	3,753	9.6	1	100
Ethanol precipitation	8	14.40	2,859	198.0	21.6	76.2
Bio-Gel P-200	18	3.40	2,356	692.9	72.2	63.0
Phenyl-Sepharose	27	0.48	1,698	3,552.3	370.0	45.2
Carboxymethyl Bio-Gel	70	0.42	1,501	3,573.0	372.2	40.0

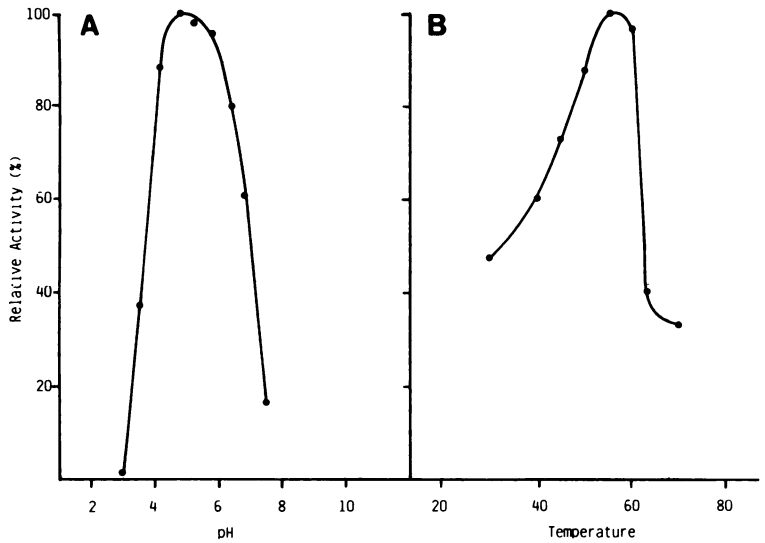

FIG. 6. Effect of pH and temperature on laminarinase activity. Laminarinase activity was measured at various pHs (A) and temperatures (degrees Celsius) (B) under standard enzyme assay conditions.

single protein band (Fig. 5). The purification scheme (Table 1) resulted in a 372.2-fold purification of laminarinase relative to the crude broth and a substantial increase in the specific activity (from 9.6 to 3,573.0 U/mg). Approximately 40% of the laminarinase activity in the crude enzyme was recovered.

Properties of the purified laminarinase. Widely differing estimations of molecular mass were obtained by gel filtration and 12.5% polyacrylamide SDS-PAGE. The values obtained were 28 and 70 kilodaltons upon gel filtration and SDS-PAGE, respectively. Gel filtration was run under nondenaturing conditions (i.e., without guanidine hydrochloride or SDS treatment of the proteins), although 0.1 M NaCl was added to the 100 mM sodium acetate elution buffer in order to minimize interactions between the P-200 gel and the protein.

Isoelectric focusing of the purified enzyme resulted in a single protein band which corresponded to an isoelectric point of 7.2 (data not shown).

The optimal pH for laminarinase activity was 4.8. Greater than 90% of the maximum activity occurring at pH 4.8 was observed up to a pH of 5.8 (Fig. 6). The optimal temperature was 55°C. Greater than 90% of the maximal activity was retained at 60°C (Fig. 6).

The enzyme demonstrated a broad pH stability, as more than 90% of the maximal activity remained following 12-h incubations in buffers within a pH range of 5.0 to 9.3 (Fig. 7). The enzyme was relatively less stable at low pHs than at high pHs. The enzyme was stable during 30-min incubations at temperatures of ≤55°C.

The K_m was reproducibly calculated to be 0.0016%. This corresponds to 0.065 mM, assuming an average degree of

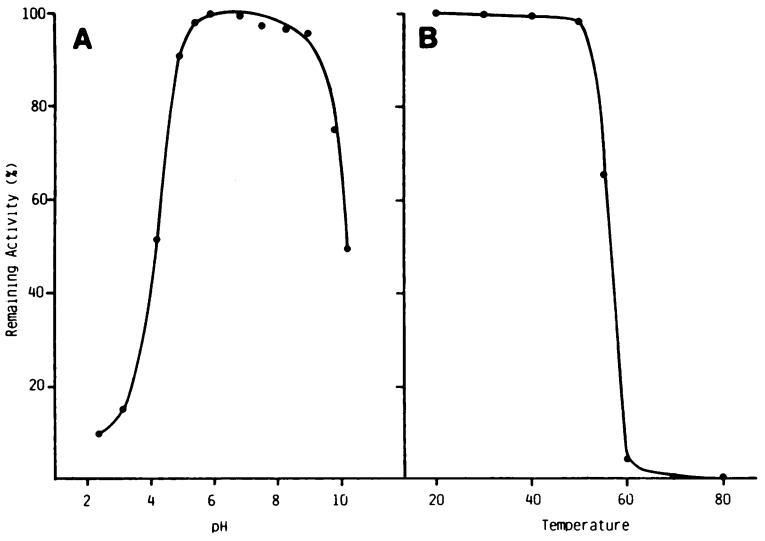

FIG. 7. Effect of pH and temperature on laminarinase stability. Laminarinase was incubated with buffers of various pHs (A) for 12 h prior to assay at pH 5.0. Laminarinase was also incubated at various temperatures (degrees Celsius) in the absence of substrate for 30 min and assayed under standard conditions.

TABLE 2. Metals and inhibitors demonstrating inhibition of laminarinase activity

Compound	Relative activity (%)
$HgCl_2$	29
$MnCl_2$	26
$KMnO_4$	6
N-Bromosuccinimide	7
Control (no addition)	100

polymerization of 15 for laminarin derived from *L. saccharina* (Sigma) (12). The V_{max} was 3,170 μmol of glucose equivalents per mg of the pure enzyme per min.

The effects of several known enzyme inhibitors and metals on the activity of the purified enzyme are shown in Table 2. Only *N*-bromosuccinimide, $KMnO_4$, $MnCl_2$, and $HgCl_2$ were found to affect enzyme activity; they resulted in 93, 94, 74, and 71% relative inhibition, respectively.

A variety of glucans and xylans were tested as substrates for purified laminarinase (Table 3). The enzyme was active only against glucans containing β-1,3 linkages; the greatest activity was against the mixed β-1,3–β-1,6 glucan, laminarin (100%). Less activity was evident against pachyman (45%) and lichenan (4%).

Incubation of laminarin with a low concentration of enzyme (5.5 U) resulted in generation of glucose and laminarin oligosaccharides (Fig. 8). Incubation of laminarin with a high concentration of enzyme (55 U) resulted in the generation of large amounts of glucose and lesser amounts of other oligosaccharides. Laminarin oligosaccharides of three or more glucose units were hydrolyzed by the enzyme (Fig. 9). Laminaripentaose was hydrolyzed to a greater extent than was the triose or the tetraose.

DISCUSSION

The yield of laminarinase upon growth of the organism on glucose was approximately one-fourth that obtained by growing it on *A. bisporus* fruiting bodies (5.2 versus 22.9 units, respectively). By culturing the fungus on glucose, repressible extracellular enzymes and contaminating material from the growth substrate are minimized. The relatively simple purification scheme used for this study resulted in a high recovery of the purified enzyme.

TABLE 3. Relative rates of hydrolysis of selected glucans and xylans

Substrate	Monomer	Linkage(s)	Relative hydrolysis rate (%)
Laminarin (*L. saccharina*)	Glucose	β-1,3, β-1,6	100
Xylan (Oat spelts)	Xylose	β-1,4	0
Xylan (*P. dumetosus*)	Xylose	β-1,3	0
Pachyman (*W. cocos*)	Glucose	β-1,3	45
Lichenan (*U. barbata*)	Glucose	β-1,3, β-1,4	4
Soluble starch	Glucose	α-1,4	0
Carboxymethyl cellulose	Glucose	β-1,4	0
Dextran	Glucose	α-1,6	0
Pullulan (*A. pullulans*)	Glucose	α-1,4, α-1,6	0
Pustulan (*U. papullosa*)	Glucose	β-1,6	0

Disparity in molecular masses determined by gel filtration and SDS-PAGE has been observed for a number of extracellular enzymes (1, 8, 24, 46; Royer and Nakas, in press) and may result from interaction of the enzyme with the gel-filtration medium. The molecular mass estimated by SDS-PAGE (70 kilodaltons) was similar to that observed in other fungi (42, 47). The isoelectric point of *T. longibrachiatum* laminarinase (7.2) was intermediate between those of 3.7 (42, 47) and 9.3 (14), which were determined for other laminarinases.

The optimal pH for enzyme activity (4.8) was similar to that found for endo-β-1,3 glucanases from a variety of organisms (18, 19, 39, 40).

The measured K_m of the purified enzyme (0.0016% for laminarin) was substantially lower than those reported for many endo-type β-1,3 glucanases (6, 9, 12, 19, 39) and was approximately 80-fold lower than that of the crude enzyme from *T. longibrachiatum* grown on *A. bisporus* fruiting bodies (34). This may indicate that different laminarinases

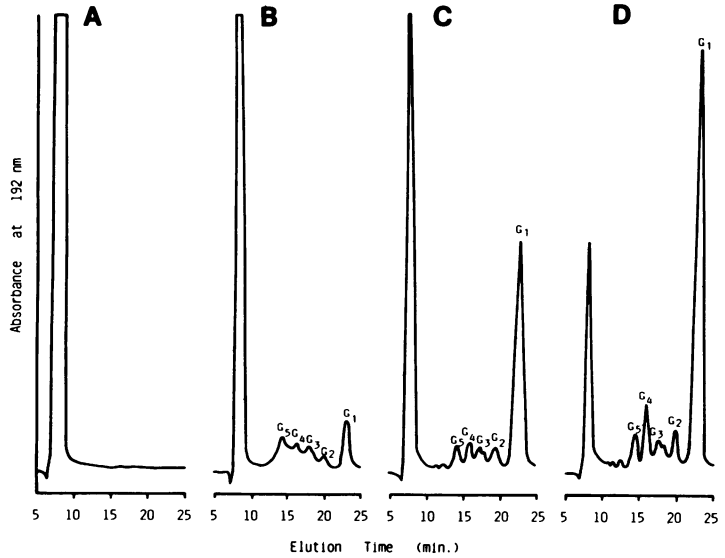

FIG. 8. HPLC analysis of laminarinase action on laminarin using various concentrations of enzyme. Laminarin (0.5%) was incubated with 0.0 (A), 5.5 (B), 22.5 (C), and 55.0 (D) U of purified laminarinase; incubation was for 1 h.

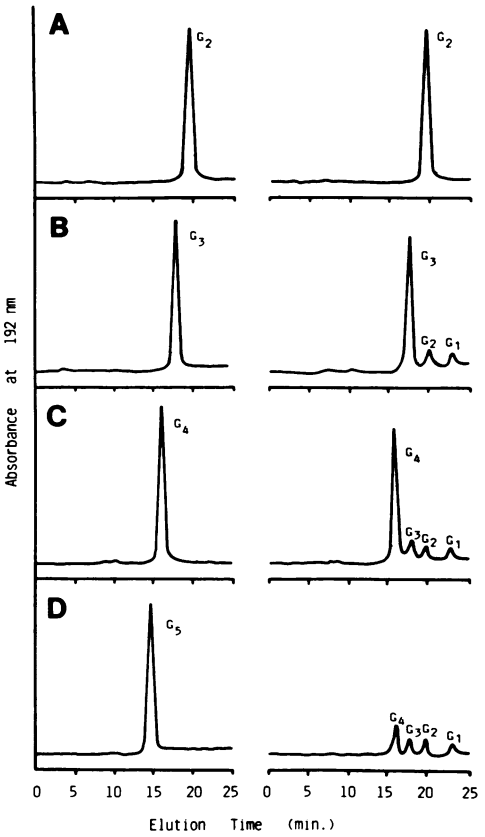

FIG. 9. HPLC analysis of laminarinase action on laminarin oligosaccharides. Enzyme (0.8 μg) was incubated with laminaribiose (A), laminaritriose (B), laminaritetraose (C), or laminaripentaose (D) for 1 h under the standard assay conditions.

are synthesized when *T. longibrachiatum* is grown on different carbon sources or may indicate the presence of inhibitors in the crude enzyme produced from *A. bisporus*. It is conceivable that the enzyme under study may be one which is involved in a morphogenetic role (e.g., acting in conjunction with β-1,3-glucan biosynthetic enzymes for the synthesis of cell wall material [43]). Therefore, prior to the application of such an enzyme it is necessary to investigate the possibility of fungal cell disruption resulting from overproduction of the enzyme (44).

Enzyme activity was inhibited by several of the compounds tested. Inhibition by mercuric ions may indicate the importance of indole amino acids in enzyme function (27), as has been demonstrated for a *Penicillium* cellulase (5). *N*-Bromosuccinimide has several modification functions; among these are reactivity with tyrosine, histidine, tryptophan, thiol, and disulfide groups (45) and cleavage of proteins (13). A β-1,3-glucanase from *Streptomyces* sp. strain W19-1 has been found to have a similar inhibitor susceptibility; inhibition by both Hg^{2+} and *N*-bromosuccinimide has been reported (14). Both $MnCl_2$ and $KMnO_4$ were found to be inhibitory. To our knowledge, inhibition by Mn^{2+} is rare among laminarinases and may be unique to this laminarinase.

None of the compounds tested led to a significant enhancement of laminarinase activity, a result similar to that obtained for a β-1,3-glucanase isolated from *Rhizoctonia solani* (41). However, this result differs from that obtained with a β-1,3-glucanase purified from malted barley, which was shown to be enhanced in the presence of Ba^{2+}, Mn^{2+}, and Co^{2+} (19), and the β-1,3-glucanase mentioned previously (14), which is stable in the presence of Ca^{2+}.

The purified enzyme was active only toward glucans containing β-1,3 linkages, such as laminarin, pachyman, and lichenan. No cleavage was apparent for a β-1,3-linked xylan. β-1,3-Glucan specificity is the general rule for laminarinases (3). Inactivity of the enzyme toward pustulan suggests that the enzyme is unable to cleave β-1,6 linkages. It is likely that this inability extends to β-1,6-glucose interchain linkages within the laminarin molecule. Lack of activity against pachyman was surprising, since pachyman is a straight-chain β-1,3-linked glucan. The lower degree of catalysis may be the result of the low solubility of pachyman or the presence of inhibitory contaminants.

The enzyme appeared to act in an endoglucanase-like fashion, as indicated by the generation of a mixture of oligosaccharides in addition to glucose following incubation on laminarin and laminaripentaose (Fig. 8 and 9). Owing to the specificity for β-1,3 linkages and the generation of smaller laminarin oligosaccharides, it may be included within the enzyme class EC 3.2.1.6. It differed from the laminarinase of *Rhizopus arrhizus*, which also cleaves β-1,4 linkages adjacent to β-1,3 linkages in cereal glucans (28).

Transglycosylase activity was not observed for the enzyme, as indicated by the lack of increase in oligosaccharide chain length following incubation with the pure enzyme. This result is in contrast to that determined for a β-1,3-glucanase from *Rhizoctonia solani* (41), which was shown to produce larger oligosaccharides following incubation of the enzyme with laminaripentaose.

The enzyme was unable to cleave laminaribiose (Fig. 9), a characteristic which has been ascribed to several other laminarinases (12, 19, 29, 39). The inability of the enzyme to cleave laminaribiose is further evidence that the enzyme is an endoglucanase (6). The limited hydrolysis of the triose and tetraose relative to that of the pentaose may indicate a preference for the pentamer as a substrate.

Laminarin is abundant in nature, particularly among algae of the families *Phaeophyceae* and *Chrysophyceae*. In marine macroalgae of the genus *Laminaria*, it typically represents 22 to 34% of the dry weight of the alga (25). Consequently, laminarin represents an important potential source of renewable carbohydrate that may be utilized as a substrate for fuel, solvent, and chemical feedstock production. Previous work in our laboratory has involved the bioconversion of acid-hydrolyzed *L. saccharina* to neutral solvents such as butanediol, butanol, acetone, and ethanol. Potential for utilization of laminarinase of *T. longibrachiatum* for hydrolysis of the crude alga to fermentable sugars is currently being evaluated.

ACKNOWLEDGMENTS

We thank the New York SEA GRANT Institute, the National Oceanic and Atmospheric Administration, the U.S. Department of Commerce, and the McIntire-Stennis Cooperative Forestry Research Program of the U.S. Department of Agriculture for their financial assistance.

LITERATURE CITED

1. **Baker, C. J., C. H. Whalen, and D. F. Bateman.** 1977. Xylanase from *Trichoderma pseudokoningii*. Phytopathology **67**:1250–1258.
2. **Boucaud, J., J. Bigot, and J. Devaux.** 1987. Partial purification and characterization of exo- and endo-1,3-β-D-glucanases from *Atriplex* leaf tissues, their involvement in cell extension growth

and the effects of salinity. J. Plant Physiol. **128**:337–349.
3. **Bull, A. T., and C. G. C. Chesters.** 1966. The chemistry of laminarin and the nature of laminarinase. Advances Enzymol. Relat. Areas Mol. Biol. **28**:325–364.
4. **Del Rey, F., T. Santos, I. Garcia-Acha, and C. Nombela.** 1979. Synthesis of 1,3-β-glucanases in *Saccharomyces cerevisiae* during the mitotic cycle, mating and sporulation. J. Bacteriol. **139**: 924–931.
5. **Eriksson, K.-E., and G. Petersson.** 1968. Studies on cellulolytic enzymes. V. Some structural properties of the cellulase from *Penicillium notatum*. Arch. Biochem. Biophys. **124**:160–166.
6. **Fleet, G. H., and H. J. Phaff.** 1974. Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall-associated β-(1→3)-glucanases. J. Biol. Chem. **249**:1717–1728.
7. **Giulian, G. B., R. L. Moss, and M. Greaser.** 1984. Analytical isoelectric focusing using a high voltage vertical slab polyacrylamide gel system. Anal. Biochem. **142**:421–436.
8. **Hammes, B. D., and D. Rickwood.** 1981. Gel electrophoresis of proteins: a practical approach. IRL Press, Ltd., Oxford.
9. **Hiura, N. M., M. Kobayashi, T. Nkajima, and K. Matsuda.** 1986. Purification and some properties of two wall-associated (β-1,3) glucanases from *Neurospora crassa* cells. Agric. Biol. Chem. **50**:2461–2467.
10. **Jones, D., A. H. Gordon, and J. S. D. Bacon.** 1974. Cooperative action by endo- and exo-β-(1→3)-glucanases from parasitic fungi in the degradation of cell-wall glucans of *Sclerotinia sclerotiorum* (Lib.) de Bary. Biochem. J. **140**:47–55.
11. **Kaufman, S.** 1971. Fractionation of protein mixtures with organic solvents. Methods Enzymol. **22**:233–238.
12. **Kitamoto, Y., R. Kono, A. Shimortori, N. Mori, and Y. Ishikawa.** 1987. Purification and some properties of an exo-β-1,3-glucanase from *Trichoderma harzianum*. Agric. Biol. Chem. **51**: 3385–3386.
13. **Kronman, M. J., F. M. Robbins, and R. E. Andreotti.** 1967. Reaction of N-bromosuccinimide with lysozyme. Biochim. Biophys. Acta **143**:462–472.
14. **Kusama, S., I. Kusakabe, and K. Murakami.** 1986. Purification and some properties of β-1,3-glucanase from a *Streptomyces* sp. Agric. Biol. Chem. **50**:1101–1106.
15. **Lachance, M., and H. J. Phaff.** 1979. Comparative study of molecular size and structure of exo-β-glucanases from *Kluyveromyces* and other yeast genera: evolutionary and taxonomic implications. Int. J. Syst. Bacteriol. **29**:70–78.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Manners, D. J., A. J. Masson, and J. C. Patterson.** 1973. The structure of a β-(1–3)-D-glucan from yeast cell walls. Biochem. J. **135**:19–30.
18. **Manners, D. J., and G. Wilson.** 1973. Studies on β-glucanases. Some properties of a bacterial endo-β-(1,3)-glucanase system. Biochem. J. **135**:11–18.
19. **Manners, D. J., and G. Wilson.** 1974. Purification and properties of an endo-(1–3)-β-D-glucanase from malted barley. Carbohydr. Res. **37**:9–22.
20. **McIlvaine, T. C.** 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. **49**:183–185.
21. **Mitchell, R.** 1963. Addition of fungal cell-wall components to soil for biological disease control. Phytopathology **53**:1068–1071.
22. **Mitchell, R., and M. Alexander.** 1963. Lysis of soil fungi by bacteria. Can. J. Microbiol. **9**:169–176.
23. **Nielson, B. L., and L. R. Brown.** 1984. The basis for colored silver-protein complex formation in stained polyacrylamide gels. Anal. Biochem. **141**:311–315.
24. **Paice, M. G., L. Jurasek, M. R. Carpenter, and L. B. Smillie.** 1978. Production, characterization and partial amino acid sequence of xylanase A from *Schizophyllum commune*. Appl. Environ. Microbiol. **36**:802–808.
25. **Percival, E., and R. H. McDowell.** 1967. Chemistry and enzymology of marine algal polysaccharides. Academic Press, Inc., New York.
26. **Philpott, W. A., and J. M. Chapman.** 1977. A new improved method for the assay of endo-β-1,3-glucanase. Anal. Biochem. **79**: 257–262.
27. **Ramachandran, L. K., and B. Witkop.** 1964. The interaction of mercuric acetate with indoles, tryptophan, and proteins. Biochemistry **3**:1603–1611.
28. **Reese, E. T., and M. Mandels.** 1959. β-D-1,3 Glucanases in fungi. Can. J. Microbiol. **5**:173–185.
29. **Rombouts, M., and H. J. Phaff.** 1974. Lysis of yeast cell-walls. Lytic β-(1–3)-glucanases from *Bacillus circulans* WL-12. Eur. J. Biochem. **63**:121–130.
30. **Saito, H., A. Misaki, and T. Harada.** 1968. A comparison of the structure of curdlan and pachyman. Agric. Biol. Chem. **32**: 1261–1269.
31. **Scott, J., and R. Schekman.** 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. **142**:414–423.
32. **Shallenberger, R. S., C. Searles, and B. Lewis.** 1974. Laminarinase activity in the crystalline style of the surf clam (*Spissula solidissima*). Experientia **30**:597–598.
33. **Shan, W. Y., and G. A. MacLachan.** 1979. 1,3-β-D-Glucanases from *Pisum sativum* seedlings. I. Isolation and purification. Biochim. Biophys. Acta **571**:244–255.
34. **Sharma, A., and J. P. Nakas.** 1987. Preliminary characterization of laminarinase from *Trichoderma longibrachiatum*. Enzyme Microb. Technol. **9**:89–93.
35. **Shinshi, H., and K. Kato.** 1983. Physical and chemical properties of β-1,3-glucanase from cultured tobacco cells. Agric. Biol. Chem. **47**:1455–1460.
36. **Skujins, J. J., H. J. Potgieter, and M. Alexander.** 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1–3) glucanase. Arch. Biochem. Biophys. **111**:358–364.
37. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
38. **Sorenson, S. P. L.** 1909. Uber die Messung und die Bedeutung der Wasserstoffionen-konzentration bei enzymatishen Prozzen. Biochem. Z. **21**:131–200.
39. **Takahashi, M. J., M. Komuro, and S. Souatome.** 1978. Purification and properties of β-1,3-glucanase from *Lentinus lepideus*. J. Ferment. Technol. **56**:499–505.
40. **Totani, K., S. Harumiya, F. Nanjo, and T. Usui.** 1983. Substrate affinity chromatography of β-1,3-glucanase from *Basidiomycetes* species. Agric. Biol. Chem. **47**:1159–1162.
41. **Totsuka, A., and T. Usui.** 1986. Separation and characterization of the endo-β-(1–3)-D-glucanase from *Rhizoctonia solani*. Agric. Biol. Chem. **50**:543–550.
42. **Tsujisaka, Y., N. Hamada, and R. Kobayashi.** 1981. Purification and some properties of an exo-β-1,3-glucanase from *Basidiomycetes* species. Agric. Biol. Chem. **45**:1201–1208.
43. **Villaneuva, J. R., V. Notaio, T. Santos, and T. G. Villa.** 1976. β-Glucanases in nature: biochemistry and function of β-glucanases in yeasts, p. 323–355. *In* J. F. Peabody, A. H. Rose, H. J. Rogers, and E. C. Cocking (ed.), Microbial and plant proplasts. Academic Press, Inc., New York.
44. **Wessels, J. G. H., and Y. Koltin.** 1972. R-glucanase activity and susceptibility of hyphal walls to degradation in mutants of *Schizophyllum* with disrupted nuclear migration. J. Gen. Microbiol. **71**:471–475.
45. **Witkop, B.** 1961. Nonenzymatic methods for the preferential and selective cleavage and modification of proteins, p. 221–321. *In* C. B. Anfinsen, Jr., M. L. Anson, K. Bailey, and J. T. Edsall (ed.), Advances in protein chemistry, vol. 16. Academic Press, Inc., New York.
46. **Wood, T. M., and S. I. McCrae.** 1986. Studies of two low-molecular weight endo-(1–4)-β-D-xylanases constitutively synthesized by the cellulolytic fungus *Trichoderma koningii*. Carbohydr. Res. **148**:321–330.
47. **Zaichina, I. V., N. A. Tiunova, and N. Y. Kobzeva.** 1985. Purification and study of the properties of 1,3-β-glucanases from *Geotrichum candidum*. Prikl. Biokhim. Mikrobiol. **21**:203–212.