Purification and Characterization of Laminaran Hydrolases from *Trichoderma viride*

**Rika NOBE,**¹ Yoichi SAKAKIBARA,¹ Nobuhiro FUKUDA,¹ Naoto YOSHIDA,¹
Kihachiro OGAWA,² and Masahito SUIKO¹,†

¹Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, Miyazaki University, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan
²Department of Food Technology, Faculty of Horticulture, Minami Kyushu University, 11609 Minamitakanabe, Takanabe 884-0003, Japan

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At least three extracellular laminaran hydrolases which hydrolyzed laminaran (β-1,3:1,6-glucan) from *Eisenia bicyclis* were secreted in wheat bran solid medium by *Trichoderma viride* U-1. These three enzymes, lam AI, AII, and B, were purified to electrophoretic homogeneity. Their molecular masses were estimated to be 70.1, 70.4, and 45.0 kDa for lam AI, AII, and B, respectively, by SDS-PAGE. Whereas both lam AI and AII could hydrolyze laminarin from *Laminaria digitata*, lam AII showed higher activity against *Laminaria* laminarin rather than *Eisenia* laminaran. On the other hand, lam B preferentially hydrolyzed pustulan, a β-1,6-glucan. Laminarioligosaccharide was hydrolyzed by lam AI and AII but not B, whereas gentiooligosaccharide was hydrolyzed by only lam B. It showed that lam AI and AII were specific for β-1,3-linkages, but lam B was specific for β-1,6-linkages. These results indicated that *T. viride* U-1 has a multiple glucanolytic enzyme system.

**Key words:** *Trichoderma viride*; β-1,3-glucanase; β-1,6-glucanase; extracellular enzyme; *Eisenia bicyclis*

Soilborne fungi of the genus *Trichoderma* are well known biocontrol agents for several phytopathogenic fungi.¹⁵ *Trichoderma* species produce various hydrolytic enzymes, including chitinases, β-1,3-glucanases, β-1,6-glucanases, and proteases, when grown on laminaran, chitin, or fungal cell walls as the sole carbon source.²⁻⁴ Because chitin and β-1,3-glucan form the main structural components of the fungal cell wall, chitinases and β-1,3-glucanases have been proposed as the key enzymes in the degradation of cell walls during mycoparasitism against phytopathogenic fungi.⁵ Although the existence of β-1,6-glucan polymers is not common in most living cells, β-1,6-linkages occur frequently in the cell walls of yeast, mushrooms and filamentous-fungal cell walls.⁶⁻⁹

β-1,3-glucanases are classified into exo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase [EC 3.2.1.58]), and endo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase [EC 3.2.1.6 or EC 3.2.1.39]). All β-1,3-glucanases of the genus *Trichoderma* so far sequenced have been classified as family 55 of glycosyl hydrolases, based on amino acid sequence similarities.¹⁰⁻¹³ Many cell-wall lysing microorganisms secrete multiple enzymes and complete degradation of cell walls is often accomplished by synergistic action of endo- and exo-enzymes of chitinase and β-1,3-glucanase.¹²,¹³ The genus *Trichoderma* has been shown to produce multiple glucanolytic enzymes under various conditions.⁴,¹⁴⁻¹⁶

We have previously reported on hyper-hydrolytic enzymes produced by *Trichoderma viride* when grown on wheat bran solid culture.¹⁷ A commercial mycological enzyme preparation, Usukizyme, was thus prepared from a wheat bran solid culture of *T. viride* U-1, originally isolated from a bed log of *Lentinus edodes* (Shiitake mushroom). The enzyme preparation is a mixture of various mycological enzymes, including chitinases and glucanases, and is widely used in protoplast preparations of fungi such as molds, yeasts, and mushrooms for cell fusion and gene manipulation.¹⁸⁻²⁰ In our continuing elucidation of the mode of action of this industrial enzyme preparation, we describe the detailed characterization of its glucanolytic components.

Usukizyme has a high hydrolytic activity against laminaran, a β-1,3:1,6-glucan derived from *Eisenia bicyclis*. Although several β-1,3:1,6-glucan hydrolases have been described,²¹,²² sufficient information on characterization of the similar enzymes from the genus *Trichoderma* is lacking. Besides, β-1,3-
Materials and Methods

Chemicals. Laminaran (from Eisenia bicyclis) was purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). Laminarin (from Laminaria digitata), lichenan (Cetraria islandica), β-glucan (from barley), xylan (from oat spelt), and inulin (chicory root) were products of Sigma Chemical Co. (St. Louis, MO, USA). Pustulan (from Umbilicaria papullosa) was from Calbiochem (La Jolla, CA, USA). Laminariligosaccharides (β-1,3-linkages, DP = 2–7) and gentiooligosaccharide (β-1,6-linkages, DP = 9) were from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Curdlan (from Alcaligenes faecalis), CM-cellulose, and soluble starch were from Wako Pure Chemical Industries (Osaka, Japan). Colloidal chitin was prepared from powdered chitin (Ajinomoto Co., Ltd.) by the method of Jeuniaux.28 Usukizyme, a mycolytic enzyme preparation derived from Trichoderma viride U-1, was purchased from Kyowa Kasei Chemical Co., Ltd. (Osaka, Japan). All other chemicals were obtained commercially and were of analytical grade.

Enzyme and protein assays. Laminaran hydrolase activity was assayed in a 1-mL reaction mixture containing 0.5 mL of 1.0% (w/v) laminaran in 0.1 M acetate buffer (pH 5.0) and 0.5 mL of an appropriately diluted enzyme solution in the same buffer. The mixture was incubated at 45°C for 30 min. The amount of reducing sugar produced was measured as described by Somogyi and Nelson.27,28 One unit of laminaran hydrolase activity was defined as the amount of enzyme that liberated 1.0 μmol of reducing sugar, equivalent to glucose, from laminaran per minute in the 1.0-mL assay mixture in 30 min. Protein concentrations were measured by the method of Lowry,29 using bovine serum albumin (Sigma) as the standard. During purification, protein concentrations were estimated by measuring absorbance at 280 nm, with bovine serum albumin as the standard.

Enzyme purification. All purification procedures were done at 4°C. Usukizyme preparation (1.2 g) was dissolved in 15 mL of 50 mM acetate buffer (pH 5.0) and centrifuged at 8,000 × g for 10 min. The supernatant was put onto a DEAE Sepharose CL-6B (Pharmacia Biotech AB, Uppsala Sweden) column (2.5 × 30 cm) previously equilibrated with the same buffer. Elution with a linear gradient of 0 to 1.0 M NaCl for 600 min at a flow rate of 0.6 mL/min resulted in two laminaran hydrolase peaks. Two peaks of the active fractions at 0 and 0.6 M NaCl were designated as peaks A and B, respectively. Each peak was pooled and concentrated in 50 mM acetate buffer (pH 5.0) containing 1.0 M ammonium sulfate by using an Ultrafree-15 centrifugal filter (Millipore, MA, USA).

Peak A was then put onto a phenyl Sepharose HP column (1.6 × 20 cm) equilibrated with the same buffer. The column was eluted at a flow rate of 1 mL/min in a stepwise gradient composed of 90 mL each of 0.6, 0.5, 0.4, 0.3, and 0 M ammonium sulfate in the same buffer. Elution with a stepwise gradient resulted in two laminaran hydrolase peaks at 0.5 and 0.3 M ammonium sulfate. Each peak, designated as peak A1 and peak AII, was pooled and concentrated in 50 mM acetate buffer (pH 5.0) by using an Ultrafree-15 centrifugal filter (Millipore). The peak A1 was then put onto a Superdex 200 pg column (2.6 × 60 cm) at a flow rate of 0.5 mL/min with 10 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. The peak AII was put onto a Mono Q HR column (0.5 × 5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was eluted at a flow rate of 1 mL/min in a linear gradient of 0 to 1.0 M NaCl for 20 min in the same buffer. The peak AII was then eluted at 0.25 M NaCl.

The peak B was then put onto a phenyl Sepharose HP column (1.6 × 20 cm) equilibrated with 1 M ammonium sulfate in 50 mM acetate buffer (pH 5.0). The column was eluted at a flow rate of 1 mL/min in a stepwise gradient composed of 90 mL each of 1.0, 0.5, 0.4, 0.3, and 0 M ammonium sulfate in the same buffer. The peak of laminaran hydrolase activity at 0.4 M ammonium sulfate was pooled and concentrated in 50 mM acetate buffer (pH 5.0) by using an Ultrafree-15 centrifugal filter (Millipore). Finally, the concentrated laminaran hydrolase peak was put onto a HiLoad 26/60 Superdex 75 pg column (2.6 × 60 cm) at a flow rate of 0.5 mL/min with 10 mM acetate buffer (pH 5.0) containing 0.15 M NaCl.

Each laminaran hydrolase was eluted as a single protein peak that coincided with the peak of enzyme activity.

Electrophoresis and analysis of N-terminal amino acid sequences. SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli.30 Protein bands in the gel were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing was done on a Multiphor II electrophoresis system (Pharmacia
Biotech) using an Ampholine PAGplate (pH 3.5 to 9.5) according to the manufacturer’s instructions. An IEF calibration Low and Broad p/ k hit (Pharmacia Biotech) was used to estimate the isoelectric point. After SDS-PAGE of the purified enzyme, the protein band was blotted onto a polyvinylidene difluoride (PVDF) membrane (BioRad Laboratories, Hercules, CA, USA). The N-terminal amino acid sequences were analyzed with a Procise 492 gas phase sequencer (Applied Biosystems Division, Perkin-Elmer).

Effects of pH and temperature on the activity and stability of three purified laminaran hydrolases. The effect of pH on activity was observed in 0.1 M sodium acetate-HCl (pH 2.0 to 4.5), 0.1 M sodium acetate (pH 4.5 to 6.0), and 0.1 M potassium phosphate (pH 6.0 to 8.5) and 0.1 M glycine-NaOH (pH 9.0 to 11.0) at 45°C for a 30-min incubation. To measure the pH stability, each enzyme solution was maintained at 30°C for 24 h in the respective buffer solution (pH 2.0 to 11.0) and the residual activity was measured at 45°C and pH 5.0.

The effects of temperature on the purified laminaran hydrolases activities were measured under the standard conditions, except that the reaction mixtures were maintained at various temperatures ranging from 10 to 70°C for 30 min at each respective optimum pH in 0.1 M acetate buffer. To measure the thermal stability, each enzyme solution in 0.1 M acetate buffer was incubated at various temperatures for 30 min, after which the residual activity was assayed as described above.

Hydrolysis of laminaran, laminarin, pustulan, laminarioligosaccharide, and gentiooligosaccharide. The reaction mixtures consisted of 0.5% (w/v) substrate and the enzyme solution (30 mU/ml) in 50 mM acetate buffer, pH 5.0. Alternatively, each oligosaccharide was dissolved in 50 mM acetate buffer, pH 5.0, to make a 0.1% (w/v) solution, and the enzyme solution (60 mU/ml) was added. Each mixture was incubated at 45°C for 0–24 h. The mixture was then heated in a boiling water bath for 10 min to stop the reaction. High-performance thin-layer chromatography (HPTLC) of hydrolysis products was done on pre-coated silica gel 60 HPTLC plates (Merck AG, Darmstadt, Germany). The plates were developed twice at room temperature with a solvent system of chloroform, acetic acid, and water (3:10:1). Spots were stained by spraying the plate with aniline-diphenylamine-phosphoric acid reagent and then heating at 120°C for 5 min. Sugars were identified using laminarioligosaccharides (DP = 2–7) mixture and gentiooligosaccharides as the standard. Gentiooligosaccharide standards were prepared by hydrolyzing pustulan as previously described by Katohda et al. 32)

Results

Purification of three laminaran hydrolases

In this study, three purified laminaran hydrolases, designated as lam AI, AII, and B, were found upon the elution on DEAE Sephacrose CL-6B and phenyl Sepharose HP columns. Table 1 summarizes the purification of lam AI, AII, and B from Trichoderma viride U-1. The lam AI, AII, and B were purified 46.1-, 3.63-, and 14.8-fold, with a yield of 1.83, 0.02, and 1.37%, respectively. The purified enzymes had a specific activity of 108 U/mg for lam AI, 8.50 U/mg for lam AII, and 34.8 U/mg for lam B against laminaran from Eisenia fetida (Table 2).

Molecular mass and general properties of three purified laminaran hydrolases

The molecular masses of three purified enzymes were estimated to be 70.1, 70.4, and 45.0 kDa for lam AI, AII, and B by SDS-PAGE (Fig. 1). The isoelec-
Three purified enzymes (10 μg each) were electrophoresed on a 12.5% SDS polyacrylamide gel. Lane M, molecular mass markers; Gel(1), purified lam AI; Gel(2), purified lam AII; Gel(3), purified lam B.

**Table 2.** Comparison of Biochemical Properties of Lam AI, AII, and B from *T. viride* U-1

<table>
<thead>
<tr>
<th></th>
<th>Molecular mass (kDa)</th>
<th>Specific activity (U/mg)</th>
<th>Optimum pH</th>
<th>Optimum temperature (°C)</th>
<th>pH stability*</th>
<th>Temperature stability (°C)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>lam AI</td>
<td>70.1</td>
<td>108</td>
<td>5.0</td>
<td>45</td>
<td>5.0</td>
<td>&lt;40</td>
<td>7.5</td>
</tr>
<tr>
<td>lam AII</td>
<td>70.4</td>
<td>8.50</td>
<td>5.5</td>
<td>50</td>
<td>5.5</td>
<td>&lt;55</td>
<td>6.3</td>
</tr>
<tr>
<td>lam B</td>
<td>45.0</td>
<td>34.8</td>
<td>5.0</td>
<td>55</td>
<td>6.0</td>
<td>&lt;55</td>
<td>3.0</td>
</tr>
</tbody>
</table>

One unit (U) was defined as the amount of enzyme that liberated 1.0 μmol of reducing sugar, equivalent to glucose, from substrate per minute.

* Each molecular mass was measured by SDS-PAGE.

* Activity was assayed against laminarin from *Eisenia fetida*.

* The pH ranges showed these enzymes retained greater than 80% of the original activity in the table. Other experimental details are described in the text.

The optimum points of the purified enzymes were 7.5, 6.3, and 3.0 for lam AI, AII, and B, respectively, by isoelectric focusing on an Ampholine PAGplate (pH 3.5 to 9.5) (Pharmacia Biotech).

The enzymatic properties were examined by using these purified enzymes, and laminaran as the substrate. Table 2 summarizes the general characteristics of the purified lam AI, AII, and B.

The optimum pHs for the three enzyme activities were similar, and showed maximum activity at the pH range from 5.0 to 5.5. For studying the effects of pH on the stability of three enzymes, the enzyme solution was incubated for 24 h under various conditions. Lam B activity was stable over a broad pH range from 4.0 to 8.0. On the other hand, lam AI and AII showed narrow pH stability ranges of 4.0 to 6.0 and 5.0 to 6.0, respectively. At these pH ranges, the enzymes retained greater than 80% of their original activity.

The optimum temperature range for the three laminaran hydrolases was from 45°C to 55°C. After a 30-min preincubation at different temperatures, the thermal stability was measured. Lam AI remained stable up to 40°C, but lam AII and B were stable up to 55°C. Complete inactivation was observed when the three enzymes were held at 60, 65, and 70°C for lam AI, AII, and B, respectively.

The three laminaran hydrolases activities were measured under standard conditions in the presence of various 1 mM metal ions and EDTA. Hg²⁺ strongly inhibited lam AI and B activity, but not lam AII. The three enzymes were relatively tolerant to Zn²⁺, Mn²⁺, Cd²⁺, Co²⁺, Ni²⁺, Cu²⁺, and Mg²⁺. EDTA had no significant effect on the three enzymes activities (Table 3).
**Substrate specificity of the three laminaran hydro- lases**

The activity of the three enzymes toward various substrates is summarized in Table 4. Whereas both lam AI and AII could hydrolyze laminarin from *L. digitata*, lam AII showed higher activity against *Laminaria* laminarin rather than *Eisenia* laminaran. On the other hand, lam B preferentially hydrolyzed pulstulan, a β-1,6-glucan polymer. The three enzymes had no activity against insoluble β-1,3-glucan, β-1,3;1,4-glucan and other substrates, as shown in the Table 4.

**Products of enzyme action and mode of substrate cleavage**

The hydrolysis of *Eisenia* laminarin by the purified enzymes over a 24-h period was analyzed by thin-layer chromatography. All of the three enzymes hydrolyzed laminarin, yielding glucose, gentiobiase (β-1,6-oligosaccharide, DP = 2), and a series of oligosaccharides (Fig. 2, A). Products from *Laminaria* laminarin by lam AI and AII, and pulstulan by lam B were examined by thin-layer chromatography. The hydrolysis products of *Laminaria* laminarin by lam AI and AII also yielded glucose, gentiobiase, and a series of oligosaccharides (Fig. 2, B). On the other hand, the hydrolysis products of pulstulan by lam B gave mainly gentiobiase, gentiotriose (β-1,6-oligosaccharide, DP = 3), and two unidentified spots (Fig. 2, C).

The hydrolysis of gentiooligosaccharide (DP = 9), which consists of a β-1,6-linkage of glucose and laminarioligosaccharide (DP = 7), which consists of a β-1,3-linkage of glucose by three purified enzymes, were examined as described in materials and methods. Laminarioligosaccharide (DP = 7) was hydrolyzed by lam AI and AII, to only glucose and laminaribiose, eventually (Fig. 3, A). These enzymes also react with laminaritriose and laminaritetraose (DP = 3 and 4) and finally produce only glucose and laminaribiose, but laminaribiose was not hydrolyzed (data not shown). The hydrolysis of gentiooligosaccharide (DP = 9) yielded only gentiobiase and gentiotriose as the final products by only lam B (Fig. 3, B).

**N-terminal amino acid sequences**

The N-terminal amino acid sequences of laminaran hydrolases from *T. viride* U-1 were analyzed. As these sequences had no homology to each other, homology to other sequences was investigated by a BLAST search (Fig. 4). The lam AI sequence had high homology to bgn1, β-1,3-glucanase of *T. viridescens*, and lam AII sequence showed high similarity to LAM1.3, an exo-β-1,3-glucanase of *T. harzianum*. These genes have been cloned, and shown to belong to glycosyl hydrolase family 55. Lam B sequence shared a high degree of homology with β-1,3-glucanases from *Pichia anamala*, *Yarrowia lipolytica*, and *Candida oleophila*, and with a cellulase from *Flabilidella neoformans*. All these sequences belong to glycosyl hydrolase family 5. As shown in Fig. 4, a BLAST search of the amino acid sequence showed the conserved K-I-R-G-V-N-L-G region.

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**Table 3.** Effects of Metal Ions and Enzyme Inhibitors on the Activity of Lam AI, AII, and B from *T. viride* U-1

<table>
<thead>
<tr>
<th>Effector</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AI</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>110</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>84</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>86</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>89</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>80</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>92</td>
</tr>
<tr>
<td>EDTA</td>
<td>100</td>
</tr>
</tbody>
</table>

The enzyme solution was incubated with each of the reagents at 1 mM concentrations at room temperature for 30 min. The residual activity was measured as described in the text.

**Table 4.** Substrate Specificity of the Purified Lam AI, AII, and B from *T. viride* U-1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Main linkage type (monomer)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AI</td>
</tr>
<tr>
<td>Laminarin from <em>Eisenia bicyclica</em></td>
<td>β-1,3;β-1,6(Glc)</td>
<td>108.0</td>
</tr>
<tr>
<td>Laminarin from <em>Laminaria digitata</em></td>
<td>β-1,3(Glc)</td>
<td>24.8</td>
</tr>
<tr>
<td>Pustulan from <em>Umbilicaria papulosa</em></td>
<td>β-1,6(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Curdlan from <em>Alcaligenes faealis</em></td>
<td>β-1,3(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Lichenan from <em>Cetraria islandica</em></td>
<td>β-1,3;β-1,4(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>β-Glucan from barley</td>
<td>β-1,3;β-1,4(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>β-1,4(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>α-1,4;α-1,6(Glc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>β-1,4(GlcNAc)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Xylan</td>
<td>β-1,4(Xyl)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Inulin</td>
<td>β-2,1(Fru)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The reaction mixture containing 0.5% (w/v) the respective substrate and an appropriate dilution of each respective enzyme in 0.1 M acetate buffer, pH 5.0, was held at 45°C for 30 min. Activity was measured as described in the text. N.D., Not detected.
A) Laminaran from *Eisenia bicyclis*

![Reaction time (hr)]

B) Laminarin from *Laminaria digitata*

![Reaction time (hr)]

C) Pustulan

![Reaction time (hr)]

**Fig. 2.** Thin-layer Chromatogram of Laminaran, Laminarin, and Pustulan Hydrolyzates by the Purified Laminaran Hydrolases.

The reaction mixtures consisted of 0.5% (w/v) of each substrate and the purified enzyme solution (30 mU/ml) in 50 mM acetate buffer, pH 5.0. The hydrolyzates of (A) laminaran from *Eisenia bicyclis*, (B) laminarin from *Laminaria digitata*, and (C) pustulan were subjected to TLC as described in the text. Standards (M1) consisted of glucose (G1) and laminarioligosaccharides mixture (from G2 to G5). Standards (M2) were gentiooligosaccharides as described in the text. G1-5: glucose oligomers, DP = 1-5.

**Discussion**

*T. viride* U-1 is a mycoparasitic fungus that secretes various mycolytic enzymes, including chitinases and laminarinases (β-1,3-glucanases) in wheat bran solid medium. Some β-1,3-glucanases have previously been isolated from different strains and species of the genus *Trichoderma* in various conditions, and characterized. Vazquez-Garcidueñas *et al.* biochemically showed *T. harzianum* produced at least seven extracellular β-1,3-glucanases when grown on some polysaccharides or cell walls, by the activity staining after two-dimensional gel separation, but characterization of these enzymes have been incomplete. In this study, it was found that at least three laminaran hydrolase activities were secreted in the culture filtrate, and these enzymes were purified and characterized.

In terms of molecular mass, the three laminaran hydrolases purified from *T. viride* U-1 separated into lam AI and AII, which were about 70 kDa, and lam B, which was about 45 kDa. Lam AI and AII were very similar in size, but their isoelectric points were different. The activities of lam AI and B were significantly inhibited by Hg²⁺, implying the importance of a sulfhydryl group for expression of the enzyme activity. On the other hand, lam AII was insensitive to Hg²⁺.

Both lam AI and AII were specific toward β-1,3-linkages of oligosaccharides and produced glucose and biose from laminarioligosaccharide (DP = 7) but the substrate specificity against the β-glucan of each enzyme showed differences. *Laminaria* laminarin is
**Laminaran Hydrolases of Trichoderma viride**

### A) Laminarioligosaccharide (DP = 7)

![Image of A) Laminarioligosaccharide (DP = 7)]

### B) Gentiooligosaccharide (DP = 9)

![Image of B) Gentiooligosaccharide (DP = 9)]

**Fig. 3.** Thin-layer Chromatograms of Laminarioligosaccharide and Gentiooligosaccharide Hydrolyzates by the Purified Laminaran Hydrolases.

Each oligosaccharide was dissolved in 50 mM acetate buffer, pH 5.0, to make a 0.1% (w/v) solution, and the enzyme solution (60 mM/ml) was added. (A) laminarioligosaccharide (β-1,3-linkage, DP = 7) and (B) gentiooligosaccharide (β-1,6-linkage, DP = 9) were hydrolyzed by three purified enzymes and subjected to TLC. Standards (M1) were laminarioligosaccharides mixture. Standards (M2) were gentiooligosaccharides as described in the text. G$_1$-7: glucose oligomers, DP = 1-7. Lane C, no enzyme; Lane AI, purified lam AI; Lane AII, purified lam AII; Lane B, purified lam B.

**Protein** | **Origin (GenBank protein accession number)**
--- | ---
Lam AI | T. viride : 1 -AGTSFWYAMDDHTQQYKETAP 21
β-1,3-glucanase precursor bgn1 | T. virens (AAL84694.1) : 32 RASTSFYWYAMDDHTQQYKETAP 53
β-1,3-glucanase precursor bgn2 | T. virens (AAL84695.1) : 33 RAYTYYPNDDHVNPAYKETAP 53
endo-β-1,3-glucanase | T. harzianum (CAA58889.1) : 35 RAYTYYPNDDHVNPAYKETAP 53
Lam AII | T. viride : 1 -HS-SYXYENIAXXAXAPX-A 18
exo-β-1,3-glucanase | T. harzianum (CAA05375.1) : 35 RAS-SXYWENIAXXFXAPX-AP 54

**Fig. 4.** Comparison of N-Terminal Amino Acid Sequences for Three Laminaran Hydrolases with Some Other Fungi β-Glucanases.

Numbers show the number of amino acid residues from the N-terminus of the total ORF. Amino acids identical in at least four of the sequences are marked in black.

Laminaran and laminaran are composed of a linear polymer of β-1,3-linked glucose residues and may contain small amounts of β-1,6-linkages. On the other hand, *Eisenia* laminaran is composed of β-1,3- and β-1,6-linkages in the ratio 3:2, and it has overall repeated sequences like 1→3, 1→6 with occasional branching points. Thus, it is suggested that the activity of these enzymes may depend on not only the type of glucose linkage but also the conformation of polysaccharide. Similar results have been observed with β-1,3-glucanases of *Bacillus circulans* and *Hordeum vulgare*. In addition, laminaribiose was not produced from laminarin and laminaran by lam AI and AII. Therefore future detailed investigation is essential for evaluating the more detailed structure of substrates and oligosaccharides produced by lam AI and AII in order to confirm and study the lack of laminaribiose in laminarin and laminaran hydrolyzates.

All reported enzymes that hydrolyze *Eisenia*...
laminaran were \( \beta-1,6 \)-glucanases that effectively hydrolyzed pustulan, a \( \beta-1,6 \)-glucan. A \( T. \ virens \) \( \beta-1,3 \)-glucanase found to have a high degree of identity to lam AI has not been characterized, because the gene sequence was cloned using degenerate primers against homology regions based on amino acid sequences of the glycoside hydrolase family (GH) 55.38 Therefore, lam AI from \( T. \ viride \) is considered a novel enzyme that recognized the \( \beta-1,3 \)-linkages of \( \beta-1,3;1,6 \)-glucan, and hydrolyzed it. The N-terminal amino acid sequence of lam AI was almost identical to that of \( T. \ harzianum \) exo-\( \beta-1,3 \)-glucanase, the molecular mass of which was 110 kDa.69

On the other hand, the results of substrate specificity and oligosaccharide hydrolyses indicated that lam B was a \( \beta-1,6 \)-glucanase that recognized the \( \beta-1,6 \)-linkages of laminaran and pustulan. Although very few microbial \( \beta-1,6 \)-glucanases have so far been characterized, comparison of their properties with lam B showed similarities in molecular mass and optimal pH. In fungi, the molecular masses of \( \beta-1,6 \)-glucanases are usually in the range from 30 to 45 kDa, and the optimal pHs of these enzymes are between pH 4.5 and 5.5.39,41

The major final products of pustulan were gentiobiose, gentiotriose, and two unidentified spots. Pustulan is a glucose polymer linked via \( \beta-1,6 \)-glucosidic bond with 10–20% \( O \)-acetylated, and these two spots disappeared after treatment with ammonia. Thus, it is suggested that these two spots were acetylated biose and triose. \( \beta-1,6 \)-glucanases were divided into two types in terms of hydrolyzates of pustulan. One is the type with which glucose and biose are the major final products produced from pustulan,32,41 the other has biose and triose as the major final products.21,22,42 Thus, lam B belongs to the latter type. Although two \( \beta-1,6 \)-glucanases (BGN16.1 and BGN16.2) have been purified from \( T. \ harzianum \),43,44 the pK value (3.0) of lam B was lower than that of BGN16.1 (7.4–7.7) and BGN16.2 (5.8). Besides a BLAST search of the N-terminal amino acid sequence indicated a conserved sequence, K-L-R-G-V-N-L-G, might be important for \( \beta \)-glucanases of glycoside hydrolase family 5 belongs to \( \beta-1,3 \)-glucanases, cellulases, \( \beta-1,6 \)-glucanases, and so on.16,110 The N-terminal amino acid sequence of lam B had low homology to other known \( \beta-1,6 \)-glucanases, suggesting that the purified lam B is a novel \( \beta-1,6 \)-glucanase of the genus \( Trichoderma \) which can hydrolyze \( E. \) laminaran.

These results showed that \( T. \ viride \) U-1 secreted multiple glucanolytic enzymes, including \( \beta-3,1;3,1 \)-glucanase (lam AI), \( \beta-1,3 \)-glucanase (lam AI1), and \( \beta-1,6 \)-glucanase (lam B), indicating that it enables \( T. \ viride \) U-1 to degrade the cell walls of a variety of fungi. Further understanding of the genes of these three enzymes is needed to clarify the correlation between structure and function of these enzymes. These results may explain the mechanism of cell-wall degradation of a variety of fungi.

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