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Purification and Characterization of β-Glucosidase from *Penicillium verruculosum*

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The β-glucosidase was purified to homogeneity from the culture filtrate of *P. verruculosum* by column chromatography. The enzyme was a glycoprotein with a relative size of approximately 220 kDa with an isoelectric point of 4.8, which was composed of dimeric protein of 105 kDa. The enzyme was stable up to 60°C and the presence of glycerol significantly increased its thermostability. The enzyme was found to hydrolyze both β-aryl and β-alkyl-glucosides in addition to β-glucosyl glucose and catalyzed glucosyl transfer to cellobiose. The enzyme attacked laminarin in an exotype-like fashion. The apparent Km's of the enzyme toward cellobiose, laminaribiose, laminarin were 0.53 mM, 0.35 mM and 1.11 mM, respectively. Glucose and glucono-δ-lactone were competitive inhibitors for the enzyme. Copper (Cu^{2+}), mercury (Hg^{2+}) and p-chloromercuribenzoate were strong inhibitors of the enzyme. The immunoblotting result revealed that one form of β-glucosidase was biosynthesized, irrespective of carbon sources used. Polyacrylamide gel electrophoresis analysis of the *in vitro* translated product of total RNA from avicel grown mycelium established that the *P. verruculosum* β-glucosidase precursor was approximately 95 kDa in size. The amino acid composition and N-terminal amino acid sequence are given.

Cellulose, the world's most abundant glucose polymer, may be hydrolyzed to glucose by cellulolytic fungi. The attack on highly crystalline cellulose has been shown to involve the cooperative action of three kinds of enzymes such as the endo- and exo-1,4-β-glucanases and β-glucosidase (43, 44, 46). Two types of hydrolytic reaction seem to operate during the enzymatic degradation of cellulose; endo-1,4-β-glucan glucanohydrolase (EC 3.2.1.4) cleaves a linear cellulose chain in a supposedly random fashion and 1,4-β-D-glucan cellobiohydrase (EC 3.2.1.9) in turn splits off cellobiose unit one at a time from a shrinking chain while β-glucosidase (EC 3.2.1.21) hydrolyzes cellobiose to glucose. The cellobiose released from cellulose hydrolysis is an inhibitor to both of exoglucanase and endoglucanase (1, 11, 42). Thus, the removal of cellobiose from reaction solution allows both exo- and endo-glucanase to function more efficiently. β-Glucosidase, therefore, plays an important role in cellulolysis.

Trichoderma species are the most intensively investigated organisms because of their favored sources of cellulase system important in industrial saccharification of cellulosic materials (25, 26). However, they have a low activity of β-glucosidase. To supplement the low activity of β-glucosidase of *Trichoderma* species, a number of studies have been performed on the isolation of other cellulolytic fungi (5, 6, 40) or mutants (32, 36) that can produce large quantities of β-glucosidase and that are more active in cellobiose hydrolysis (40, 46). Current attention has turned toward molecular cloning of gene encoding β-glucosidase for better understanding of its role in cellulolysis (16, 24, 28, 44, 45).

Three types of cellulase secreted by *P. verruculosum* (2, 3) have been reported to be higher in enzyme activity toward natural cotton than those observed for *Trichoderma* species (32). We are interested in molecular cloning of genes encoding cellulase of *P. verruculosum* and in synergistic action between three types of enzymes. As

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the first part of molecular cloning of β-glucosidase study, we describe the purification and characterization of β-glucosidase from the culture filtrate of *P. verruculosum* grown in Avicel medium.

MATERIALS AND METHODS

Chemicals

Microcrystalline cellulose (Avicel), carboxymethylcellulose (CMC) and metal compounds were obtained from E. Merck (Darmstadt, FRG), IEF carrier ampholites were from LKB (Bromma, Sweden). Sophorose was kindly provided by Dr. I. Susumu, Manager, Chief Research Biologist, Tochigi Research Laboratories, KAO Corp., Tochigi, Japan. All other chemicals were purchased from Sigma Chemical Co., (St. Louis, USA) and Amersham (Buckinghamshire, England).

Organism and Culture Conditions

The organism used was *Penicillium verruculosum* F-3 isolated by Chung *et al.* (2). The conidia for batch culture inoculum were allowed to germinate in 10% malt extract for 4 days at 23℃ and the mycelium germinated from conidia were inoculated into 1 *l* of defined medium containing 0.5% Avicel, 0.25% cellobiose, 1% cellobiose octaacetate and 0.2% CMC. The cultures were grown under shaking conditions at 125 rpm for 14 days at 30℃ (New Brunswick Scientific Co., Psychrotherm TM, NJ, USA).

Enzyme Purification

All purification steps were carried out at 4℃. The enzyme solution was concentrated and dialyzed by using ultrafiltration system (Vision Scientific Co., Korea) with 10,000 molecular weight cut-off membrane under 60 lb/in^2 of nitrogen unless otherwise described. The crude enzyme was obtained by centrifugation (8,000×g, 60 min) of the culture broth. To the supernatant, ammonium sulfate was added to give 75% saturation and the resulting pellet was obtained by centrifugation (20,000×g, 60 min). The pellet was dissolved in 50 mM citrate buffer (pH 5.0) and dialyzed exhaustively against the same buffer. The dialyzed protein was concentrated and loaded on a Sephacryl S-200 column (60×2.6 cm) equilibrated with 50 mM citrate buffer (pH 5.0) and eluted with the same buffer at a flow rate of 15 m*l*/h. The active fractions were pooled for total volume of 64 m*l* and dialyzed against 20 mM phosphate buffer (pH 7.0). A portion of the active fractions from gel filtration chromatography was dialyzed and applied to DEAE-Sephadex A-50 column (35×1.6 cm) equilibrated with 20 mM phosphate buffer (pH 7.0). The column was washed with 200 m*l* of the same buffer and then eluted with a 400 m*l* linear gradient of NaCl from 0.0 to 0.4 M in the same buffer. The active fractions (40 m*l*) were pooled and dialyzed exhaustively against 50 mM citrate buffer (pH 4.2). The dialyzed enzyme from anion exchange chromatography was concentrated and then applied to SP-Sephadex C-50 column (32×1.0 cm) equilibrated with 50 mM citrate buffer (pH 4.2). The column was washed with 100 m*l* of same buffer and then eluted with 100 m*l* of linear gradient of NaCl (0.0 to 0.5 M) in the same buffer. The active fractions were pooled and concentrated. The concentrated enzyme was suspended in 50 mM citrate buffer (pH 5.0) and stored at −20℃.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in vertical slab gel by the method of Laemmli (19). The isoelectric point of purified enzyme was determined on slab gel with ampholite covering the pH range of 3.5 to 9.0 by the general procedure of Giulian *et al.* (8). Staining for glycoprotein was performed by the periodic acid Schiff's base technique described by Glossmann and Neville (9).

Gel Filtration

The relative molecular weight of native enzyme was estimated by a molecular sieve chromatography on a Sephacryl S-300 column (60×1.6 cm; void volume, 42 m*l*) equilibrated with 50 mM citrate buffer (pH 5.0) and calibrated with apoferritin (443,000), β-amylase (20, 000), alcohol dehydrogenase (150,000) and bovine serum albumin (66,000). The proteins were eluted with the same buffer at a flow rate of 10 m*l*/h, and the eluate as fractionated (2 m*l* per fraction).

Enzyme Assay

The substrate for enzyme assay was cellobiose except the estimation of Ki value for glucose and glucono-δ-lactone where p-nitrophenyl-β-glucoside (PNPG) was used as the substrate. Cellobiase activity was estimated by determining the glucose released from cellobiose. Glucose was measured with a commercial assay kit (Sigma, Technical Bulletin No. 510). Enzyme solution was incubated with 10 mM citrate buffer (pH 5.0) for 30 min at 70℃. After incubation, the reaction mixture was boiled for 5 min and then cooled to room temperature. Five mililiter of the glucose oxidase reagent was added to the mixture and incubated for 30 min at 37℃. The amount of released glucose was determined spectrophotometically at 450 nm. PNPGase activity was measured by the same method as described above except stopping the reaction by the addition of 1.0 M sodium carbonate and reading the absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole glucose min^{-1} or 1 μmole

p-nitrophenol min^{-1}.

Protein Estimation

Protein concentration was routinely estimated according to the method of Lowry *et al.* (22) with bovine serum albumin as a standard.

pH Optimum and Stability

Optimal pH was determined by measuring the enzyme activity in the pH range of 2.0 to 8.0. The effect of pH on enzyme stability was measured over a range of 2.0 to 8.0 by using a different buffer system. After incubation of the enzyme over the pH range of 2.0 to 8.0 for 20 h at 30℃, the residual enzyme activity was measured under enzyme assay conditions.

Temperature Optimum and Stability

The temperature optimum was determined within the range of 20 to 90℃. Thermal stability was determined by the assay of residual enzyme activity after preincubation of enzyme at the temperature range of 20 to 90℃ for 2 hours.

Determination of Carbohydrate Content

The carbohydrate content of enzyme was determined with the phenol-sulfuric acid assay method of Dubois *et al.* (7) by using glucose as a standard.

Effect of Various Chemical Agents

The effect of metal ion on enzyme activity was determined by the addition of enzyme to the reaction mixture containing 1 mM cation in 50 mM acetate buffer (pH 5.0) and the residual enzyme activity was determined. The metals used were $CaCl_2$, $HgCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $ZnSO_4$, $Pb(NO_3)_2$ and $MnCl_2$. The enzyme inhibitors were EDTA, p-chloromercuribenzoate sulfonic acid (PCMBS), and glucono-δ-lactone. As anionic detergent and reducing agent, SDS and 2-mercaptoethanol were used.

Determination of K_m and V_{max}

K_m and V_{max} were determined by Lineweaver-Burk plots (21) using cellobiose, salicin, PNPG, gentiobiose, laminaribiose, sophorose, methyl-β-glucosides and laminarin as substrates.

Transglycosylase Activity

Two units of purified enzyme (1.4 μg) were incubated with cellobiose (10-40 mM) or cellobiose plus glucose (each of 10-40 mM) in 10 ml of 10 mM citrate buffer (pH 5.0). After start of incubation, a 100 μl aliquot was withdrawn at indicated times, and the products were analyzed with HPLC system (Waters Associates Ins/Milford, Massachusetts, USA) with Aminex HPX-87C carbohydrate column (Bio Rad Lab., Richmond, Calif. USA). Deionized water was used as solvent at flow rate of 0.5 ml/min at 75℃.

Amino Acid Composition

The amino acid composition of the purified enzyme was determined by using an applied Biosystem automatic amino acid analyzer (Millign Biosearch prosequencer 6600) after hydrolysis of the enzyme with 6 N HCl at 155℃ for 45 min. Cysteine and cystine were determined as cysteic acid after oxidation with performic acid.

Antiserum Preparation and Immunoblotting

Antiserum to β-glucosidase was prepared as described elsewhere (15). Immunoblotting of the samples prepared from various cultures was conducted as described by Kubicek *et al.* (18) except using horse-radish peroxidase coupled secondary antibody.

***In vitro* Translation of RNA**

Total RNA was isolated by the procedures established by McDonald *et al.* (23). Total RNA was translated *in vitro* by using a rabbit reticulocyte lysate (Amersham Corp.) according to the procedure described by Pelham *et al.* (33).

RESULTS

Enzyme Purification and Properties

A four step purification of the culture filtrates was performed by ammonium sulfate precipitation, gel filtration, anion- and cation-exchange chromatography. Gel filtration on Sephacryl S-200 was found to be essential in removing xylanase and redish pigments present in ammonium sulfate-precipitated pellet. Since the presence of these in pellet resulted in both degradation and contamination of DEAE-Sephadex A-50 polymer. The overall purification procedures for the *P. verruculosum* β-D-glucosidase is summarized in Table 1. The enzyme resulted in approximately 127-fold purification with a 15% activity yield. The enzyme purity at each purification step was analyzed by SDS-PAGE (Fig. 1), and the active peaks after cation exchange chromatography gave a single protein band with a molecular weight 105 kDa (Fig. 1; lane 5). The enzyme was found to be a glycoprotein (Fig. 1; lane 5). The relative molecular weight (Mr) of the purified enzyme was estimated to be approximately 220 kDa on a calibration column of Sephacryl S-300 gel filtration. Since the enzyme contains 12% carbohydrate by weight, it was difficult to obtain an accurate size by gel filtration. Thus, its Mr obtained here was provided for comparison with other enzymes. The pH optimum for the enzyme was 5.0. The enzyme was stable in a pH range of 4.5 to 6.0 (Fig. 2A). An estimate of the pI of the enzyme was approximately 4.8 as judged with the calibration markers (Fig. 2B). The enzyme activity increased as a linear function of temperature from 40 to 70℃, which was the temperature optimum (Fig. 3), and decreased rapidly above 70℃. The temperature stability of the enzyme in the absence of substrate decreased rapidly above 60℃. The thermal stability of the purified enzyme was significantly affected by glycerol. When

Table 1. Summary of purification of β-glucosidase from *P. verruculosum*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Culture filtrates	1,208	1,385	1.1	1.0	100
Ammonium sulfate precipitation	535	1,108	2.1	1.8	80
Sephacryl S-200 gel filtration	47	887	18.9	17	64
DEAE-Sephadex A-50 Ion-exchange chromatography	3.6	321	89.2	78	23
SP-Sephadex C-50 Ion-exchange chromatography	1.48	214	144.6	127	15

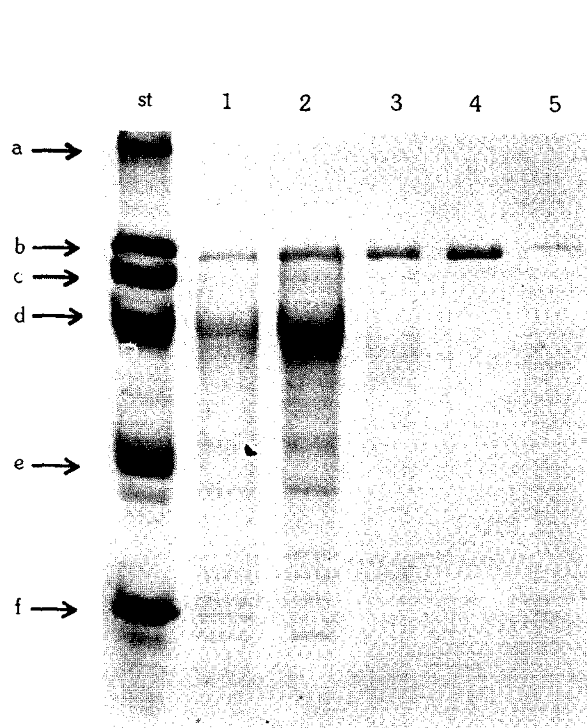

Fig. 1. SDS-PAGE of β-glucosidase. An acrylamide concentration of 8% was used, and gels were stained with 0.25% Coomassie blue R-250 (Lane, 1-4), and periodic acid Shiff's reagent (Lane, 5).

Lanes: 1, culture filtrates (100 μg protein); 2, after Sephacryl S-200 gel filtration (100 μg protein); 3, after DEAE-Sephadex A-50 chromatography (20 μg protein); 4 and 5, after SP-Sephadex C-50 chromatography (20 μg protein). Markers used were: a, Myosin (205 kDa); b, β-galactosidase 9116 kDa); c, phosphorylase (97.4 kDa); d, bovine serum albumin (66 kDa); e, albumin egg (45 kDa); f, carbonic anhydrase (29 kDa)

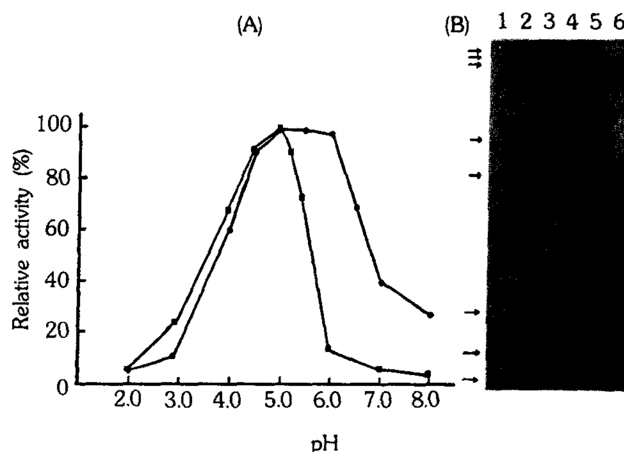

Fig. 2. Effect of pH on activity (■—■) and stability (●—●) of β-glucosidase from *P. verruculosum* (A) and analytical isoelectricfocusing of β-glucosidase (B).

The enzyme activity and stability was measured under conditions described in the text. pI markers used were: lane 1, amyloglucosidase (pI, 3.60); 2, glucose oxidase (pI, 4.2); 3, trypsin inhibitor (pI, 4.6); 4, myoglobin (pI, 6.8 and 7.2); 5, lactodehydrogenase (pI, 8.3, 8.4 and 8.6); 6, β-glucosidase.

the enzyme was incubated at 70℃ in the absence of glycerol, the enzyme was inactivated by 90% within 2 h. However, more than 80% of enzyme activity remained in the presence of 25% glycerol (Fig. 3). Effects of various compounds and bivalent metal ions on the activity were investigated. The activity of the enzyme was reduced to 89% by 1 mM Hg^{2+} and 73% by 1 mM Cu^{2+}. The enzyme activity was also inhibited 73% by 0.5-1.0% SDS, 76% by 1% mercaptoethanol and 96% by 1 mM PC-MBS, respectively. No other chemicals significantly affec-

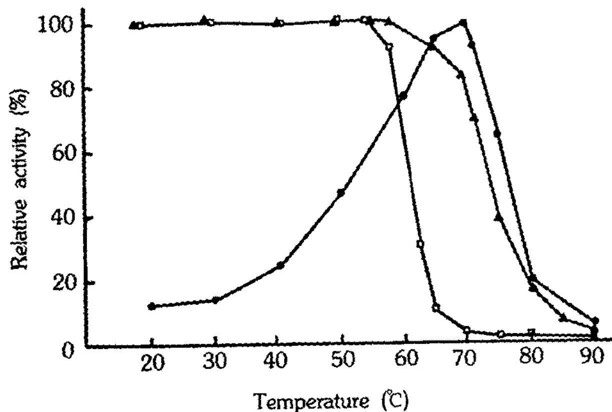

Fig. 3. Effect of temperature on activity (●—●) and stability of purified β-glucosidase from *P. verruculosum*.

The enzyme activity was assayed at the temperature ranging from 20 to 90℃. To establish the thermostability, the enzyme was assayed after incubation at the indicated temperature for 2 h in the absence of substrate, with (▲—▲) or without (□—□) the addition of 25% glycerol to reaction mixture.

ted the enzyme activity (Data not shown).

Kinetics

Values for Michaelis-Menten type saturation kinetics were obtained with cellobiose, PNPG, gentiobiose, salicin, methyl-β-glucosde, laminaribiose, sophorose and laminarin (Table 2). The K_m of the enzyme for laminarin was estimated to be 3.3 mg/m*l*. Assuming a degree of polymerization of 15 for laminarin, this is equivalent to a substrate concentration of approximately 1.11 mM. The enzyme produced only glucose without the release of laminaribiose or laminarioligosaccharides (Fig. 4), suggesting that the enzyme attacked laminarin in an exotype-like fashion. Substrate inhibition was observed when the concentration of cellobiose or PNPG was higher than 10 mM and 5 mM, respectively. Glucose and glucono-δ-lactone were competitive inhibitors of the enzyme as judged by a common intercept on 1/V axis of Lineweaver-Burk plot (25). The dissociation constant (K_i) obtained from the secondary plot was 1.1 mM for glucose and 19.4 μM for gluconolactone. When PNPG was used as the substrate, the K_m/K_i values for glucose and glucono-δ-lactone were 0.48 and 27.3, respectively.

Transglycosylase Activity

The result from the study of transglycosylase activity of β-glucosidase is shown in Fig. 5. Incubation of cellobiose (40 mM) with enzyme (2U) resulted in the generation of trisaccharide (G_3) which corresponded to the retention time of the cellotriose as a standard. Trisaccharide was detected 15 min after incubation, reached the maxi-

Table 2. K_m and V_{max} values of various substrates for β-glucosidase from *P. verruculosum*

Substrates	K_m(mM)	V_{max} (10^{-6} mole sec^{-1})
Cellobiose	0.53	0.58
PNPG	0.20	0.29
Salicin	0.32	0.13
Gentiobiose	0.61	0.51
Laminaribiose	0.35	0.82
Methyl β-glucoside	6.49	0.31
Sophorose	0.43	0.31
Laminarin	1.11	0.11

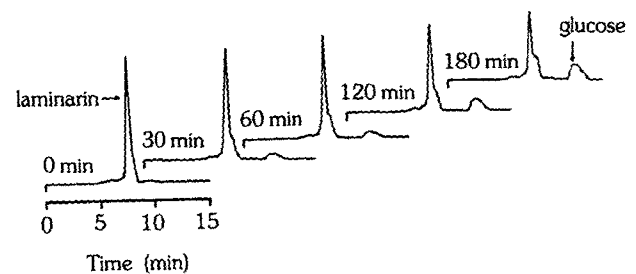

Fig. 4. HPLC analysis of the products by β-glucosidase on laminarin.

The β-glucosidase was incubated at 70℃ with laminarin (1 mg/m*l*) in 10 mM citrate buffer (pH 5.0). After start of incubation the reaction products were periodically taken and assayed.

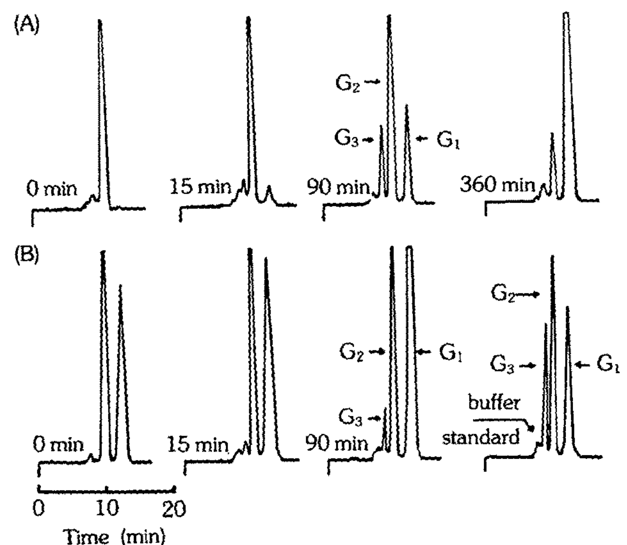

Fig. 5. HPLC analysis of the products by β-glucosidase on cellobiose alone (A) or a mixture of cellobiose and glucose (B).

β-Glucosidase was incubated at 70℃ with 40 mM cellobiose or in combination with 40 mM glucose in 10 mM citrate buffer (pH 5.0).

mum content (approximately 10% of total saccharides in the initial cellobiose of 40 mM) at the next 75 min and at the end of 6 h, was converted to glucose via disaccharide. Transglycosylation product was not detected at the initial concentration of cellobiose lower than 10 mM. Similar profile was observed in admixture of cellobiose (40 mM) and glucose (40 mM). However, the amount of transglycosylation product (G_3) in the presence of glucose was less than 40% of the maximum at the absence of glucose, suggesting that glucose inhibits transglycosylase activity.

Multiplicity of Extracellular β-Glucosidase on Various Carbon Sources

With respect to the extracellular β-glucosidase of fungi, the existence of multicomponents differing in physico and/or chemical properties has been reported (2, 30, 31). Most of these have been concerned with the culture filtrate of different ages of mycelium grown on the medium containing one type of carbon source. To examine the existence of the multicomponents of *P. verruculosum* β-glucosidase, protein samples in the culture filtrate of mycelia grown on various carbon sources were separated by SDS-PAGE (Fig. 6). The β-glucosidase corresponding to 105 kDa was detected by immunoblotting using antiserum specific to the purified β-glucosidase from Avicel-grown mycelium. A single immunoreactive band corresponding in molecular weight to 105 kDa indicates that *P. verruculosum* produces only one form of β-glucosidase (Fig 6).

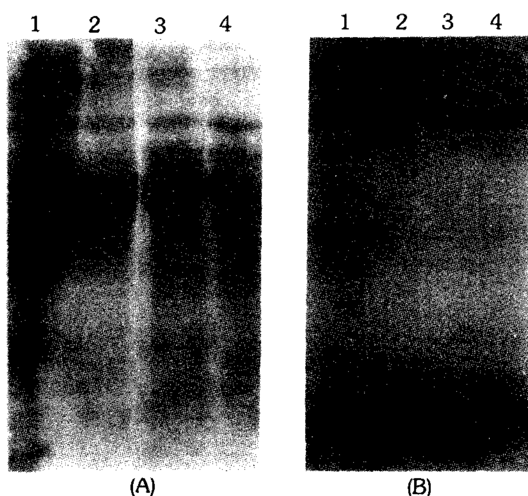

Fig. 6. SDS-PAGE (A) and immunoblot (B) of β-glucosidases secreted by *P. verruculosum* on avicel, cellobiose, COA and CMC.
Each sample was harvested after the cultivation at optimal concentration of carbon sources for 14 days at 30℃ and 1 *ml* of sample was applied. Lanes: 1, avicel; 2, cellobiose; 3, COA; 4, CMC

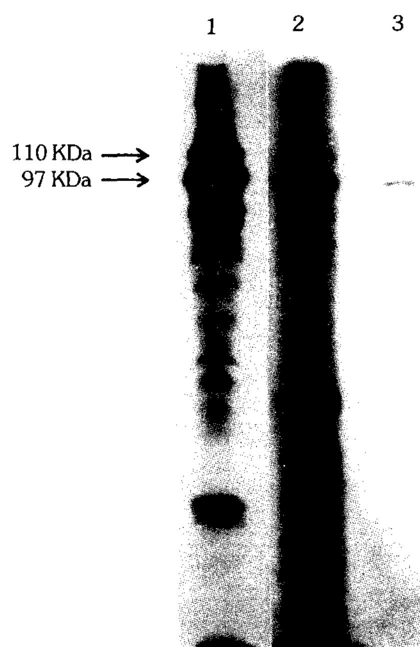

Fig. 7. Translation of total RNA in rabbit reticulocyte lysate.
The RNAs were translated in a 50 *μl* of reaction mixture for 120 minutes. The translated products were fractionated by SDS-PAGE and then subjected to fluorography. Lanes: 1, mRNA from Brome Mosaic Virus; 2, Total RNA from *P. verruculosum*; 3, Immunoprecipitation of translated product of total RNA from *P. verruculosum*.

Table 3. Amino acid composition of β-glucosidase from *P. verruculosum*

amino acids	% by weight	molar %
aspartic acid	8.4	7.8
glutamic acid	7.4	6.2
serine	7.1	8.3
glycine	12.9	21.2
histidine	1.5	1.2
arginine	4.5	3.2
threonine	7.3	7.6
alanine	10.4	14.4
proline	7.0	7.5
tyrosine	3.2	8.1
valine	7.7	1.7
methionine	2.1	3.1
cysteine	1.0	1.1
isoleucine	3.3	3.1
leucine	6.8	6.4
phenylalanine	3.5	2.6
tryptophan	2.3	1.4
lysine	3.5	3.0

Identification of the β-Glucosidase Precursor

To prove the synthesis of only one form of β-glucosidase at molecular level, we attempted the *in vitro* translation of total RNA from avicel grown mycelium of *P. verrucolosum*. The immunoprecipitation result obtained by using translation products is presented in Fig. 7. The single band corresponding to the approximate molecular weight of 95 kDa indicates that there is only one polypeptide precursor of the 105 kDa β-glucosidase.

Amino Acid Composition

The amino acid composition of β-glucosidase is given in Table 3. The enzyme contains a low level of basic and hydrophobic (except Ala, Val, and Pro) amino acids and a high level of acidic and polar (except Try) amino acids. The NH_2-terminal amino acid sequence was determined by using a purified β-glucosidase. The determined amino-terminal sequence was NH_2-Asp-Tyr-Ser-Pro-Ileu-Ala-Tyr-X-Ala-Gly, where X means unknown amino acid.

DISCUSSION

P. verruculosum secretes a complete cellulase complex containing endo- and exo-glucanase and β-glucosidase required for hydrolysis of crystalline cellulose (2, 3). When this organism was grown in the mineral medium containing cellobiose octaacetate as the cellulase inducer (3), the cellulase activity is either higher than that of *T. ressei* (3, 35, 40) or comparable to its mutants (3, 32) that are good source of extracellular cellulase.

In this study, extracellular β-glucosidase was purified to homogeneity from the culture filtrate of *P. verruculosum* grown in avicel medium. The purified enzyme was a dimeric glycoprotein with approximately molecular weight of 220 kDa consisting of two 105 kDa subunits. Irrespective of carbon sources tested, we found that *P. verruculosum* produced only one form of extracellular β-glucosidase as evidenced in the immunoblotting analysis. This result is in contrast to that observed in *Macrophomina phaseolina* (37), which was shown to produce two different forms of β-glucosidase when grown in either cellulose or noncellulose medium. The immunoprecipitation result by the β-glucosidase related translation product made in rabbit reticulocyte cell-free system revealed that there was only one polypeptide precusor of about 95 kDa in size, which confirmed the biosynthesis of one form of β-glucosidase at molecular level. The difference in the molecular weight of 10 kDa from its final 105 kDa product could arise from the combined processes of both glycosylation and proteolysis. The enzyme is glycosylated protein in which carbohydrate content accounts for 12%. Proteolysis might occurs as well for the remoal of β-glucosidase secretion signal peptide during secretion as in other β-glucosidase (24).

The physico-chemical properties of β-glucosidases from different fungi have been summarized by Coughlan (4) as well as Woodward and Wiseman (45). Compared with the properties of β-glucosidases from different fungi, the enzyme consists of two identical subunits as the β-glucosidases from *Aspergillus funigatus* (45), *A. oryzae* (4), and *Alternaria alternata* (27), and its Mr is similar to that of *A. oryzae* (4).

Other chemical properties of *P. verruculosum* β-glucosidase were found to be similar to those of other fungal β-glucosidase (4, 27, 45). The optimal activity of enzyme at 70℃ exhibited the highest optimal temperature among other enzymes of mesophilic fungi. This value has been observed in β-gluosidases from thermophilic fungi; *Talaromyces emersonii* (4, 28) and *Thermoascus aurantiaca* (41, 45). The purified enzyme was stable up to 60℃, similar to other enzymes from mesophilic fungi (4, 27, 54). The thermal stability was increased by the addition of glycerol and the enzyme was stable up to 70℃ at the concentration of 25% glycerol. The increased thermal stability of the enzyme by glycerol could in part arise from the result of stabilizing of the enzyme conformation by a preferential exclusion of glycerol from protein domain as has been examined in Chymotrypsinogen A. However, the actual mechanism by which increases the thermal stability of enzyme by glycerol is not clear.

The affinity of *P. verruculosum* β-glucosidase for cellobiose was similar to the β-glucosidases from other fungal sources (4, 38, 45, 46). However, the *P. verruculosum* β-glucosidase is able to hydrolyze laminaribiose better than cellobiose, which was reported in *Trichoderma koningii* (44) and *Sporotrichum thermophile* (30). In addition, the enzyme is also able to attack laminarin in an exotype-like fashion as has been observed in laminarinase of *Candida albicans* (31) and *Neurospora crassa* (14). The ability of the *P. verruculosum* β-glucosidase to hydrolyse laminarin was similar to β-glucosidase from autolyzed mycelium of *Alternaria alternata* (27). But the hydrolysis rate of the *P. verruculosum* enzyme was much higher than that reported for the *Alternaria alternata* enzyme with the K_m being 1.1 mM for the former and 50 mM for the latter, and the V_{max} of *P. verruculosum* β-glucosidase was three times that value of *Alternaria alternata*. These characteristics, along with high optimal temperature for the enzyme activity, was considered by authors to be important enough for futher enzymetic study of *P. verruculosum* β-glucosidase and a possible supplement source of enzyme for saccharification of laminarin.

Glucose competitively inhibited the enzyme as it did most of β-glucosidase from other sources (29, 44, 46). Gluconolactone is the more potent inhibitor of the enzyme than glucose, which is similar to the β-glucosidases

from *Alternaria alternata* (27) and *T. koningii* (44). To our knowledge, the inhibition of the enzyme activity by copper ions appears to be rare among fungal β-glucosidases. The incubation of enzyme in the presence of Cu^{+2} caused significant loss in the activity. This result was similar to β-glucosidase from *Pyricularia oryzae* (13) and *T. reseei* QM 414 (39), in which the relative inhibition has been reported to be 91% (1 mM Cu^{+2}) and 21% (0.2 mM Cu^{+2}), respectively. The inhibition of enzyme by Cu^{+2} and 2-mercaptoethanol may indicate a pssible involvement of sulfhydryl groups at or near enzyme active sites, as supported by strong inhibition of enzyme activity by PCMBS.

Transglycosylation product was observed at higher concentration of cellobiose than 10 mM whereas only glucose was formed at lower concentration. In this respect the *P. verruculosum* enzyme was very similar to the β-glucosidase from *A. foetidus* (11) where the initial cellobiose concentration for formation of transglycosylation product was also higher than 10 mM. The amino acid composition of *P. verruculosum* β-glucosidase is similar to that found in a β-glucosidase from *Schizophyllum commune* (6) in that both enzymes contain a low level of basic amino acids. Comparison of the 11-amino-terminal sequence of *P. verruculosum* β-glucosidase with the corresponding sequence of other microorganism (10, 17, 20, 24, 34) showed that there is no discernable homology between them. But the data presented in this paper could be useful for the comparison of the amino terminal sequence of other fungal β-glucosidases. We are in progress toward the β-glucosidase expression and regulation at the level of stable mRNA and posttranslation, and the cDNA gene cloning of the *P. verruculosum* β-glucosidase.

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