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Characterization of Recombinant Yeast Exo-β-1,3-Glucanase (Exg 1p) Expressed in *Escherichia coli* Cells

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Yeast exo-β-1,3-glucanase gene (*EXG1*) was expressed in *Escherichia coli* and the recombinant enzyme (Exg1p) was characterized. The recombinant Exg1p had an apparent molecular mass of 45 kDa by SDS-PAGE and the enzyme has a broad specificity for β-1,3-linkages as well as β-1,6-linkages, and also for other β-glucosidic linked substrates, such as cellobiose and pNPG. Kinetic analyses indicate that the enzyme prefers small substrates such as laminaribiose, gentiobiose, and pNPG rather than polysaccharide substrates, such as laminaran or pustulan. With a high concentration of laminaribiose, the enzyme catalyzed transglucosidation forming laminarioligosaccharides. The enzyme was strongly inhibited with high concentrations of laminaran.

Key words: exo-β-1,3-glucanase gene (*EXG 1*); *Saccharomyces cerevisiae*; recombinant exo-β-1,3-glucanase (Exg1p)

Glucans containing β-1,3-linkages are the main structural components responsible for the strength and integlity of yeast cell walls. The dynamic structural reorganization of cell wall structure during the morphogenetic process may require the concerted action of enzymes capable of breaking existing bonds and forming new covalent bonds in a highly controlled process.[1,2] β-1,3-glucanases are located in the fungal cell wall and may be important in wall metabolism during growth and morphogenesis.[3,4] *EXG1* encodes the major exo-β-1,3-glucanase of yeast, *Saccharomyces cerevisiae*[5] and an *EXG1* deletion mutant showed marked resistance to yeast killer toxin.[6] The *S. cerevisiae EXG1* product (Exg1p) has not been well characterized.[7,8,9]

To know the precise enzymatic properties and function of Exg1p, *Saccharomyces cerevisiae EXG1* was expressed in *Escherichia coli* cells and the recombinant enzyme was purified and characterized.

Materials and Methods

Materials. DNA restriction and modification enzymes were purchased from Takara Shuzo. Laminaran, pachyman, pustulan, schizophyllan, laminaribiose, and gentiobiose were obtained from the laboratory collection.[10,11] All other materials were available from the usual commercial sources.

Microbial strains, plasmids, and media *Saccharomyces cerevisiae* A451 (*MATa aro7 can1 leu2 trp1 ura3*) was donated by Dr. F. Hishinuma (Mitsubishi Kagaku Institute of Life Science). *Escherichia coli* DH5α, *E. coli* BL21 (DE3), and *E. coli* XL1- Blue were purchased from Takara Shuzo. *E. coli* cells were grown in LB medium (1% BactoTrypton, 1% Bacto yeast extract, and 1% NaCl), and 50 μg/ml ampicillin was added when necessary. Plasmids pUC119, pET-32a were purchased from Takara Shuzo.

Preparation of EXG1 and Expression. The *EXG1* gene was prepared by PCR using the following primers designed for adapting the *Bam*HI site: 5′-CGGGATCCCGATGCTTTCGCTTAAAACGTTA-3′ (sense) and 5′-TGTGGCACAATTTCTAACTAACGGGATCCCG-3′ (antisense), based on *S. cerevisiae EXG1* gene.[5] The underlined letters in the sequences are *Bam*HI sites. Genomic DNA of *S. cerevisiae* A451 was used as the template DNA for PCR. The synthesized DNA was subcloned into pUC119 and sequenced to confirm the absence of any undesired mutation.

To express the *EXG1* product in *E. coli*, the restriction enzyme *Nco*I cleavage site was introduced into the *E. coli* expression vector, pET-15b, and a *Nco*I-*Bam*HI fragment encoding the mature sequence of *EXG1* was inserted into the vector. The resulting vector (pETEXG) was transfered to *E. coli* BL21 (DE3). The transformed cells ware cultured at

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Abbreviations: Exg 1p, exo-β-1,3-glucanase of *Saccharomyces cerevisiae*; CMC, carboxymethyl cellulose; pNPG, p-nitrophenyl-β-D-glucoside; Glc, Glc2, Glc3, etc; D-glucose, glucobiose. glucotriose etc,. PCR, polymerase chain reaction

37°C in LB medium containing 50 μg/ml ampicillin, until the O.D. (A600nm) reached 0.5; then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was continued for 20 h at 15°C. The cells were harvested by centrifugation.

Purification of recombinant Exg1p. The harvested cells were resuspended in 50 mM sodium acetate buffer, pH 5.5, and ruptured by sonication. The cell homogenate was centrifuged and the supernatant was dialyzed against the same buffer. The supernatant solution was put on a DEAE-Toyopearl column (Toso Co.) and the enzyme activity was eluted with the same buffer with a linear gradient of 0–0.4 M NaCl. The fractions containing exo-β-1,3-glucanase activity were pooled. The pooled fraction was further purified by FPLC on a HiTrapQ anion-exchange column (Amersham Pharmacia Biotech). The active fraction was eluted with 50 mM the same buffer with a linear gradient of 0–0.5 M NaCl. Finally, the active fraction was purified by gel filtration on a Hi-Load Superdex 75 column (Amersham Pharmacia Biotech). The enzyme was eluted with 50 mM sodium acetate buffer, pH 5.5, and used as a purified enzyme preparation.

Enzyme assays β-1,3-Glucanase activity was measured by the following method in a 100 μl reaction mixture consisting of 50 mM sodium acetate buffer (pH 5.5), 0.5 mg of laminaran, and enzyme solution, 30 μl. The mixture was incubated at 30°C for 20 min and the released reducing sugar was measured by the method of Somogyi.[12] One kat of the activity is defined as the amount of enzyme required to produce 1 mol of glucose per 1 sec.

Products analysis, relative activity assay, and transglucosydation assay were done by HPLC (TSK-gel G2000PW, Toso Co.) analysis of incubations of various substrates (50 mg/ml for polysaccharides; laminaran, pustulan, schizophyllan, CMC), (10 mg/ml for oligosaccharides; laminaribiose, cellobiose, gentiobiose, and pNPG) in 50 mM sodium acetate buffer (pH 5.5) at 30°C for the appropriate time with recombinant Exg1p.

N-Terminal sequence analysis. The purified enzyme was put through amino acid sequence analysis on an Applied Biosystems 473A protein sequencer with 610A data analysis system.

Results

Properties of the recombinant Exg1p

The recombinant Exg1p was purified through three steps and the purified enzyme gave a single 45 kDa protein band on SDS-PAGE (Fig. 1) The purified protein showed a specific activity of 426.7 mkat/kg toword laminaran. In N-terminal amino acid analysis, the initial 10 residues was GRYYDYDHGS, which was identical to the predicted sequences of mature protein.[5] The highest activity of the Exg1p toword laminaran was found at pH 4.0 under standard conditions and stable in the pH range 3 to 10 (Fig. 2). The temperature optimum of this enzyme was 40°C and stable up to 50°C on heating for 30 min. (Fig. 3)

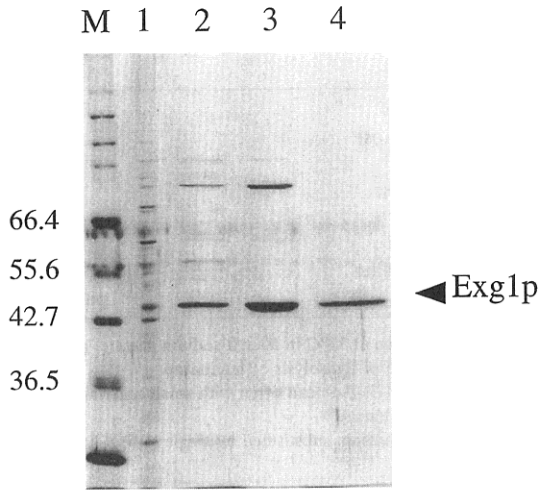

Fig. 1. SDS-PAGE Analysis of Recombinant EXG1p.
SDS-PAGE was done by the method of Laemmli.[16] The gel was stained with a silvest stain kit (Nakarai tesque Co.). M, protein standards; 1, cell extract; 2, preparation from DEAE-Toyopeal 650S; 3, preparation from Hitrap-Q; 4, preparation from Superdex-75

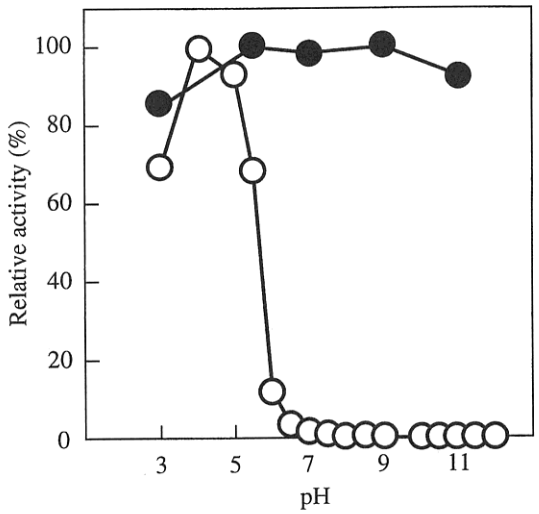

Fig. 2. Effectσ of pH on the Exg1p Avtivity.
pH optimum (○); The enzyme activity was asayed under standard condition except that various buffers. pH stability (●); The enzyme activity was assayed under the standard conditions except that each enzyme was treated with various pH for 30 min.

Substrate specificity

Relative rates of hydrolysis of various saccharide substrates by recombinant Exg1p (Table 1) showed that the enzyme was active on both β-1,3- and β-1,6-

Table 1. Relative Rate for the Exg1p Hydrolytic Reaction[a]

Substrate	Structure	Relative rate (%)[b]
laminaran[c]	[-3)Glc(β1-3)Glc(β1-]n	100
laminaribiose	Glc(β1-3)Glc	376
pustulan[d]	[-6)Glc(β1-6)Glc(β1-]n	41
gentiobiose	Glc(β1-6)Glc	21
CMC	[-4Glc(β1-4)Glc(β1-]n	0
cellobiose	Glc(β1-4)Glc	3
schizophyllan[e]	Glc(β1-6)[-3)Glc(β1-3)Glc(β1-3)Glc(β1-]n	0
pNPG	para-Nitrophenyl-β-D-Glucoside	22

[a] Reactions were done at 30°C in 50 mM sodium acetate, pH 5.5 for 5 min and liberated glucose was measured by HPLC analysis (Details are in the text).
[b] Relative to the rate of hydrolysis of laminaran
[c] Essentially linear (1-3)-β-glucan often with small and variable amounts of side brancing through (1-6)-linkages[17]
[d] A linear (1-6)-β-D-glucan[18]
[e] (1-3)-β-Glucan mainchain substituted by single (1-6)-β-linked glycosyl residues in every three (1-3)-β-linked glucosyl residues[19]

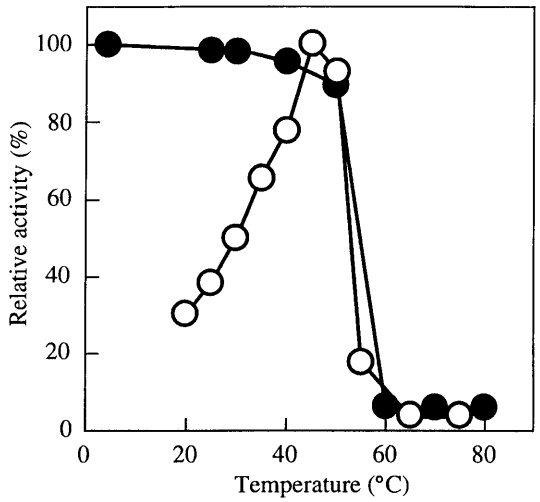

Fig. 3. Effects of Temperature on the Exg1p Activity.
Temperature optimum (○); The enzyme activity was assayed under standard conditions except for various temperature. Temperature stability (●); The enzyme activity was assayed under standard conditions except for the enzyme being heated for 30 min at various temperature.

glucans, but had no activity with CMC. It is noted that the enzyme did not hydrolyze schizophyllan (β-1,3-glucan with β-1,6-linked single branches). Among oligosaccharide substrates, laminaribiose gave the highest activity and gentiobiose showed about one thirties of that of laminaribiose. The enzyme also hydolyzed to some extent, pNPG. Kinetic parameters (Table 2) support these results. The enzyme has a significant preference for β-1,3-glucosidic linkages, while activity toword β-1,6-glucosidic linkages was much lower affinity (higher *K*m) and catalytic efficiency (*k*cat). When Exg1p was incubated with various concentration of laminaran, a Michaeris-Menten plot showed substrate inhibition at high substrate concentrations (Fig. 4).

Mode of action

The HPLC profiles (Fig. 5) demonstrated that the enzyme hydolyzed laminaran and pustulan in an exo-type manner, releasing D-glucose.

Table 2. Kinetic Constants for Exg1p Hydrolytic Reaction[a]

Substrate	*V*max (μM/sec)	*K*m (mM)	kcat (sec-1)	kcat/*K*m ($sec^{-1}mM^{-1}$)
laminaran	1.24	13.0[b]	17.1	—
laminaribiose	33.5	9.8	184.6	18.8
pustulan	1.03	24.8[b]	14.2	—
gentiobiose	1.01	11.1	2.80	0.25
pNPG	0.20	2.82	5.51	1.95

[a] Reactions were done at 30°C in 50 mM sodium acetate, pH 5.5. Liberated glucose was measured by HPLC analysis (Details are in the text).
[b] mg/ml.

We checked transglucosidase activity of this enzyme. Figure 6 shows the effects of transglucosidase on hydrolysis of laminaribiose by recombinant Exg1p. Under the low substrate concentration (2.9 mM), laminaribiose was hydrolyzed completely to glucose, while a high concentration (14.6 mM) of the substrate caused a transglucosidation reaction to form trisaccharide and tetrasaccharide during the first 20 min. The transglucosidase products of disaccharide and trisaccharide were laminaribiose and laminaritriose which were identified by ^{1}H- and ^{13}C-NMR respectively (data not shown), and trisaccharide was completely hydrolyzed to glucose by the *Aspergillus saitoi* β-1,3-glucosidase treatment.[13]

Discussion

Exo-β-1,3-glucanase genes (*EXG*) in yeast have been detected in *Saccharomyces cerevisiae*[5] and *Candida albicans*.[14] The *Candida* exo-β-1,3-glucanase (Exg) has been well characterized since the enzyme protein was secreted into the medium.[14,15] On the other hand, *S. cerevisiae* usually does not secrete the exo-β-1,3-glucanase in the medium[7,9]. The enzyme must be bound to cell walls.[9] We tried to prepare this enzyme by expression of *E. coli* using *S. cerevisiae* exo-β-1,3-glucanase gene (*EXG1*).[5]

The recombinant exo-β-1,3-glucanase had very in-

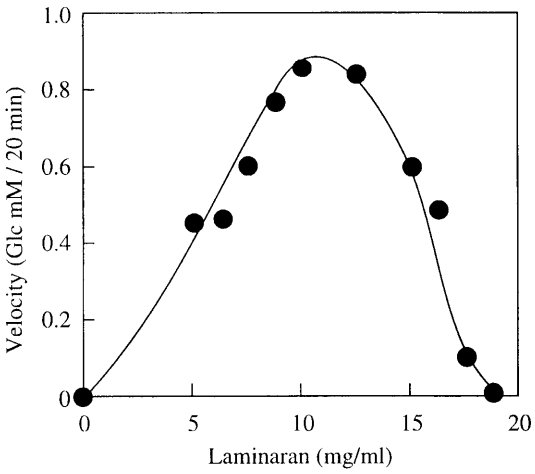

Fig. 4. Effects of Laminaran Concentration on Velocity of the Exg1p.

Reactions were done under the standard condition except for substrate concentration. Each reaction contained 0.34 μg (0.4 nkat) Exg1p and varying amount of laminaran. Liberated glucose was measured by the Somogyi-Nelson method.

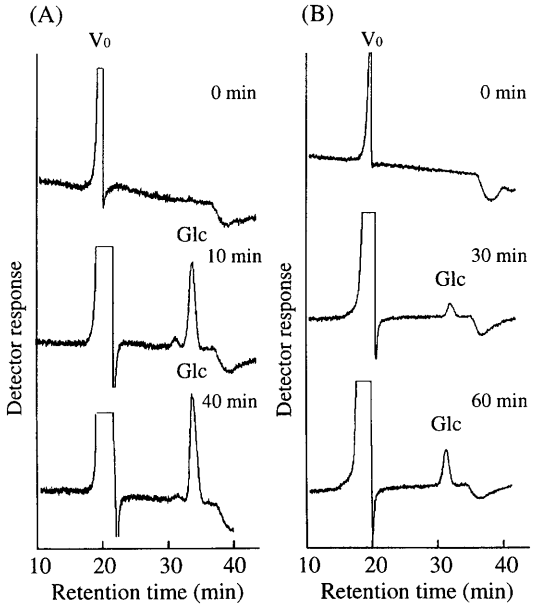

Fig. 5. The course of Hydrolysis of β-glucan Substrates Follwed by Separation on TSKgel 2000 PW.

(A) laminaran, (B) pustulan, Reactions were done undr standard conditions except for reaction time.

teresting properties. The enzyme has very broad specificity for β-glucosidic linkages and strong transglucosidase activity. The enzyme is able to hydrolyze β-1,3-glucan as well as β-1,6-glucan by an exo-type mode of action, yielding only D-glucose. The enzyme did not act on schizophyllan, the structure of which is a branched β-1,3-glucan with one unit of three glucose residues of the repeat unit being substituted at O-6 with a single β-D-glucose residue as shown in Table 1. The reason why this β-1,3- and β-1,6-branched structure was not a substrate for this enzyme is not known.

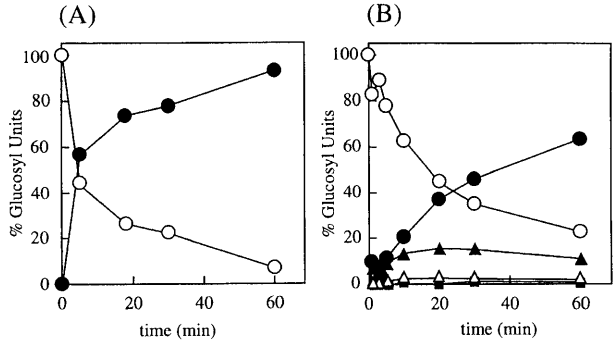

Fig. 6. The Course of the Degradation of Laminaribiose and the Formation of Transglucosidation Products at Low (A) and High (B) Concentrations of Laminaribiose.

Reaction mixture contained (A) 2.9 mM or (B) 14.6 mM of laminaribiose, with 0.17 μg (0.2 nkat) of Exg1p in 20 μl of sodium acetate buffer (50 mM, pH 5.5). Reactions were done at 30°C. laminarobiose (○), Glc (●), Glc3 (▲), Glc4 (△), Glc5 (■).

Kinetic analyses indicate that this enzyme is a kind of β-glucosidase that prefers small β-glucosidic linked substrates including pNPG. At the present time, the Exg1p seems to belong to the category of exo-β-1,3-glucanase (EC 3.2.1.58), but the results of this study indicate that the yeast Exg1p may be classified as a new type of β-glucanase or β-glucosidase that has not been described before.

It was also noted that the enzyme was strongly inhibited at high concentrations of laminaran. These characteristic of this enzyme such as broad specificity, strong transglucosidation activity, and inhibition of high concentrations of laminaran suggests that Exg1p is involved in yeast cell wall morphogenesis, in the process of assembly and rearrangement of β-1,3- and β-1,6-glucans.

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References

1) Nombela, C., Molina. M., Cenamor, R., and Sanches, M., Yeast β-glucanases: a complex system of secreted enzymes. *Microbiol. Sci.*, **5**, 328–332 (1988).
2) Goldman, R. C., Sullivan, P. A., Zakula, D., and Capoblanco: Kinetics of β-1,3-glucan interaction at the donor and acceptor sites of the fungal glucosyltransferase encoded by the BGL gene. *Eur. J. Biochem.*, **227**, 372–378 (1995).
3) Muthukumar, G., Suhng, S., Magee, P. T., Jewell,

R. D., and Primerano, D. A., The *Saccharomyces cerevisiae SPR1* gene encodes a sporulation-specific exo-β-1,3-glucanase which contributes to ascospore thermoresistance. *J. Bacteriol.*, **175**, 386–394 (1993).
4) Hien, N. H., and Fleet, G. H., Variation of 1,3-β-glucanases in *Saccharomyces cerevisiae* during vegetative growth, conjugation and sporulation. *J. Bacteriol.*, **156**, 1214–1220 (1983).
5) Vazquez de Aldana, C. R., Correa, J., Segundo, P. S., Bueno, A., Nebreda, A. R., Mendez, E., and del Rey. F., Nucleotide sequence of the exo-1,3-β-glucanase-encoding gene, *EXG1*, of the yeast *Saccharomyces cerevisiae. Gene*, **97**, 173–182 (1991).
6) Jung, R., Ram, A. F., Sheraton, J., Klis, F. M., and Bussey, H., Regulation of cell wall β-glucan assembly, *PTC1* negatively affects *PBS2* action in a pathway that includes modulation of *EXG1* transcription. *Mol. Gen. Genet.*, **248**, 260–269 (1995).
7) Brock, T. D., β-Glucanase of yeast. *Biochem. Biophys. Res. Commun.*, 19, 623–629 (1965).
8) Santos, T., delRey, F., Conde, J., Villanueva, J. R., and Nombela, C., *Saccharomyces cerevisiae* mutant defective in exo-1,3-β-glucanase production. *J. Bacteriol.*, 139, 333–338 (1979).
9) Hien, N. H., and Fleet, G. H., Separation and characterization of six 1,3-β-glucanases from *Saccharomyces cerevisiae. J. Bacteriol.*, 156, 1204–1213 (1983).
10) Shiota, M., Nakajima, T., Satoh, A., Shida, M., and Matsuda, K., Comparison of β-glucan structures in a cell wall mutant of *Saccharomyces cerevisiae* and the wild type. *J. Biochem.*, (Tokyo), **98**, 1301–1307 (1985).
11) Hiura, N., Kobayashi, M., Nakajima, T., and Matsuda, K., Purification and some properties of two wall-associated β-1,3-glucanases from *Neurospora crassa* cells. *Agric. Biol. Chem.* **50**, 2461–2467 (1986).
12) Somogyi, N. J., Notes on sugar determination. *J. Biol. Chem.*, **195**, 19–23 (1952).
13) Kasahara, S., Nakajima, T., Miyamoto, C., Wada, K., Furuichi, Y., and Ichishima, E., Characterization and mode of action of exo-β-1,3-glucanase from *Aspergillus saitoi. J. Ferment. Bioeng.*, **74**, 238–240 (1992).
14) Chambers, R. S., Broughton, M. J., Cannnon, R. D., Carne, A., Emerson, G. W., and Sullivan, P. A., An exo-β-1,3-glucanase of *Candida albicans*: purification of the enzyme and molecular cloning of the gene. *J. Gen. Microbiol.*, **139**, 325–334 (1993).
15) Stubbs, H. J., Brasch, D. J., Emerson, G. W., and Sullivan, P. A., Hydrolase and transferase activities of β-1,3-glucanase of *Candida albicans. Eur. J. Biochem.*, **263**, 889–895 (1999).
16) Leammli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
17) Nelson, T. E., and Lewis, B. A., Separation and characterization of the soluble and insoluble components of laminaran. *Carbohydr. res.* **33**, 63–74 (1974).
18) Hiura, N., Nakajima, T., and Matsuda, N., Purification and some properties of an endo-β-1,6-glucanase from *Neurospora crassa. Agric. Biol. Chem.*, **51**, 3315–3321(1987).
19) Kikumoto, S., Miyajima, T., Kimura, K., Okubo, S, and Komatsu, K., Polysaccharide produced by *Schizophyllum commune*. Pt.II. Chemical structure of an extracellular polysaccharide. *Nippon Nougeikagaku Kaishi (in Japanese).* **45**, 162–168 (1971).