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Expression of Thermostable α-Glucosidase from *Thermus caldophilus* GK24 in Recombinant *Saccharomyces cerevisiae*

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Abstract A gene (GenBank AF096282) coding for a α-glucosidase (TcaAG, EC 3.2.1.20) from *Thermus caldophilus* GK24 was expressed in *Saccharomyces cerevisiae*, a generally recognized as safe (GRAS) host. The thermostable α-glucosidase was produced inside of the GRAS host at 0.04 unit/mg-dry cell by the constitutively expressing ADH1 promoter and at 1.2 unit/mg-dry cell by the inductively expressing GAL10 promoter, respectively. No α-glucosidase activities were found in the medium when the MF-alpha signal sequence from *S. cerevisiae* or α-amylase signal sequence from *Aspergillus oryzae* were fused before the α-glucosidase gene for the secretion.

Key words: α-Glucosidase, protein expression, recombinant enzyme, *Saccharomyces cerevisiae*, *Thermus caldophilus* GK24

Thermostable proteins and enzymes from *Thermus* spp. have inherent stability against various protein denaturants, a desirable property for industrial enzyme applications [5, 6, 9]. A thermostable α-glucosidase (EC 3.2.1.20) has been cloned and purified from *Thermus caldophilus* GK24 [11], which thereafter released glucose from isomaltose, panose, isomaltotriose, and isomaltotetraose, and not from starch or pullulan. The biochemical characteristics of the recombinant enzymes from *E. coli* were revealed to be the same as for the native enzyme from *T. caldophilus* GK24 [11]. The *E. coli* expression system, however, raises safety concerns in food and medical applications. *Saccharomyces cerevisiae* has long been considered as a generally recognized as safe (GRAS) host organism for the expression of recombinant proteins because *S. cerevisiae* does not produce endotoxins and pyrogens. *S. cerevisiae* can process a precursor protein to be made into a mature form by resident proteases, and then secrete the heterologous proteins into the culture media. Expression in yeast of the genes from *T. caldophilus*, however, has not been reported as yet.

In this study, we investigated the expression in *S. cerevisiae* of the thermostable α-glucosidase from *T. caldophilus* GK24, and the extracellular production of the enzyme, by using a mating factor α (MFα) signal sequence from *S. cerevisiae*.

E. coli MV1184 harboring an expression vector with the α-glucosidase gene (pET-TcaAG) was received from Dr. D. S. Lee, KRIBB, Korea. The yeast host strain was *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ can1 GAL2 his3 ura3-52*). The *E. coli*-yeast shuttle vectors, pVT-103U [15] containing the yeast alcohol dehydrogenase isozyme I (*ADHI*) promoter and pYEGA [1] containing the yeast *GAL10* promoter, were used for gene cloning. Two expression vectors were developed for the production of α-glucosidase in *S. cerevisiae* (Fig 1). To construct pVT-TcaAG (8.3 kbp), the 1.6-kbp XbaI-HindIII fragment containing the *T. caldophilus* α-glucosidase (TcaAG) gene from pET-TcaAG (5.5 kbp) was introduced into the same site of pVT-103U (6.8 kbp), a constitutive expression vector that has the *ADH1* promoter (Fig. 1A). In addition, the pYEGA-MF-TcaAG vector with signal sequence was constructed for the secretory production of α-glucosidase. Amplified MFα signal sequences from a *Pichia pastoris* expression vector, pICZαB (Invitrogen, V195-20, San Diego, CA, U.S.A.), by polymerase chain reaction (PCR) and TcaAG were inserted into the EcoRI and HindIII sites of pYEGA (6 kbp) that has the galactose-inducible *GAL10* promoter, resulting in pYEGA-MF-TcaAG. (7.8 kbp) (Fig 1B). In this study, we chose to use the MFα signal sequence because it has been successfully used for secretory production of various recombinant proteins in yeast [4, 8, 12, 16]. The pVT-TcaAG and pYEGA-MF-TcaAG were proved to be correctly

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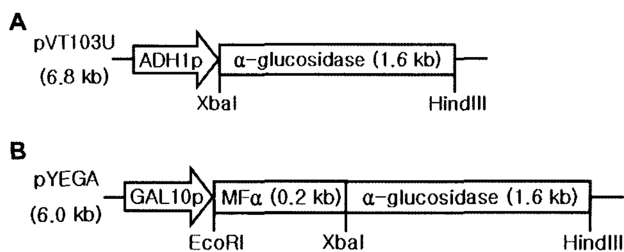

Fig. 1. Schematic diagram of vector constructions for the expression of thermostable α-glucosidase in *S. cerevisiae*.
A. pVT-TcaAG for constitutive expression of the enzyme. **B.** pYEGA-MF-TcaAG for inductive and secretory production of the enzyme with galactose-inducible *GAL10* promoter and the MFα signal sequence.

and properly constructed, by electrophoresis (data not shown).

To investigate the expression of a thermostable α-glucosidase from the recombinant yeasts, batch cultivations were carried out in a 5-l jar fermentor. Batch cultivation of the recombinant yeast for enzyme expression under the control of the *ADH1* promoter was performed using a YPD medium (1% yeast extract, 2% polypeptone, 2% dextrose). Batch cultivation for enzyme expression under the control of the *GAL10* promoter was performed using the YPDG medium (4% yeast extract, 1% polypeptone, 2% dextrose, 3% galactose). A jar fermentor (KF-2.5L, KoBioTech, Incheon, Korea) was maintained at 30°C and pH 5.8 with 600 rpm agitation and 0.5 vvm aeration in a 2-l working volume. The dry cell weight was estimated by a predetermined conversion factor of 0.27 g dry cell weight/l optical density at 600 nm. The total reducing sugar content of the culture broth was determined by a dinitrosalicyclic (DNS) method of that described by Miller [10]. *S. cerevisiae* cells (100 mg) were suspended in 50 mM potassium phosphate buffer (pH 7.2). Cells were disrupted with 425–600 μm glass beads (Sigma, G9268, St. Louis, MO, U.S.A.) using a vortexer for 2 min at room temperature and then chilled quickly in an ice bath and the process repeated three times. Cell debris was removed by centrifugation at 12,500 ×*g* for 10 min, and the supernatant (100 μl) was used as an intracellular enzyme solution for the activity assay. The extracellular enzyme solution was prepared by the 10-fold concentration of the culture broth using ultrafiltration. α-Glucosidase activity was measured as the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) [11]. The reaction mixture (0.5 ml) that contained 50 mM phosphate buffer (pH 7.2), 10 mM *p*NPG, and the enzyme was incubated at 70°C for 10 min. One unit of enzyme activity was defined as the amount of 1 μmol *p*NP produced per min under the above conditions [14]. In the time profile of batch culture for *S. cerevisiae*/pVT-TcaAG, cells grew for 12 h by the uptake of glucose as a carbon source (Fig. 2A). The intracellular α-glucosidase activity was observed to yield a pattern similar to a cell growth curve, and was 0.26 unit/ml-broth (0.04 unit/mg-dry cell and 85 unit/mg-protein) at 48 h.

In the time profile of batch culture for *S. cerevisiae*/pYEGA-MF-TcaAG aimed at the secretory production of α-glucosidase, the cell growth phase could be divided into a glucose consumption stage until 12 h and a galactose consumption stage until 32 h (Fig 2B). The galactose-inducible promoter in *S. cerevisiae*, *GAL10*, was strongly repressed by glucose and can achieve maximum induction of target protein after depletion of glucose in the medium. The stationary phase of cell growth was observed as the entire carbon source was depleted thereafter. Extracellular α-glucosidase activity was not found in the cell-free supernatant of the culture broth, which means that the MFα signal sequence failed to secrete a *Thermus* α-glucosidase in *S. cerevisiae*. The presence of internal signal sequences, hydrophobic domains, and membrane domains in the amino acid sequence of target proteins prevents secretion because of adherence in the membrane compartments of the late Golgi on the secretory pathway [6, 13]. The molecular character of *Thermus* α-glucosidase as related to these factors needs to be further elucidated. Intracellular α-glucosidase activity instead was shown to increase sharply by galactose induction during the 12

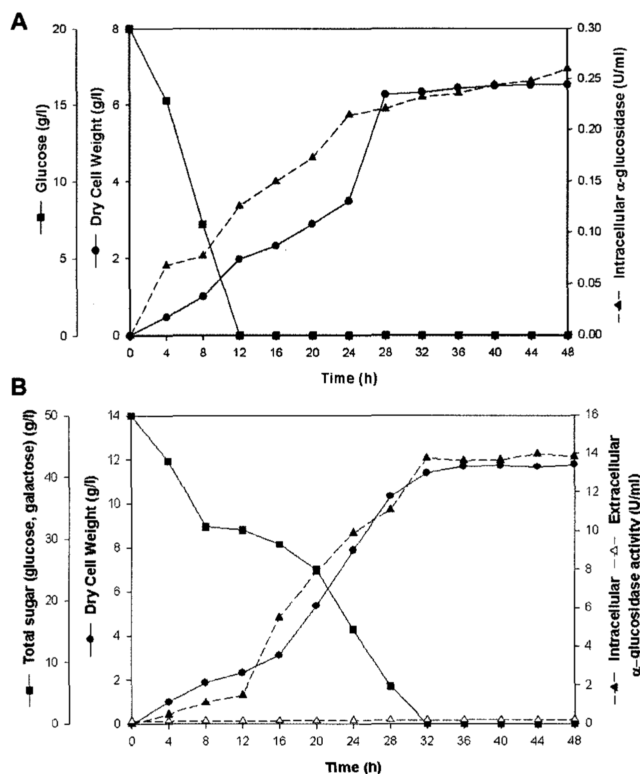

Fig. 2. Time profiles for batch cultivations of recombinant *S. cerevisiae*.
A. *S. cerevisiae*/pVT-TcaAG. **B.** *S. cerevisiae*/pYEGA-MF-TcaAG.

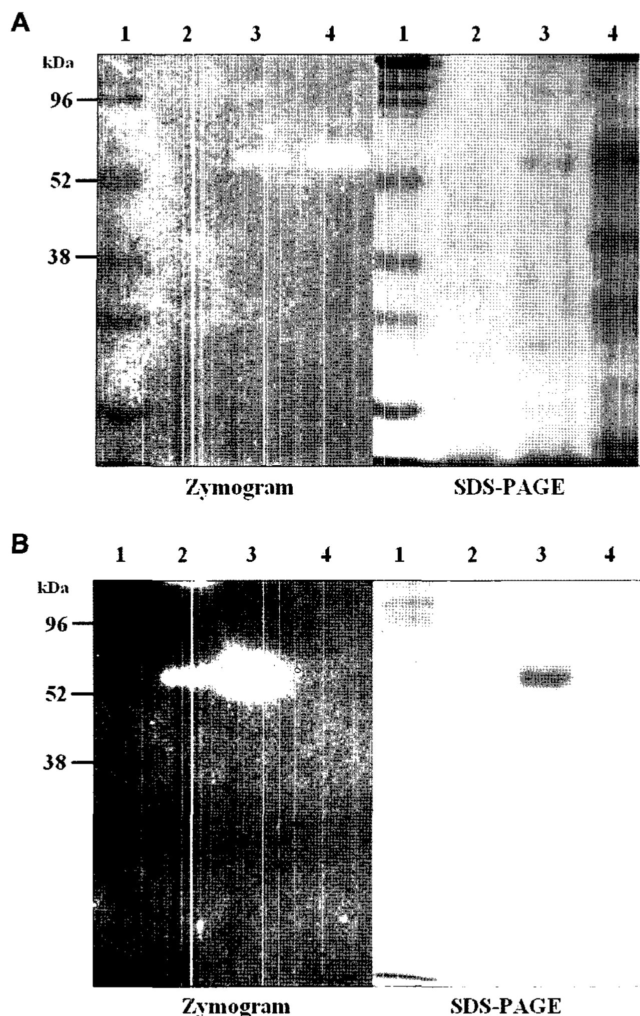

Fig. 3. Zymogram and SDS-PAGE to identify thermostable α-glucosidase produced from recombinant yeast. All samples were purified partially by heat treatment (70°C, 20 min) [9].
A. Lane 1, marker protein; lane 2, cell lysates of *S. cerevisiae* not harboring plasmid; lane 3, cell lysates of *S. cerevisiae*/pVT-TcaAG; lane 4, cell lysates of *E. coli*/pET-TcaAG. **B.** Lane 1, marker protein; lane 2, cell lysates of *E. coli*/pET-TcaAG; lane 3, cell lysates of *S. cerevisiae*/pYEGA-MF-TcaAG; lane 4, cell-free supernatant of *S. cerevisiae*/pYEGA-MF-TcaAG culture broth.

to 32 h interval (Fig. 2B). At the end of *S. cerevisiae*/pYEGA-MF-TcaAG cultivation, intracellular α-glucosidase activity was up to 14 unit/ml-broth (1.2 unit/mg-dry cell and 4,600 unit/mg-protein) and significantly higher than that of the *S. cerevisiae*/pVT-TcaAG. (0.04 unit/mg-dry cell). Mass production of thermostable α-glucosidase for food application may be available by optimization of the yeast expression system using a strong promoter, such as *GAL10*.

Thermostable α-glucosidase prepared from yeast cell lysates in 48 h of batch culture was identified by SDS-PAGE and zymogram analysis (Fig. 3). Zymogram analysis was performed using 12% SDS-PAGE, as modified from Bibel *et al.* [2]. After SDS-PAGE, the gel was renatured with 40 mM phosphate buffer (pH 7.0) containing 2% Triton X-100 at room temperature for 30 min, and incubated in the buffer (pH 7.0) containing 3 mM 4-methylumbelliferyl-α-D-glucoside at 65°C for 20 min. The α-glucosidase activity on the gel was identified by fluorescence under a UV lamp at 366 nm. The molecular weight of the enzyme was estimated at approximately 58 kDa, equal to that produced by recombinant *E. coli*/pET-TcaAG. [11]. The thermostability and thermal activity of the recombinant *Saccharomyces* enzyme has remained unaltered from the original *Thermus* spp. (data not shown). The cell-free supernatant of *S. cerevisiae*/pYEGA-MF-TcaAG culture broth did not show an active α-glucosidase band on either SDS-PAGE or the zymograms.

From these results, it was confirmed that thermostable α-glucosidase from *T. caldophilus* GK24 was produced in a yeast expression system. The enzyme molecules expressed from the pYEGA-MF-TcaAG construct, however, were not successfully secreted by the incorporated MFα signal sequence. We also observed that thermostable α-glucosidase expressed in *S. cerevisiae*/pYEGA-AM-TcaAG from a pYEGA-AM [1] containing the α-amylase signal peptide from *Aspergillus oryzae* was not expressed extracellularly (data not shown). For the secretory production of thermostable α-glucosidase from *T. caldophilus* GK24, the use of other secretion signals, including the putative alkaline phosphatase signal sequence from *T. caldophilus* GK24 and various tagging systems, are underway in our laboratory.

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References

1. Ahn, J. O., H. W. Jang, H. W. Lee, E. S. Choi, S. J. Han, T. K. Oh, and J. K. Jung. 2003. Overexpression of thermoalkalophilic lipase from *Bacillus stearothermophilus* L1 in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **13:** 451–456.
2. Bibel, M., C. Brett, U. Gosslar, G. Kriegshauser, and W. Liebl. 1998. Isolation and analysis of genes for amylolytic enzymes of the hyperthermophilic bacterium *Thermotoga maritima*. *FEMS Microbiol. Lett.* **58:** 9–15.
3. Bitter, G. A., K. K. Chen, A. R. Banks, and P.-H. Lai. 1984. Secretion of foreign proteins from *Saccharomyces cerevisiae* directed by α-factor gene fusions. *Proc. Natl. Acad. Sci. USA* **81:** 5330–5334.
4. Brake, A. J. 1990. Alpha-factor leader-directed secretion of heterologous proteins from yeast. *Methods Enzymol.* **185:** 408–421.

5. Choi, J. J., J. W. Park, H. Shim, S. Lee, M. Kwon, J. S. Yang, H. Hwang, and S. T. Kwon. 2006. Cloning, expression, and characterization of a hyperalkaline phosphatase from the thermophilic bacterium *Thermus* sp. T351. *J. Microbiol. Biotechnol.* **16:** 272–279.
6. Conesa, A., P. J. Punt, N. van Luijk, and C. A. M. van den Hondel. 2001. The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Gen. Biol.* **33:** 155–171.
7. Duffield, M. L. and D. Cossar. 1995. Enzymes of *Thermus* and their properties, pp. 93–141. *In* R. Sharp and R. Williams (eds.), *Thermus Species*. Plenum Press, New York.
8. Hashimoto, Y., N. Koyabu, and T. Imoto. 1998. Effects of signal sequences on the secretion of hen lysozyme by yeast: Construction of four secretion cassette vectors. *Protein Eng.* **11:** 75–77.
9. Kang, S. K., K. K. Cho, J. K. Ahn, S. H. Kang, K. H. Han, H. G. Lee, and Y. J. Choi. 2004. Cloning and expression of thermostable β-glycosidase gene from *Thermus filiformis* Wai33 A1 in *Escherichia coli* and enzyme characterization. *J. Microbiol. Biotechnol.* **14:** 584–592.
10. Miller, G. C. 1959. Use of the dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* **31:** 420–428.
11. Nashiru, O., S. Koh, S. Y. Lee, and D. S. Lee. 2001. Novel α-glucosidase from extreme thermophile *Thermus caldophilus* GK24. *J. Biochem. Mol. Biol.* **34:** 347–354.
12. Ramchuran, S. O., V. A. Vargas, R. Hatti-Kaul, and E. N. Karlsson. 2005. Production of a lipolytic enzyme originating from *Bacillus halodurans* LBB2 in the methylotrophic yeast *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **12:** 1–10.
13. Schuster, M., E. Wasserbauer, G. Aversa, and A. Jungbauer. 2001. Transmembrane-sequence-dependent overexpression and secretion of glycoproteins in *Saccharomyces cerevisiae*. *Protein Expr. Purif.* **21:** 1–7.
14. Suzuki, Y., T. Yuki, T. Kishigami, and S. Abe. 1976. Purification and properties of extracellular alpha-glucosidase of a thermophile, *Bacillus thermoglucosidus* KP 1006. *Biochim. Biophys. Acta* **445:** 386–397.
15. Vernet, T., D. Daniel, and D. Y. Thomas. 1987. A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52:** 225–233.
16. Yebra, M. J., A. Blasco, and P. Sanz. 1999. Expression and secretion of *Bacillus polymyxa* neopullulanase in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **170:** 41–49.