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Note

Characterization of *Bacillus halodurans* α-Galactosidase Mel4A Encoded by the *mel4A* Gene (BH2228)

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A family-4 α-galactosidase Mel4A of *Bacillus halodurans* was expressed in *Escherichia coli* and characterized. Recombinant enzyme rMel4A depended on NAD^+, some divalent cations such as Mn^{2+}, and reducing reagents such as dithiothreitol. rMel4A was active on small saccharides such as raffinose but not on highly polymerized galactomannan. Immunological analysis indicated that raffinose induced the production of Mel4A in *B. halodurans*.

Key words: *Bacillus halodurans*; α-galactosidase; melibiase; glycoside hydrolase; raffinose

α-Galactosidases (α-Gals; EC 3.2.1.22) are enzymes that catalyze hydrolysis of the α-1,6-galactosidic linkages from the non-reducing end in galactose-containing oligosaccharides, liposaccharides, and polysaccharides. Based on amino acid sequence similarity, α-Gals have been classified in families 4, 27, 36, 57, and 110 of glycoside hydrolases (The CAZy database, http://www.cazy.org/).[1)] Bacterial α-Gals are grouped mainly into families 4, 36, and 110 with some exceptions.

The complete genome sequence of *Bacillus halodurans* C-125 revealed the presence of three putative α-Gal genes: BH2228 encoding an enzyme of family 4, BH1870 encoding an enzyme of family 27, and BH2223 encoding an enzyme of family 36.[2)] Family 4 of the glycoside hydrolases includes maltose-6-phosphate glucosidase (EC 3.2.1.122), α-glucosidase (EC 3.2.1.20), 6-phospho-β-glucosidase (EC 3.2.1.86), and α-glucuronidase (EC 3.2.1.139) in addition to α-galactosidase (EC 3.2.1.22) (http://www.cazy.org/). Among family-4 enzymes, however, there is little information on α-Gal. Although MelA from *Escherichia coli* K-12 was purified and the analysis of its structure such as amino acid composition and N-terminal amino acid sequence was carried out, no fundamental enzyme properties of MelA were reported.[3)] Hence, we chose BH2228, named *mel4A*, for biochemical characterization.

The *mel4A* gene (BH2228, DDBJ accession no. BA000004) was amplified by PCR using KOD DASH DNA polymerase (Toyobo, Osaka, Japan) and a primer set, 5′-GCGCGCTAGCATGGGAAAGATTACATTTCTTGG-3′ and 5′-CGCGGTCGACTTATTTGAAGGACTGTAGCCAGC-3′, containing *Nhe*I and *Sal*I recognition sites (underlined) from *B. halodurans* C-125 (=JCM9153) genomic DNA as the template. The amplified DNA fragment was ligated into pT7Blue and introduced into *E. coli* XL1-Blue by a standard transformation protocol. A plasmid purified from a recombinant clone was digested with *Nhe*I and *Sal*I and the inserted DNA fragment was recovered from an agarose gel and ligated between the *Nhe*I and *Sal*I sites of pET-28a(+), yielding pET28a(+)-Mel4A. *E. coli* BL21(DE3) harboring pET28a(+)-Mel4A was used in the production of the recombinant Mel4A (rMel4A). *E. coli* BL21(DE3) harboring pET28a(+)-Mel4A was grown overnight at 37 °C in 500 ml of LB broth containing kanamycin (60 µg/ml). Isopropyl-β-D-thiogalactopyranoside was added to the culture to give a final concentration of 1 mM, and the culture was incubated for a further 20 h at 20 °C. Cell-free extract was prepared by ultrasonication of the cells, and was used as the crude enzyme solution in the purification of rMel4A with a HiTrap Chelating HP column (Amersham Biosciences,

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Abbreviations: DTT, dithiothreitol; α-Gal, α-galactosidase; *p*NP, *p*-nitrophenol; *p*NP-Gal, *p*-nitrophenyl-α-D-galactopyranoside; PCR, polymerase chain reaction; Tris, Tris(hydroxymethyl)aminomethane

Piscataway, NJ) according to the supplier's protocol. Active fractions were collected and desalted by dialysis against 100 mM Tris–HCl buffer (pH 7.4) containing 60 µM NAD^+, 1 mM Mn^{2+}, and 1 mM dithiothreitol (DTT). Purified enzyme showed a single band with a molecular mass of 51 kDa on a gel of SDS-polyacrylamide gel electrophoresis (data not shown).

Enzymes classified in family 4 of the glycoside hydrolases are known to require NAD^+, Mn^{2+} ion, and reducing agents for maximum activity.[4–6] To determine the nucleotide, metal ion, and reducing agent requirements of rMel4A, the enzyme activity was measured with *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-Gal) as the substrate in 100 mM Tris–HCl buffer (pH 7.4) containing combinations of these reagents. The reaction mixture was incubated at 37 °C for 5 min, and the reaction was stopped by the addition of borate buffer (pH 9.8). The amount of *p*NP released from *p*NP-Gal was measured by the absorbance at 405 nm. One unit of activity was defined as the amount of enzyme that liberates 1 µmol of *p*NP per min from *p*NP-Gal under the given assay conditions. Among NAD^+, $NADP^+$, NADPH, and NADH, NAD^+ strongly activated rMel4A activity and $NADP^+$ showed a slight activating effect, in that the effect of the latter was about 5% of that of the former (data not shown). Table 1 shows the specific activities of rMel4A in the reaction mixtures containing NAD^+, the Mn^{2+} ion, and reducing agents at constant concentrations. Without the reagents, rMel4A showed no catalytic activity on *p*NP-Gal. Although the addition of one of these reagents slightly activated rMel4A, the enzyme was highly activated by the addition of these three reagents. The activating effect of DTT on rMel4A was stronger than that of mercaptoethanol. Although it was not possible to determine the effect of certain metal ions in the presence of DTT, since precipitates were formed, the Co^{2+} and Fe^{2+} ions showed a strong activating effect, as compared to that of Mn^{2+} in the presence of NAD^+. The Ni^{2+} and Zn^{2+} ions moderately enhanced rMel4A activity. As shown in Fig. 1, when rMel4A activity was assayed in reaction mixtures containing different amounts of one of NAD^+, Mn^{2+}, and DTT and constant amounts of the other two reagents, the enzyme was found to be heavily dependent on the concentration of each of these. The concentrations of NAD^+, Mn^{2+}, and DTT required for the maximal activity of rMel4A were determined to be 0.2, 2, and 50 mM, respectively, indicating that NAD^+, Mn^{2+}, and DTT were prerequisites for rMel4A activity. In all of the family-4 enzymes, a Cys residue located around the 160–175 positions of their respective sequences is conserved. Crystallographic study of α-glucosidase AglA from *Thermotoga maritima* has

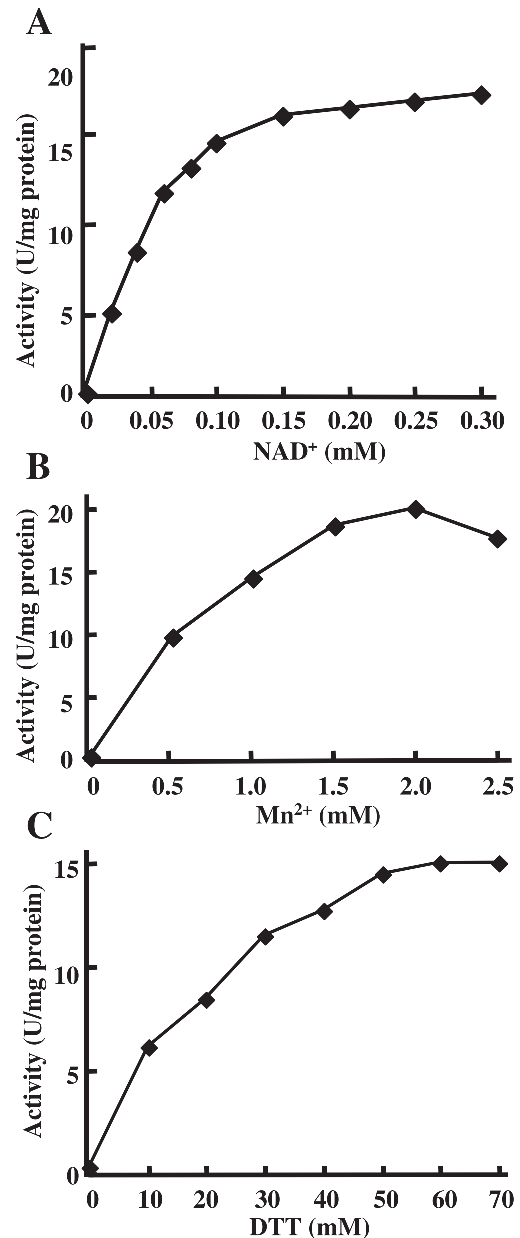

Fig. 1. Dependence of rMel4A Activity on NAD^+ (A), Mn^{2+} (B), and DTT (C).

rMel4A activity was assayed with *p*NP-Gal in the presence of various concentrations of one of three reagents, NAD^+, Mn^{2+}, and DTT, and constant concentrations of two other reagents, *viz.*, 60 µM for NAD^+, 1 mM for Mn^{2+}, and 50 mM for DTT.

Table 1. Effects of NAD^+, Mn^{2+}, and Reducing Agents on Enzyme Activity of rMel4A

NAD^+ (60 µM)	Mn^{2+} (1 mM)	DTT (50 mM)	ME[a] (50 mM)	Activity (U/mg)
−	−	−	−	ND[b]
+	−	−	−	0.12
−	+	−	−	0.0003
−	−	+	−	ND[b]
−	−	−	+	0.0023
+	+	−	−	0.29
+	+	+	−	15.4
+	+	−	+	2.05

[a] Mercaptoethanol
[b] Not detectable

indicated that a reduction in activity in the absence of DTT was due to oxidation of the SH group of the Cys-174 residue to Cys-SO_2H, and that DTT was probably necessary to maintain Cys-174 in the reduced form.[7] Crystal structure of phospho-α-glucosidase GlvA from *Bacillus subtilis* indicated that Cys-171 was linked to Mn^{2+}.[8] rMel4A showed weak activity toward *p*NP-α-D-glucopyranoside (0.6 U/mg) in addition to *p*NP-Gal. rMel4A hydrolyzed melibiose, raffinose, and stachyose, but not guar gum (highly polymerized galactomannan), and the K_m and k_{cat} values for these substrates were determined to be as follows: 1.6 mM and 16.3 s^{-1} for pNP-Gal, 16.1 mM and 9.2 s^{-1} for melibiose, 5.0 mM and 0.93 s^{-1} for raffinose, and 39.1 mM and 1.3 s^{-1} for stachyose. The values for stachyose were apparent, since raffinose is produced during hydrolysis of the original substrate, stachyose. rMel4A showed the highest k_{cat} value on melibiose among the natural substrates. On the other hand, it showed higher affinity for raffinose than for melibiose or stachyose. the optimum pH and temperature of rMel4A were 7.4 and 37 °C. The enzyme was stable between pH 7 and 8 at 4 °C upon 24-h of incubation and stable at 40 °C for 10 min.

Western blot analysis using an anti-rMel4A antiserum identified an immunoreactive protein in intracellular proteins of *B. halodurans* grown in a medium containing raffinose as the carbon source (Fig. 2). Localization of Mel4A in the intracellular fraction was consistent with the apparent absence of a signal peptide sequence in Mel4A and the maximum activity of rMel4A in the neutral pH range despite the alkaliphilic property of *B. halodurans*. It is interesting that only raffinose, and not melibilose or stachyose, induced expression of the *mel4A* gene although *B. halodurans* grew on these three saccharides and purified rMel4A was active toward these substrates. Although this bacterium also utilized guar gum as a carbon source, the presence of guar gum did not induce production of Mel4A. It is likely that the hydrolysis of guar gum is carried out by α-Gal(s) other than Mel4A, since at least two possible α-Gal genes other than *mel4A* can be identified in the *B. halodurans* genome, BH1870 encoding an enzyme of family 27, and BH2223 encoding an enzyme of family 36. Since some α-Gals classified in family 27 are known to hydrolyze highly polymerized galactomannan,[9] guar gum might be hydrolyzed by the family-27 α-Gal. Complete degradation of galactomannas requires the combined action of endo-1,4-β-mannanase and β-mannosidase, which hydrolyze the backbone of mannan, and α-Gal, which releases the galactosyl groups. *B. halodurans* must produce mannan-degrading enzymes as well as α-Gals.

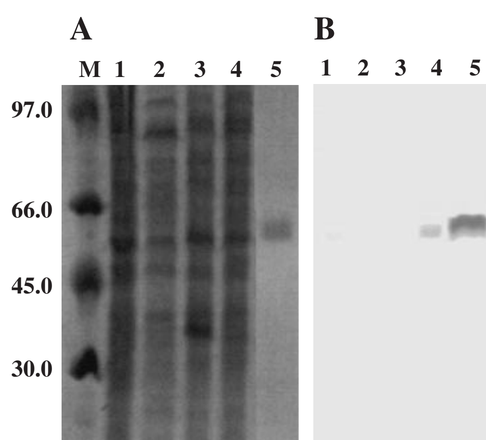

Fig. 2. Expression of Mel4A in *B. halodurans*.

Gels were stained with Coomassie brilliant blue (A). Mel4A on Western blot analysis was detected with a polyclonal mouse antiserum raised against purified rMel4A (B). Lane M, standard markers; lane 1, proteins in the culture supernatant of *B. halodurans* grown on melibiose; lane 2, grown on guar gum; lane 3, grown on glucose; lane 4, grown on raffinose; lane 5, purified rMel4A.

In this study, we successfully expressed and characterized *B. halodurans* Mel4A as a recombinant enzyme. Studies of the structure-function relationship of family-4 α-Gal should be helpful for a better understanding of these unique enzymes.

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