An NAD^+, Mn^{2+} and DTT-dependent α-galactosidase from *Bacillus halodurans*

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The α-galactosidase *mel4A* (previously called *melA*) gene of *Bacillus halodurans* was recombinantly expressed in *Escherichia coli*, purified and characterized. The *mel4A* gene consists of 1305 nucleotides encoding a protein of 434 amino acids with a predicted molecular weight of 49,761. According to its primary structure as deduced from the nucleotide sequence of the gene, Mel4A was assigned to family 4 of glycoside hydrolases. Almost all of the enzyme was produced as inclusion bodies at 37°C in *E. coli*. In order to reduce the expression level, cultivation temperature was decreased to 20°C so that the enzyme could be collected from soluble fraction. Recombinant α-galactosidase Mel4A was purified to homogeneity in a single step using His-binding metal affinity chromatography. *B. halodurans* Mel4A has the unusual property, i.e., absolutely depending on NAD^+ and Mn^{2+} for activity. Co^{2+} and Ni^{2+} also activated Mel4A, albeit less efficiently than Mn^{2+}. In addition, Mel4A activity required reducing condition which met by the addition of dithiothreitol (DTT). In the presence of all cofactors, optimum activity was achieved at 37°C and pH 7.4. The enzyme hydrolyzed *p*-nitrophenyl-α-D-galactopyranoside, melibiose, raffinose, and stachyose but not guar gum, indicating that this enzyme preferred small saccharides to highly polymerized galactomannans. Western immunoblots of intracellular and extracellularproteins of *B. halodurans* revealed that raffinose induced the expression of intracellular Mel4A of *B. halodurans*. This bacterium was also able to utilize guar gum as the carbon source, but Western blot analysis indicated that the production of Mel4A was not enhanced by the addition of guar gum.

INTRODUCTION

α-Galactosidases (α-Gal; α-D-galactoside galactohydrolase; melibiase; 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, lipo saccharides and/or polysaccharides. α-Gals widely occur in bacteria (5, 6), filamentous fungals (3), yeasts (11), plants (2), animals (7) and human (1). Some of them have been purified and characterized.

Based on the amino acid sequence similarity, α-Gals have been classified under the families 4, 27, 36 and 57 of glycoside hydrolases (4). Almost all of the eukaryotic α-Gals showed a significant degree of amino acid sequence homology and they have been assigned to family 27. To date, some bacterial α-Gals have been characterized and grouped mainly into families 4, 36 and 57.

Galactose is the constituent of oligosaccharides (melibiose, raffinose, and stachyose) and polysaccharides such as galactomannan. These oligosaccharides are present in beans and mushrooms. Because monogastric animal and human body lack of α-galactosidase, these oligosaccharides end up in the large intestine undigested and cause flatulence. The use of microbial α-galactosidase treatment prior consumption has been proposed to prevent flatulence. α-Galactosidase have been used in soymilk production to reduce the content of undigested oligosaccharides. In the sugar beet industry, α-galactosidases have been used to increase the sucrose yield by eliminating raffinose, which prevents normal crystallization of beet sugar.

Bacillus halodurans C-125 was isolated in 1977 and reported as a β-galactosidase and xylanase producer. *B. halodurans* is a haloalkaliphilic bacteria which grow well in the pH range of 6.8 – 10.8. It is the strain most thoroughly characterized, physiologically, biochemically and genetically along with *B. subtilis*. This bacterium produces extracellular enzymes of industrial interest: protease, pectinase, amylase, xylanase. Extracellular enzymes produced by *B. halodurans* show relatively high optimum temperatures (50-70° C) and alkaline pH optima (8).

The complete genome sequence of *B. halodurans* has been determined (9) and it revealed the presence of three putative α-galactosidase genes; BH2228 belonging to glycoside hydrolases family 4, BH1870 belonging to family 27 and BH2223

belonging to family 36 (4).

MATERIALS AND METHODS

Materials. Melibiose and raffinose were purchased from Nacalai Tesque (Japan). *p*NP-galacturonic acid and *p*NP-glucoronic acid were kindly provided by Dr. Motomitsu Kitaoka (NFRI, Japan). Stachyose, guar gum, *p*NP-Gal, other *p*NP glycosides, and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Restriction endonucleases and other enzymes were purchased from the Takara (Kyoto, Japan) and used in accordance with the manufacture's instructions.

Bacterial strains, plasmids, media and cultivation condition. *Bacillus halodurans* C-125 was obtained from the Japan Collection of Microorganism (JCM) and cultured aerobically in the Horikoshi-I medium pH 10 at 37^{o} C overnight (?). *Escherichia coli* strains XL1-Blue and BL21(DE3) were used as the cloning and gene expression host. Recombinant *E. coli* strains were cultured at 37^{o} C in Luria-Bertani (LB) medium supplemented with ampicillin (75 μg ml^{-1}) or kanamycin (60 μg ml^{-1}). Plasmid pT7Blue and pET-28a(+) (Novagen, USA) were used for the TA cloning and gene expression experiments.

Construction of recombinant plasmid. Open reading frame BH2228 (*mel4A*) was amplified by PCR using Taq polymerase master mix (Qiagen, USA). The PCR-derived DNA fragments were ligated into the TA cloning vector pT7Blue and introduced to *E. coli* XL1-Blue. Plasmids were purified and inserted DNA fragments were ligated into expression vector pET-28a(+). Recombinant plasmid pET28-Mel4A was expressed in E. coli BL21(DE3).

Protein purification. *E. coli* BL21 (DE3) harboring pET28-Mel4A was used as a source for recombinant enzyme. The recombinant strain was grown overnight at 37^{o} C in 500 mL of LB broth containing 60 μg ml^{-1} kanamycin. IPTG of 1 mM final concentration was added and the culture was incubated for further 20 h at 20^{o} C. The cells were harvested by centrifugation and resuspended in lysis buffer. The cells were disrupted by sonication and cell debris was removed by centrifugation. The supernatant was used as the crude enzyme solution and purified to homogeneity in a single step using HiTrap Chelating HP (Amersham) column chromatography.

RESULTS AND DISCUSSION

The *mel4A* gene of *B. halodurans* encodes a α-galactosidase. The *B. halodurans* Mel4A consists of 434 amino acids with a calculated molecular size of 49,761 kDa (Figure 1). According to amino acid sequence similarity-based classification of glycoside hydrolases, Mel4A belongs to glycoside hydrolase family 4 (GHF4). It is similar to *E. coli* MelA but distinct from reported eukaryotic α-Gal belonging to family 27 and bacterial α-Gal belonging to family 36.

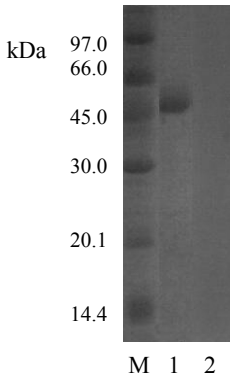

Fig. 1. SDS-PAGE of purified Mel4A. Lane M, protein molecular mass standard (molecular masses shown at the left); lane 1, Mel4A; lane 2, protein purified from *E-coli* BL21(DE3) harboring pET28-a(+).

Mel4A lost almost all of its activity when assayed in the absence of NAD^+. The highest activity was achieved when the enzyme incubated with NAD^+, Mn^{2+} and DTT (Figure 3). Almost all of the GHF4 enzymes were reported to required NAD^+ and Mn^{2+} for activity. In the presence of NAD^+ or Mn^{2+}, some of them require a high concentration of reducing agent (DTT or mercaptoethanol) for activity (6, 10).

Multiple alignments of the GHF4 enzymes revealed many conserved glycine-rich residues (Figure 2). The presence of putative NAD^+ binding protein in N-terminal (residue 1 to around 50) of GHF4 proteins has been suggested by Thompson *et al* (10). The NAD^+ binding motifs are conserved in *B. halodurans* Mel4A. The result of this research also supports the assumption that the presence of

conserved N-terminal NAD^+ binding motif is related with NAD^+ binding affinity.

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                      <----- NAD+-binding domain ------>
                          • ••                    •
MELA_BACHD      ---MGKITFLGAGSTIFAKNVLGDCMLTPSLQEFEFALFDIDLDRLHDSETMLKHLKETL 57
AGAL_BACSU      ---MKKITFIGAGSTIFAKNVLGDCLLTEALNGFEFALYDIDPKRLQESQLMLENLRDRY 57
AGAL_ECOLI      MMSAPKITFIGAGSTIFVKNILGDVFHREALKTAHIALMDIDPTRLEESHIVVRKLMDSA 60
AGAL_SALTY      MMTAPKITFIGAGSTIFVKNILGDVFHREALKSAHVALMDIDETRLEESHIVVRKLMDSA 60
AGAL_RHIME      -MASFKIAIIGAGSIGFTKKLFTDILSVPELRDVEFALTDLSEHNLAMIKSILDRIVEAN 59
                     **:::****  *.*::: * :    *.  ..** *:.  .*   . :: .: :
MELA_BACHD      QANVKIEAYTDRKEALRGAKYVVNAIQVGGYDPCTITDFEIPKKYGLRQTIADTVGIGGI 117
AGAL_BACSU      NPSVAINSYDDRKLALQNAGYVINAIQVGGYKPSTVIDFEIPKRYGLRQTIADTVGIGGI 117
AGAL_ECOLI      GASGKITCHTQQKEALEDADFVVVAFQIGGYEPCTVTDFEVCKRHGLEQTIADTLGPGGI 120
AGAL_SALTY      GASGRITCHTNQKAALQDADFVVVAFQIGGYEPCTVTDFEVCKRHGLEQTIADTLGPGGI 120
AGAL_RHIME      ELPTRVTATTDRRKALEGARYIISCVRVGGLEAY-ADDIRIPLKYGVDQCVGDTICAGGI 118
                     : .  ::: **..* ::: ..::** ..    *:.:  ::*: * :.**:  ***
                                            ••              •
MELA_BACHD      FRSLRTIPVMLDFATDMEEVCP-DAWLLNYTNPMATLTGTMLR-YTGVKTVGLCHSVQVC 175
AGAL_BACSU      FRSLRTIPVLFDIAKDMEEMCP-DAWFLNYTNPMATLTGAMLR-YTNIKTIGLCHSVQVC 175
AGAL_ECOLI      MRALRTIPHLWQICEDMTEVCP-DATMLNYVNPMAMNTWAMYARYPHIKQVGLCHSVQGT 179
AGAL_SALTY      MRALRTIPHLWRICEDMTEVCP-KATMLNYVNPMAMNTWAMYARYPHIKQVGLCHSVQGT 179
AGAL_RHIME      LYGQRNIPVILDFCKDIREVAEPGAKFLNYANPMAMNTWAAIE-YGKVDTVGLCHGVQHG 177
                : . *.** :  :. *: *:.   * :***.****  * :    *  :. :****.**
                              • ••
MELA_BACHD      APELLDRLGMEKDNIQWKIAGINHMAWLLEITREGED-----LYPEIKRRAKEKQQN--- 227
AGAL_BACSU      TKDLFKALGMEHDGIEERIAGINHMAWLLEVKKDGTD-----LYPEIKRRAKEKQK---- 226
AGAL_ECOLI      AEELARDLNIDPATLRYRCAGINHMAFYLELERKTADGSYVNLYPELLAAYEAGQAPKPN 239
AGAL_SALTY      AEELARDLNIDPTSLRYRCAGINHMAFYLELERKTADGTYVNLYPELLAAYDAGQAPKPN 239
AGAL_RHIME      AEQIAEILGARDGELDYICSGINHQTWFVDVRLNGRK----VGKDELVAAFEAHPVFS-- 231
                : ::   *.     :    :**** :: :::  .  .        *:    .
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Fig. 2. Multiple alignments of the GHF4 α-galactosidases. The amino acid sequences were aligned using the ClustalW algorithm. Identical amino acids are indicated by asterisks. Colon means that conserved substitutions have been observed and dot means that semi-conserved substitutions are observed. Numbers at right denote residue positions. The arrows above the sequence at the N terminus indicate a portion of the NAD^+-binding domain, including the conserved GXGS motif of GHF4 proteins. Filled circle indicates that the residues are positionally conserved in GHF4 proteins. Abbreviations in alphabetical order (Swiss-Prot accession numbers in parentheses) are as follows: MELA_BACHD, α-galactosidase from *B. halodurans* (Q9KAQ9); AGAL_BACSU, α-galactosidase from *B. subtilis* (O34645); AGAL_ECOLI, α-galactosidase from *E. coli* (P06720); AGAL_SALTY, α-galactosidase from *S. typhimurium* (P30877); AGAL_RHIME, α-galactosidase from *R. meliloti* (Q9X4Y0).

The purified Mel4A was most active at 37° C and the maximum activity of the enzyme was observed at pH 7.4. The enzyme was stable between pH 7 and 8 at 4° C for 24 hr incubation. Purified enzymes were stable up to 40° C for 10 minutes incubation. Incubation at 50° C for 10 min resulted in a loss of approximately 40% of the enzyme activity, thus displaying distinct thermal instability.

Based on substrate specificity, α-Gals can be classified into two groups; i.e., one group is specific for small saccharides such as *p*NP-Gal, melibiose, raffinose and stachyose, and the other group can liberate galactose from highly polymerized galactomannans such as guar gum in addition to small molecular mass substrates. Most of eukaryotic α-Gals generally specific to highly polymerized galactomannans in addition to small substrate, whereas prokaryotic α-Gals prefer small substrates. In this context, *B. halodurans* Mel4A also prefers small substrates.

Western immunoblots of intracellular and extracellular *B. halodurans* proteins revealed that raffinose induced the expression of Mel4A in intracellular *B. halodurans*. *B. halodurans* grown on raffinose showed an immunoreactive protein with the size of ~51 kDa in agreement with Mel4A size. These

data suggested that Mel4A was a non-secretory protein. The purified recombinant Mel4A required neutral pH for hydrolytic activity. It also indicates that the enzyme is an intracellular enzyme of *B. halodurans*.

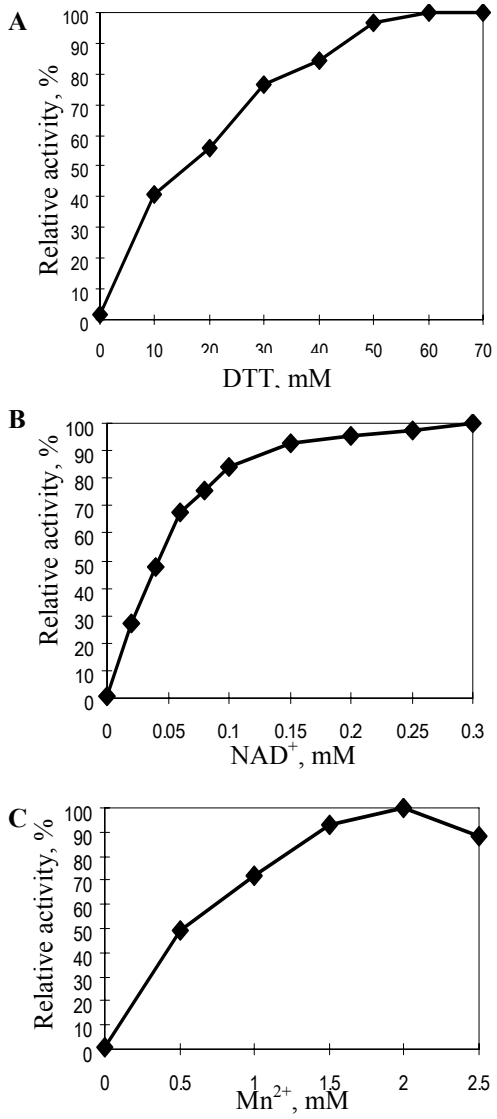

Fig. 3. DTT (A), NAD^+ (B) and Mn^{2+} (C) requirements of Mel4A. **A**: The effect of DTT was measured in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM *p*NP-Gal, 60 µmM NAD^+ and 1 mM Mn^{2+}. **B**: NAD^+ optimum concentration was studied in the presence of 50 mM DTT and 1 mM Mn^{2+}. **C**: In case of Mn^{2+} concentrations, the levels of DTT and NAD^+ were kept constant at 50 mM and 0.2 mM, respectively.

This bacterium was also able to utilize guar gum as the carbon source, but Western blot analysis indicated that the production of Mel4A was not enhanced by the addition of guar gum.

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