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Cloning, Sequencing, and Expression of the Gene Encoding the *Clostridium stercorarium* α-Galactosidase Aga36A in *Escherichia coli*

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The α-galactosidase gene *aga36A* of *Clostridium stercorarium* F-9 was cloned, sequenced, and expressed in *Escherichia coli*. The *aga36A* gene consists of 2,208 nucleotides encoding a protein of 736 amino acids with a predicted molecular weight of 84,786. Aga36A is an enzyme classified in family 36 of the glycoside hydrolases and showed sequence similarity with some enzymes of family 36 such as *Geobacillus* (formerly *Bacillus*) *stearothermophilus* GalA (57%) and AgaN (52%). The enzyme purified from a recombinant *E. coli* is optimally active at 70°C and pH 6.0. The enzyme hydrolyzed raffinose and guar gum with specific activities of 3.0 U/mg and 0.46 U/mg for the respective substrates.

Key words: *Clostridium stercorarium*; α-galactosidase

α-Galactosidase (α-Gal; EC 3.2.1.22) hydrolyzes α-galactosidic linkages at non-reducing ends in galactose-containing oligosaccharides, galactolipids, and/or galactomannan.[1] α-Gals have the potential for usage in various fields, *e.g.*, in the sugar beet industry, α-Gal was used to increase the sucrose yield by eliminating raffinose which prevents normal crystallization of beet sugar.[2] α-Gals may be used to alleviate flatulence caused by carbohydrates such as raffinose and stachyose in beans, which can not be metabolized by monogastric animals including humans.[3] α-Gals can be used to improve the properties of guar gum, a type of galactomannan, as a gelation promoter and cogelator, by enzymatic release of galactose residues from the guar gum.[4]

In this context, many genes encoding α-Gals have been isolated and characterized from various organisms including filamentous fungi, yeasts, bacteria, plants, and humans along with the enzyme properties of their translated products. On the basis of amino acid sequence homology, catalytic domains of α-Gals can be substantially classified into three groups, families 4, 27 and 36 of glycoside hydrolases.[5,6] Almost all of α-Gals from eukaryotic organisms including fungi, plants, and animals belong to family 27 and some others are classified in family 36. On the other hand, most prokaryotic enzymes have been classified into family 4 or 36 with the exceptions of *Clostridium josui* Aga27A[7] and *Saccharopolyspora erythraea* MelA (DDBJ accession no. AF061331). Furthermore, α-Gals can be classified into two groups based on their substrate specificity,[8] *i.e.*, one group is specific for small saccharides such as *p*-nitrophenyl α-D-galactopyranoside (PNP-Gal), melibiose, and raffinose, and the other group can liberate galactose from galactomannans such as guar (*Cyamopsis tetragonoloba*) gum in addition to small substrates.

Clostridium stercorarium is a spore-forming anaerobic thermophilic bacterium, which is known to ferment various kinds of polysaccharides. Therefore, this bacterium seems to be a good source of thermophilic glycoside hydrolases and their genes, and actually various genes encoding cellulases,[9] xylanase,[10] β-xylosidase,[11] α-L-arabinofuranosidase,[12] and α-L-rhamnosidase[13] have been cloned and characterized along with their gene products. In this paper, we describe the *C. stercorarium aga36A* gene encoding an α-Gal classified in family 36 of glycoside hydrolases.

Materials and Methods

Bacterial strains and plasmids. C. stercorarium strain F-9 used for isolation of the chromosomal DNA was described previously.[14] *Escherichia coli* DH5α was used as the cloning host. Plasmids pBluescript II KS (+) and SK (+) was used for cloning experiments and analysis of the DNA sequence of the *aga36A* gene.

Cloning and screening of α-galactosidase gene (aga36A) from C. stercorarium F-9. The chromosomal DNA from *C. stercorarium* F-9 was partially digested with *Sau*3AI. The 3′-recessed ends of the DNA fragments were partially filled with dG and dA by Klenow enzyme and ligated into the *Xho*I site of

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Abbreviations: α-Gal, α-galactosidase; 4MU-Gal, 4-methylumbelliferyl α-D-galactopyranoside; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PNP-Gal, *p*-nitrophenyl α-D-galactopyranoside; TLC, thin-layer chromatography

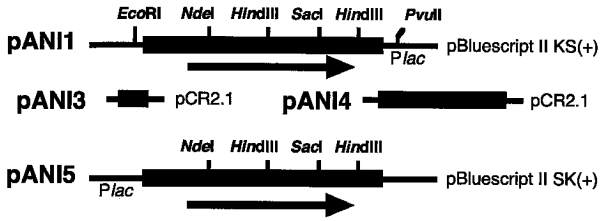

Fig. 1. Restriction Map of pANI1 Carrying the *aga36A* Gene.
Thin lines correspond to pBluescript II or pCR2.1, and bars correspond to the inserted DNA fragments. The position and direction of the ORF of *aga36A* are shown by an arrow. P*lac*, the *lac* promoter.

pBluescript II KS (+), the 3′-recessed ends of which had been also partially filled with dC and dT. Recombinant plasmids were introduced into *E. coli* DH5α. α-Gal-producing clones were screened as follows. Transformants were cultivated on LB agar (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7.0) plates at 37°C overnight and the plates were incubated at 60°C for 2 hr to inactivate the α-Gal activity of the host. Soft agar (0.5% agar in sodium phosphate buffer, pH 7.0) containing 10 mM 4-methylumbelliferyl α-D-galactopyranoside (4MU-Gal) was solidified on the plates and then incubated for 15 min at 60°C. Recombinant clones having α-Gal activity were detected as colonies showing fluorescence under UV irradiation.

Cloning of the upstream and downstream regions of aga36A. Inverse polymerase chain reaction (PCR) was used to amplify the upstream and downstream regions of *aga36A* (Fig. 1). To clone the upstream region, the *C. stercorarium* F-9 genomic DNA was digested with *Nde*I, made circular by self-ligation, and used for PCR as the template DNA with KOD Dash DNA polymerase (Toyobo, Osaka, Japan) and two primers complementary to the nucleotide sequence upstream of *aga36A*, InvN1, 5′-CGGCCATCGAACTGAAAGCG-3′, and InvN2, 5′-TAAAGACCTTATGTGGCCTC-3′ under the conditions recommended by Toyobo. The amplified DNA fragments were ligated into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, USA) using a Takara Ligation kit (Kyoto, Japan), yielding pANI3 (Fig. 1). For cloning of the downstream region, *Hin*dIII was used for digestion of the genomic DNA and primers InvC1, 5′-TCCAACCGAATACAGAACAC-3′, and InvC2, 5′-TTTATCAGATACTGCTGAGG-3′, were used for PCR. The resultant plasmid was designated as pANI4 (Fig. 1).

DNA sequencing. DNA sequencing of a plasmid pANI1 was done on a Licor (Lincoln, Nebr.) model 4000L automated DNA sequencer, with appropriate dye primers and a series of subclones. The subclones were constructed by removing specific restriction fragments from the insert DNA of pANI1 and religating it or by subcloning specific restriction fragments into pBluescript II KS (+). The nucleotide sequence data was analyzed with the GENETYX computer software (Software Development Co., Ltd., Tokyo, Japan). Homology searches in GenBank were done with a BLAST program.

Purification of Aga36A. The *Eco*RI-*Pvu*II fragment of pANI1 was ligated between the *Eco*RI and *Eco*RV sites of pBluescript II SK (+), yielding pANI5. *E. coli* DH5α(pANI5) was grown on a rotary shaker overnight at 37°C in 1000 ml of LB medium containing ampicillin (50 μg/ml) and 100 μM isopropyl β-D-thiogalactopyranoside. The cells were harvested by centrifugation and resupended in 50 ml of 20 mM Tris-HCl buffer (pH 7.5). The cells were disrupted by sonication and cell debris was removed by centrifugation. The majority of the *E. coli* proteins in the crude extract was inactivated and precipitated by heat treatment at 70°C for 15 min. This heat treatment did not decrease the total enzyme activity in the crude extract. The precipitates were removed by centrifugation to recover the supernatant. Proteins were put onto a HiPrep 16/10 DEAE FF column (1.6 × 10 cm; Amersham Pharmacia Biotech, Buckinghamshire, England) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). Adsorbed proteins were eluted with a linear gradient of NaCl (0–0.35 M) in the same buffer. Active fractions were collected, desalted, and put onto a RESOURCE PHE column (1 ml; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$. Adsorbed proteins were eluted with a decreasing gradient of $(NH_4)_2SO_4$ (1–0 M). Fractions with high activity were used for the characterization of Aga36A.

Enzyme assays. Enzyme activity was routinely measured with 10 mM *p*-nitrophenyl α-D-galactopyranoside (PNP-Gal) as a substrate. The reaction mixture (final volume of 400 μl), consisting of appropriately diluted enzyme and PNP-Gal in McIlvaine's buffer (a mixture of 0.1 M citric acid and 0.2 M $NaHPO_4$, pH 7.0), was incubated at 60°C for 15 minutes. The amount of *p*-nitrophenol released from the substrate was measured by the absorbance at 410 nm. One unit (U) of enzyme for PNP-Gal was defined as the amount of enzyme that liberates 1.0 μmol of *p*-nitrophenol per min from PNP-Gal under the given assay conditions. Raffinose (final concentration: 1%) and guar gum (0.5%) were also used as natural substrates for the enzyme assay in the same buffer and temperature described above. The amount of reducing sugars from each substrate was measured with dinitrophthalic acid reagent.[15] One unit of enzyme for these substrates was defined as the amount of enzyme that produced 1 μmol of galactose per min. The protein concentration was measured

with a Micro BCA Protein Assay Kit (Pierce, Rockland, IL) with bovine serum albumin as a standard.

Effects of temperature and pH. The temperature optimum was found under standard assay conditions with PNP-Gal as the substrate by incubating the reaction mixtures at 50°C to 80°C. Thermal stability was measured by incubating the enzyme for 10 min at different temperatures. After the enzyme solution was cooled on ice, its residual activity was measured using the standard assay. For identifying the optimum pH for enzyme activity, enzyme assays were done in Britton and Robinson's universal buffer (80 mM phosphoric acid-80 mM boric acid-80 mM acetic acid the pH was adjusted to 4 to 12 with 1 N NaOH) by a 15-min incubation at 60°C. The pH stability was measured by incubating 10 μl of the appropriately diluted enzyme in Britton and Robinson's universal buffer at 4°C for 24 h. Enzyme activity was then measured under the standard assay conditions.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was done using 10% acrylamide gel as described by Laemmli.[16]

Analysis of N-terminal amino acid sequence. Aga36A purified from the recombinant *E. coli* was fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane by electroblotting. The protein band of Aga36A was excised from the Coomassie blue-stained membrane sheet and put through N-terminal amino acid sequence analysis on an Applied Biosystems model 476A protein sequencer (Foster City, CA, USA).

Analysis of hydrolysis products. Thin-layer chromatography (TLC) of the hydrolysis products of melibiose and raffinose was done on a silica gel 60 plastic sheet (Merck, Whitehouse Station, NJ, USA) developed with a solvent of *n*-butanol-acetic acid-water (2:1:1), and mono- and oligosaccharides were made visible by spraying the plate with a diphenylamine-aniline-phosphate reagent.[17]

Nucleotide sequence accession number. The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB089353.

Results and Discussion

Cloning of the aga36A gene from C. stercorarium F-9

Approximately 5,000 transformants were obtained from the *C. stercorarium* F-9 gene library and screened for α-Gal productivity using 4MU-Gal as the substrate. As a result, two clones were selected as α-Gal-producing recombinants. Plasmids pAN1 (Fig. 1) and pAN2 were isolated from these two recombinant clones and the restriction maps of the inserted DNA fragments were constructed. Comparison of the restriction maps suggested that these plasmids contained the same inserted fragment but in the opposite directions. We chose pAN1 for further investigation. The α-Gal gene carried by pAN1 was referred to as *aga36A*.

Nucleotide sequence of aga36A and its flanking regions

When we analyzed the nucleotide sequence of the inserted fragment on the plasmid pANI1, we found an incomplete open reading frame (ORF) upstream of *aga36A*. Therefore, we amplified the upstream and downstream regions of *aga36A* by inverse PCR, cloned the amplified fragments (Fig. 1), and sequenced them. Figure 2 shows the complete nucleotide sequence of the *aga36A* structural gene along with its flanking regions. The ORF of *aga36A*, starting at nucleotide position 1,046 and ending at position 3,253, consists of 2,208 nucleotides encoding a protein of 736 amino acids with a predicted molecular weight of 84,786. Upstream of *aga36A*, a complete ORF (ORF1) encoding 276 amino acids and an incomplete ORF (ORF2) encoding 54 amino acids were identified. Downstream of *aga36A*, on the other hand, another incomplete ORF (ORF3) encoding 319 amino acids was found, but the direction of the ORF is opposite to that of *aga36A*. The putative initiation codon (ATG) of *aga36A* was preceded at a spacing of 6 bp by a potential ribosome-binding sequence (5′-AGGGGG-3′), which was homologous to the consensus Shine-Dalgarno (SD) sequence (5′-AGGAGG-3′) of *E. coli*. ORF1 was also preceded by a potential ribosome-binding sequence (5′-AGGGGG-3′). There are only 37-bp and 10-bp spacings between *aga36A* and ORF1 and between ORF1 and ORF2, respectively, suggesting that these genes are transcribed in a polycistronic mRNA.

Amino acid sequences of Aga36A and other ORFs

Comparison of the amino acid sequence of Aga36A with entries in the DDBJ database indicated that this enzyme is classified in family 36 of glycoside hydrolases.[5,6] Aga36A shows overall sequence identity with some enzymes in this family, *e.g.*, 58% sequence identity with *Clostridium perfringens* AgaN,[18] 57% and 52% identities with *G. stearothermophilus* GalA (DDBJ accession no. AF038547) and AgaN,[19] respectively, 57% identity with *Bacillus halodurans* possible α-Gal (BH2223),[20] 43% identity with *Lactobacillus plantarum* MelA (AF189765), 42% and 35% identities with *Pediococcus pentosaceus* AgaR and AgaS (L32093), respectively, and 37% identity with *Streptococcus mutans* Aga

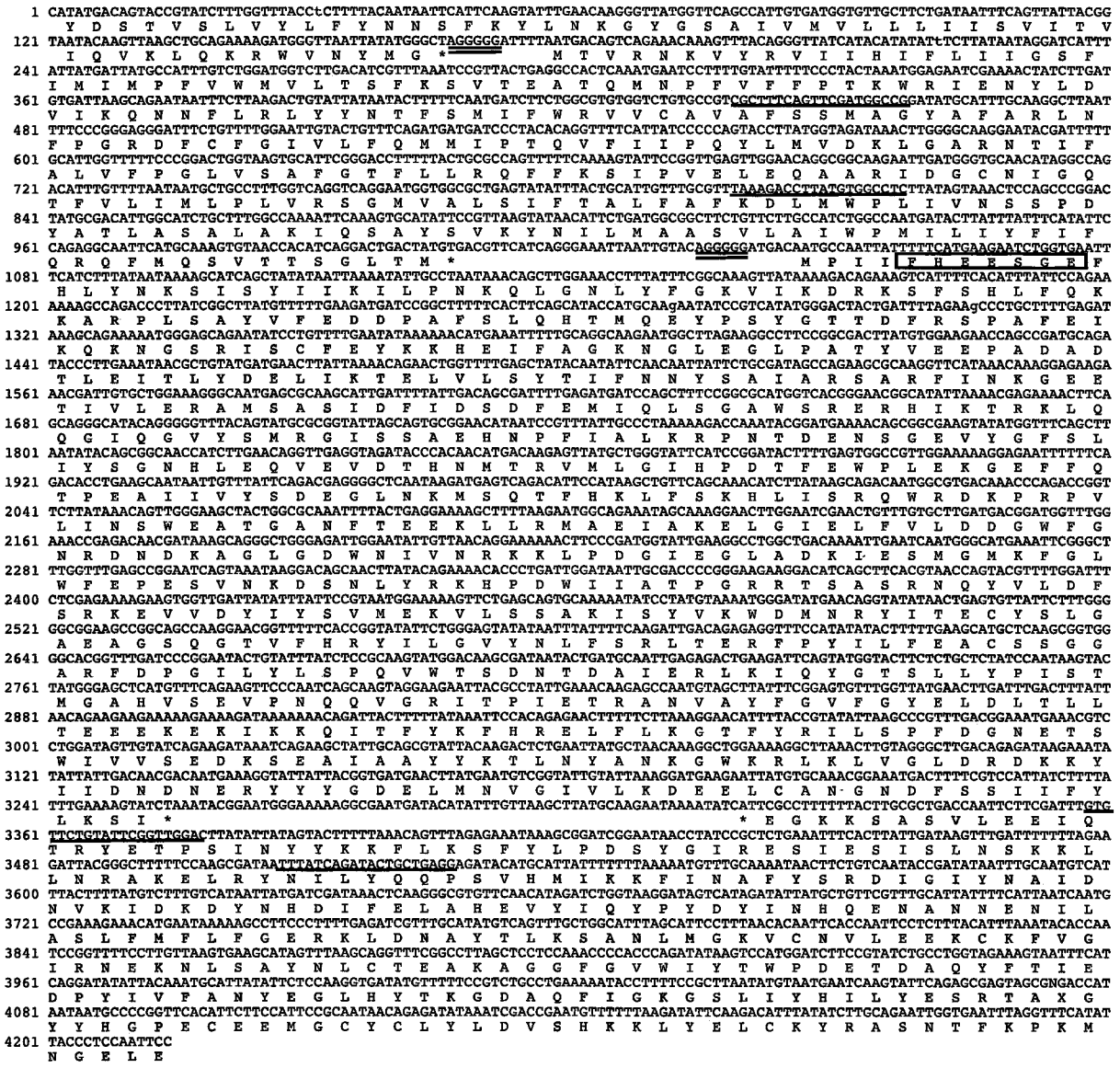

Fig. 2. Nucleotide Sequence of the *aga36A* Gene and Deduced Amino Acid Sequence of the Gene Product.
The possible Shine-Dalgarno (SD) sequences are double-underlined. The amino acid sequence of the purified enzyme experimentally analyzed is boxed. Regions corresponding to PCR primers used for amplification of the upstream and downstream regions are underlined.

(M77351). Furthermore, *C. stercorarium* Aga36A had relatively high sequence similarities with some α-Gals such as *Thermoanaerobacter ethanolicus* MelA (Y08557), *E. coli* RafA,[21] *Bifidobacterium longum* AglL (AF160969), and *Bifidobacterium adolescentis* Aga (AF124596), although their amino acid sequences are not conserved in their N-terminal and/or C-terminal regions. By contrast, Aga36A showed lower sequence identities in restricted region (about 150 amino acids in the central region of Aga36A) with the other enzymes such as *Thermus thermophlus* AgaT (AF135399), *Thermotoga neapolitana* AglA,[22] and *Thermus brockianus* AgaT.[23]

Deduced amino acid sequences of the overall region of the ORF1 and ORF2 polypeptides (ORF1p and ORF2p) were highly homologous with many sugar transport proteins, *e.g.*, ORF1p showed the highest homology (74% identity) with a probable membrane transport protein of *C. perfringens*[18] and the ORF2p (54 amino acids) showed 72% identity with a different probable membrane transport protein of *C. perfringens*.[18]

A deduced amino acid sequence (a region of about 270 amino acids) of the ORF3 polypeptide (ORF3p) showed homology with many transcriptional regulatory proteins of the AraC/XylS family, *e.g.*, 37% identity with a probable transcriptional regulator of *C. perfringens*,[18] 32% identity with the *msm* operon regulatory protein of *Streptococcus pneumoniae* (AE007480), and 33% identity with a probable transcriptional regulator protein of *B. halodurans*.[20]

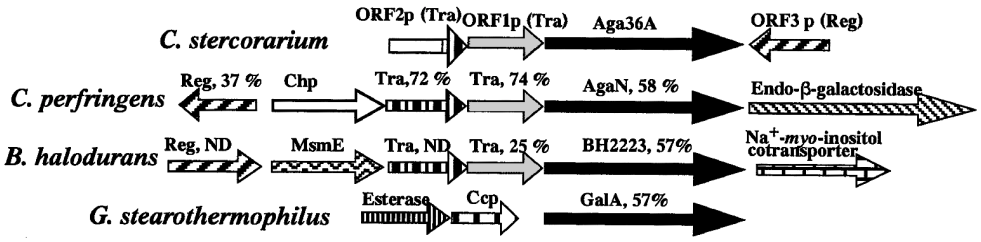

Fig. 3. Comparison of α-Gal Gene Clusters of *C. stercorarium*, *C. perfringens*, *B. halodurans*, and *G. stearothermophilus*.
Related gene products are shown in the same patterns. Tra, probable transporter protein; Reg, probable transcription regulator; Ccp, catabolite control protein, Chp, conserved hypothetical protein, MsmE, multiple sugar-binding protein. Sequence identities (%) of hypothetical gene products to Aga36A, ORF1p, ORF2p, and ORF3p of *C. stercorarium* are shown above related gene products. ND means no sequence identity detected by homology searches with the BLAST program.

Comparison of the α-Gal gene clusters

As seen in the *E. coli lac* operon, gene(s) responsible for the transport of substrates or hydrolysis products are often found in the vicinity of a glycoside hydrolase. In case of the *C. stercorarium aga36A* gene, two ORFs encoding probable transport proteins and a gene encoding a possible transcriptional regulator were found in its vicinity (Fig. 3). Therefore, we compared the organization of the α-Gal gene clusters that encoded α-Gals highly homologous with Aga36A, *i.e.*, the gene clusters of *C. perfringens aga*, *G. stearothermophilus galA*, and *B. halodurans gal* (BH2223). As shown in Fig. 3, two genes encoding probable transport proteins are found upstream of *C. perfringens aga* and *B. halodurans gal* but no gene responsible for sugar transport is identified in the vicinity of *G. stearothermophilus galA*. Amino acid sequences of ORF1p and ORF2p showed the highest homology with those of the corresponding probable transport proteins of *C. perfringens*, *i.e.*, 74% and 72% identities, respectively. ORF1p was less homologous with the corresponding protein of *B. halodurans*, *i.e.*, ORF1p showed only 25% identity with a probable transport protein and ORF2p showed no sequence identity with another probable transport protein of *B. halodurans*. Although a probable transcriptional regulator gene (ORF3) is found downstream of *aga36A* in *C. stercorarium*, a homologous gene (37% sequence identity) was conserved upstream of the *C. perfringens aga* cluster and the direction of the probable transcriptional regulator gene was opposite to that of the *C. perfringens aga* gene; a BLAST search indicated that ORF3p is the most homologous with the transcriptional regulator of *C. perfringens* among the entries in DDBJ database. These results suggest that the *C. stercorarium aga36A* gene cluster is closely related to the *C. perfringens aga* gene cluster, *i.e.*, these genes are derived from a common ancestor, and rearrangement of the gene organization occurred during their evolution.

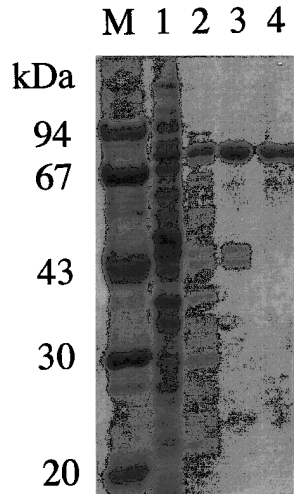

Fig. 4. SDS-PAGE of the Purified Recombinant Aga36A.
The gel was stained with Coomassie brilliant blue: lane M, protein molecular mass standard (molecular masses shown at the left); lane 1, crude extract; lane 2, proteins after heat treatent; lane 3, proteins after a HiPrep 16/10 DEAE FF column; lane 4, Aga36A after a RESOURCE PHE column.

Purification and characterization of the recombinant Aga36A

The recombinant Aga36A was purified 40-fold from the cell-free extract of *E. coli* DH5α(pANI5) by HiPrep 16/10 DEAE FF column and RESOURCE PHE column chromatographies with the yield of 18%. Specific activity of the finally purified enzyme was 450 U/mg. The purified preparation gave a single band on SDS-PAGE, and the molecular weight of the purified enzyme was estimated to be around 70,000 (Fig. 4). However, the molecular weight estimated by SDS-PAGE appeared to be smaller than that deduced from the amino acid sequence of Aga36A (84,786). The N-terminal amino acid sequence of the purified enzyme was found to be Phe-His-Glu-Glu-Ser-Gly-Glu, which is in complete agreement with the amino acid sequence (amino acid positions from 5 to 11) deduced for the DNA sequence. Therefore, it is likely that Aga36A suffered from partial proteolysis by host protease(s) at its C-terminal region. Although it is possible that the

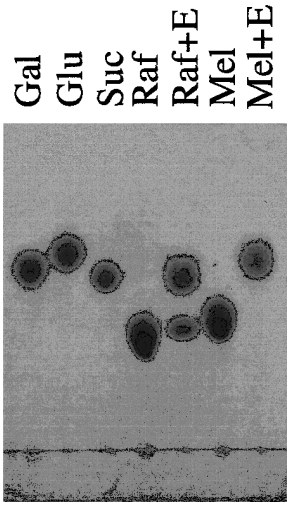

Fig. 5. Thin-layer Chromatography of Hydrolysis Products from Raffinose and Melibiose.

Raffinose (20 μg) and melibiose (20 μg) were separately incubated with the purified enzyme (0.5 unit) in 10 μl of McIlvaine buffer (pH 7.0) for 6 hr and the hydrolysates were analyzed by TLC. Gal, galactose; Glu, glucose; Suc, sucrose; Raf, raffinose; Mel, melibiose; Raf + E, raffinose digested with the enzyme; Mel + E, melibiose digested with the enzyme.

properties of the purified enzyme are not necessarily identical to those of the non-truncated enzyme, we could not purify the latter molecular species. Since the purified enzyme showed high specific activity, we used this preparation for further characterization. The purified enzyme had relatively high specific activity toward raffinose (3.0 U/mg) and low activity toward to guar gum (0.46 U/mg). The action of the enzyme on raffinose and melibiose was qualitatively analyzed by TLC. As shown in Fig. 5, the recombinant Aga36A hydrolyzed raffinose to yield galactose and sucrose, and hydrolyzed melibose to yield galactose and glucose. The enzyme was optimally active at pH 6.0 and showed high activity, *i.e.*, more than 90% of the maximum activity, in the pH range of 5 to 8 and about 95% of the maximum activity at pH 7.0 when the enzyme activity was assayed with PNP-Gal by 15-min incubation at 60°C in Britton and Robinson's universal buffer solutions at various pHs. The enzyme was stable in the pH range of 6 to 10 when incubated at 4°C for 24 h in the same buffer solutions without the substrate. The effects of temperature on the activity and stability of the enzyme were examined. The optimum temperature of the enzyme was found at 70°C. The enzyme was stable up to 70°C for 10 minutes in the absence of substrate. Enzyme assays in the presence of various metal ions (final concentration of 1 mM) such as Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} suggested that Aga36A does not have any metal ion requirement. The initial rates of the reaction were measured with various concentrations of PNP-Gal at 60°C in McIlvaine's buffer (pH 7.0). From the Lineweaver-Burk plot, the K_m and V_{max} values were calculated to be 4.3 mM and 0.43 μmol/min/μg, respectively. This K_m value was higher that that of *G. stearothermophilus* AgaN for the same substrate (0.38 mM) although the V_{max} value was comparable with that of *G. stearothermophilus* AgaN (0.52 μmol/min/μg).[19]

As described above, *C. stercorarium* Aga36A has overall sequence identity with some α-Gals of family 36 from *C. perfringens*, *G. stearothermophilus*, *B. halodurans*, *L. plantarum*, and *P. pentosaceus*. Among them, *G. stearothermophilus* AgaN was examined for its enzyme properties.[19] AgaN purified from *E. coli* was maximally active at 75°C and highly thermostable, *i.e.*, its half-life of inactivation at 70°C was 19 h. AgaN showed a high affinity for oligomeric substrates such as melibiose and raffinose. Properties of *C. stercorarium* Aga36A seem to be similar to those of *G. stearothermophilus* AgaN.

Acknowledgments

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