Expression of Rice (Oryza sativa L. var. Nipponbare) \(\alpha\)-Galactosidase Genes in Escherichia coli and Characterization

Suhong Li,1 Wook-Dong Kim,2 Satoshi Kaneko,2 Prukutty A. Prema,3 Mitsutoshi Nakajima,4 and Hideyuki Kobayashi1,4

1Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
2Food Biotechnology Division, National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8642, Japan
3Biotechnology Division, Regional Research Laboratory (CSIR), Trivandrum 695 019, India
4Food Engineering Division, National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8642, Japan

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Two putative \(\alpha\)-galactosidase genes from rice (Oryza sativa L. var. Nipponbare) belonging to glycoside hydrolase family 27 were cloned and expressed in Escherichia coli. These enzymes showed \(\alpha\)-galactosidase activity and were purified by Ni Sepharose column chromatography. Two purified recombinant \(\alpha\)-galactosidases (\(\alpha\)-galactosidase II and III; \(\alpha\)-Gal II and III) showed a single protein band on SDS–PAGE with molecular mass of 42 kDa. These two enzymes cleaved not only \(\alpha\)-D-galactosyl residues from the non-reducing end of substrates such as melibiose, raffinose, and stachyose, but also liberated the galactosyl residues attached to the O-6 position of the mannosyl residue at the reducing-ends of mannobiose and mannotriose. In addition, these enzymes clipped the galactosyl residues attached to the inner-mannosyl residues of mannopen-taose. Thus, \(\alpha\)-Gal II catalyzes efficient degalactosylation of galactomannans, such as guar gum and locust bean gum.

Key words: \(\alpha\)-galactosidase; galactomanno-oligosaccharides; galactomannans; substrate specificity; rice (Oryza sativa L. var. Nipponbare)

Galactose is found in various plant oligo- and polysaccharides present as matrix and storage components. Raffinose family oligosaccharides contain \(\alpha\)-1,6-linked D-galactopyranose units attached to sucrose. The most abundant polymers containing galactose are galactomannans. The amount and distribution of \(\alpha\)-1,6-D-galactosyl side groups along the \(\beta\)-1,4-D-mannanopyranose backbone depend on the species. For example, guar gum contains 38–40\% (w/w) galactose, whereas locust bean gum contains 22–24\% (w/w) galactose.\(\alpha\)-Galactosidase (\(\alpha\)-Gal, \(\alpha\)-D-galactoside galactohydrolase, EC 3.2.1.22) is one of the exoglycosidases widely distributed in microorganisms, plants, and animals. Some of them have been purified and characterized.\(\alpha\)-Gal cleaves single D-galactosyl residues from the non-reducing end of substrates. \(\alpha\)-Gals are of particular interest in view of their biotechnological applications. \(\alpha\)-Gal from coffee bean shows relatively broad substrate specificity, cleaving a variety of terminal \(\alpha\)-galactosyl residues, such as blood group B antigens on the erythrocyte surface. Treatment of type B erythrocytes with coffee bean \(\alpha\)-Gal results in specific removal of the terminal \(\alpha\)-galactosyl residues, generating serological type O erythrocytes.\(\) Cyamopsis tetragonoloba (guar) \(\alpha\)-Gal effectively liberates the \(\alpha\)-galactosyl residue of galactomannan. \(\alpha\)-Gal removes a large proportion of galactose moieties from guar gum, improving its gelling properties to the level of those of locust bean gum.\(\) In the sugar beet industry, \(\alpha\)-Gal has been used to increase sucrose yields by eliminating raffinose, which prevents normal crystallization of sucrose. Raffinose and stachyose in beans are known to cause flatulence. \(\alpha\)-Gal has the potential to alleviate these symptoms, in the treatment of soybean milk, for instance. We studied the substrate specificity of \(\alpha\)-Gals belonging to glycoside hydrolase family 27 using galactomanno-oligosaccharides such as Gal\(\beta\)Man\(\beta\) (\(6\)-\(\alpha\)-D-galactosyl-mannotriose) and Gal\(\beta\)Man\(\alpha\) (\(6\)-\(\alpha\)-D-galactopyranosyl-mannotetraose). Mortierella vinacea \(\alpha\)-Gal I and

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1 To whom correspondence should be addressed. Fax: +81-29-838-7996; E-mail: hkobayas@affrc.go.jp

Abbreviations: pNP-\(\alpha\)-Gal, p-nitrophenyl-\(\alpha\)-D-galactopyranoside; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; pCMB, p-chloromercuribenzoic acid; IPTG, isopropyl-\(\beta\)-D-galactopyranoside.
\( \alpha \)-Galactosidase from Rice

\( \alpha \)-Galactosidases are specific to Gal\(^5\)Man\(_3\), which has an \( \alpha \)-galactosyl residue attached to the O-6 position of the non-reducing end mannose residue of \( \beta \)-1,4-mannotriose. On the other hand, *Aspergillus niger* 5–16 \( \alpha \)-Gal\(^9\) and *Penicillium purpureogenum* \( \alpha \)-Gal\(^{10}\) show a preference for Gal\(^3\)Man\(_2\), which has an \( \alpha \)-galactosyl residue attached to the O-6 position of the third mannose from the reducing end of \( \beta \)-1,4-mannotetraose. Mortierella vinacea \( \alpha \)-Gal II,\(^{11}\) sunflower \( \alpha \)-Gal,\(^{12}\) and rice \( \alpha \)-Gal I\(^{13}\) act on both substrates to almost equal extents.

Genes encoding \( \alpha \)-Gals have been cloned from various sources, and \( \alpha \)-Gals have been divided into 4 GH families based on the nucleotide sequence database. Five putative genes (GenBank accession nos. AA085428, AA13536, BAC55816, BAD73696, and BAC79549) belong to GH family 27, and another putative gene (GenBank accession no. BAD10122) belongs to GH family 36,\(^{14}\)

The complete genome sequence of rice has been determined,\(^{15}\) and six putative \( \alpha \)-Gal genes appear in the nucleotide sequence database. Five putative genes (GenBank accession nos. AAO85428, AA13536, BAC55816, BAD73696, and BAC79549) belong to GH family 27, and another putative gene (GenBank accession no. BAD10122) belongs to GH family 36.\(^{14}\)

We have cloned the rice \( \alpha \)-Gal I (GenBank accession no. BAB12570) gene,\(^{11}\) the putative \( \alpha \)-galactosidase gene from rice and expressed them in *E. coli* to characterize the enzymes.

### Material and Methods

#### Materials

Melibiose, raffinose, stachyose, \( p \)-nitrophenyl-\( \alpha \)-D-galactoside (\( pNP-\alpha \)-Gal), guar gum, locust bean gum, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Galactomanno-oligosaccharides such as GM\(_2\) (6\(^{\text{a}}\)-D-\( \alpha \)-galactosyl mannohexose), GM\(_1\) (6\(^{\text{a}}\)-D-\( \alpha \)-galactosyl mannohexaose), and GGM\(_3\) (6\(^{\text{a}}\), 6\(^{\text{b}}\)-D-\( \alpha \)-galactosyl mannotriose) were purchased from Megazyme (Bray, Ireland). Restriction endonucleases and other enzymes were from Takara-Bio (Otsu, Japan) and were used in accordance with the manufacturer’s instructions. *E. coli* strain DH\(_5\)\(_\alpha\) and pGEM-T Easy vector from Promega (Madison, WI) and *E. coli* Origami (DE) and pET-32a(+) from Novagen (Darmstadt, Germany) were used as hosts and vectors for cloning and expression respectively.

#### Molecular cloning and gene expression

Total RNA was prepared using an RNeasy\textsuperscript{®} Plant Mini Kit (Qiagen, Hilden, Germany) from rice seedlings grown for two weeks. First-strand cDNA was synthesized directly from total RNA using a RNA LA PCR\textsuperscript{TM} Kit (AMV) Ver.1.1 (Takara-Bio). Two sets of primers were designed to amplify the two \( \alpha \)-Gal genes without signal sequence on the basis of the cDNA sequences of the putative \( \alpha \)-Gal genes from rice (*Oryza sativa* L. var. Nipponbare) available in the database.

A sense primer (5’-CCATGGCTTCGCAACG-GGCTCGG GCC-3’) containing the NeoI site and an antisense primer (5’-AACGTTCTAGCTCGCTCTTCGTG-3’) containing the HindIII site were designed for \( \alpha \)-Gal II (GenBank accession no. BAC55816), and a sense primer (5’-CCATGGCTTCACAAACGGGCTCTGCCC-3’) containing an NcoI site and an antisense primer (5’-GTCGACTCACCTATGCAA CGGTGACGG-3’) containing a SalI site were designed for \( \alpha \)-Gal III (GenBank accession no. BAC79549).

The partial fragments of \( \alpha \)-Gal genes were amplified with a Perkin-Elmer Thermal Cycler (GeneAmp PCR System 2400). Each of the 25 amplification cycles consisted of a denaturation step at 98°C for 30 s followed by annealing at 65°C for 30 s and primer extension step at 72°C for 1.5 min. Two fragments (1,125 bp and 1,116 bp) were amplified, cloned into pGEM\textsuperscript{®} TEasy vector, and then sequenced using a dRhodamine Terminator Cycle Sequencing Reaction Kit on an ABI PRISM 310 Genetic Analyzer. The obtained PCR product of \( \alpha \)-Gal II cloned in T-Easy vector was subcloned into pET-32a(+) between the NcoI and HindIII sites, and the PCR product of \( \alpha \)-Gal III was subcloned into pET-32a(+) between the NcoI and SalI sites. The resulting plasmids were then transformed into *E. coli* Origami (DE). The strain harboring the \( \alpha \)-Gal II gene was grown in LB medium containing 15\( \mu \)g/ml kanamycin, 12.5\( \mu \)g/ml tetracycline, and 50\( \mu \)g/ml ampicillin at 20°C for 48 h after induction with 0.1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG). The strain harboring the \( \alpha \)-Gal III gene was grown in the same medium at 15°C for 72 h after induction with 0.1 mM IPTG. The cells were harvested by centrifugation (6,000 \( \times \) g, 10 min), and stored at \(-30^\circ\)C.

#### Purification of the recombinant enzymes

Two recombinant enzymes were purified using the same method, as described below. Wet frozen cells were suspended in pH 7.0, 50 mM HEPES buffer with 500 mM NaCl. After sonication and centrifugation, the supernatant was passed through HisTrap HP (Amersham Biosciences, Piscataway, NJ) and the recombinant enzyme was eluted with a linear imidazole gradient (0 to 500 mM) in 50 mM HEPES, pH 7.0, containing 500 mM NaCl at a flow rate of 1 ml/min. The active fraction was desalted using a PD-10 column (Amersham Biosciences). The vector-derived protein, thioredoxin, was removed using a recombinant enterokinase kit (Novagen).
Electrophoretic analysis. SDS–PAGE was carried out using a 12.5% (w/v) acrylamide gel, and the protein bands were visualized by Coomassie Brilliant Blue (CBB) R-250 staining, and then destained with a 30% methanol and 10% acetic acid solution. The molecular weight of the enzyme was determined by SDS–PAGE using Molecular Weight Standards (Bio-Rad).

N-Terminal amino acid sequence of purified rice α-Gals. After the protein in the acrylamide gel was blotted on a polyvinylidene fluoride (PVDF) membrane, the membrane was stained with CBB R-250 to detect the protein. The protein band was cut out and used for N-terminal amino acid sequencing on a protein sequencer (G1005A; Hewlett-Packard, Palo Alto, CA).

Enzyme assay and measurement of protein concentration. The activities of rice α-Gal II and α-Gal III were assayed with 3 mM pNP-α-Gal in McIlvaine buffer, pH 5.0, at 45 °C. After incubation for 10 min, an equal volume of 0.2 M Na2CO3 was added to stop the reaction, and the absorbance at 408 nm was measured. One unit of α-Gal activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per min under the conditions described above.

The protein concentrations of the enzyme preparations were measured using a DC Protein Assay Kit (Bio-Rad) and bovine serum albumin as the standard.

Enzyme properties. The effects of pH on the activity and stability of α-Gals were investigated using McIlvaine buffers from pH 3.0 to 7.5, 0.1 M HEPES buffers from pH 7.0 to 8.5, and 0.1 M Glycine–NaOH buffers from pH 8.5 to 10.5. For determination of their pH stability, the enzymes were pre-incubated at various pHs in the absence of substrate at 20 °C for 60 min, and residual activity was then assayed by the standard assay method.

The effects of temperature on the activity of the α-Gals were determined from 20 °C to 60 °C. For determination of temperature stability, enzymes were pre-incubated at various temperatures in the buffer, pH 5.0, for 60 min, and residual activity was then determined by the standard method.

Substrate specificity. To determine specific activities against galacto-oligosaccharides, α-Gal (0.2 units) was added to 20 mM substrates (melibiose, raffinose, stachyose, GM2, GM3, and GGMS) in McIlvaine buffer, pH 5.0, and after 15 min of incubation at 45 °C, the reaction was stopped by boiling for 5 min. Then the amount of released p-galactose was determined using a Raffinose/Galactose Assay Kit (Megazyme, Bray, Ireland). One unit of activity was defined as the amount of enzyme that releases 1 μmol of galactose per min under the conditions described above.

For galactomannan hydrolysis, α-Gal (2 units) in McIlvaine buffer, pH 5.0, was added to 0.5% substrates (locust bean gum or guar gum). After incubation for 3 h, 6 h, 9 h, 12 h, 18 h, and 24 h at 30 °C, the reaction was stopped by boiling for 5 min. The amount of released D-galactose was determined using a Raffinose/Galactose Assay Kit (Megazyme), and galactose release was confirmed by TLC (HPTLC Silica Gel 60, Merck, Whitehouse Station, NJ). The chromatograms were developed with 1-propanol–nitromethane–water (5:2.3, v/v/v), with sugars on the plate being detected by heating at 140 °C for 5 min after spraying with sulfuric acid. The total sugar content was measured by the anthrone-sulfuric acid method using d-mannose as the standard.

Results and Discussion

Cloning and sequencing α-Gal I has been purified as the active enzyme from cultured rice cells and α-Gal I cDNA has been cloned from rice cells. However, α-Gal II and α-Gal III cDNAs have been cloned by reverse transcription-polymerase chain reaction (RT-PCR) from rice seedlings because it is not possible to clone genes from cultured rice cells. In the case of α-Gal II, the sequences of cDNA obtained by RT-PCR (α-Gal II) and that from the database (putative α-Gal, BAC55816) were somewhat different. This difference might have been due to flawed prediction of the intron-exon boundary sequence by computer programs. The putative sequence (BAC55816) showed a nearly 30-amino-acid deletion (from 38 to 71, shown in Fig. 1) toward α-gal II cloned by RT-PCR in this experiment and the deleted sequence is thought to be important for exhibiting the enzymatic function because this sequence is one of the homologous regions among the α-Gals belonging to GH family 27.

A conserved sequence feature at both ends of the introns was recognized as the GT-AG rule. This rule holds in most cases, but exceptions, such as GC-AG introns, have been found. The difference between the two cDNA sequences (α-Gal II and BAC55816) comes from one GC-AG intron in the genomic sequence for α-Gal II. GC-AG has also been found in many plant origins as well as in the case of α-Gal II. It is sometimes difficult to predict the function of proteins based only on sequence similarity, so it is necessary to clone and express the genes to clarify the actual activity.

Amino acid sequence alignment

The deduced amino acid sequences of recombinant rice α-Gal II and α-Gal III were compared with the available sequences from various sources using MultiAlink (http://prodes.toulouse.inra.fr/multalin). The deduced amino acid sequences of rice α-Gal II and rice α-Gal III shared high similarity with plant and yeast α-Gals, which belong to GH family 27. The rice α-Gal II and α-Gals from Coffea arabica (CAI47559), Cyanopsis teragonoloba (C3A32772), Oryza sativa α-Gal I (BAB12570), and Mortierella vinacea α-Gal II (BAA39391) showed over 63%, 61%, 62%, and 43% sequence similarity, respectively.
The rice \( /C_{11}-\text{Gal III} \) and \( /C_{11}-\text{Gals} \) from \( \text{Coffea arabica} \) (CAI47559), \( \text{Cyamopsis teragonoloba} \) (CAA32772), \( \text{Oryza sativa} /C_{11}-\text{Gal I} \) (BAB12570), and \( \text{Mortierella vinacea} /C_{11}-\text{Gal II} \) (BAA33931) showed over 63%, 62%, 56%, and 45% identity respectively. Homology was found in some regions of the \( N \)-terminal to the central domain, but the \( C \)-terminal domain showed low similarity between these enzymes (Fig. 1). There are 7 Cys residues in both rice \( /C_{11}-\text{Gal II} \) and \( /C_{11}-\text{Gal III} \), and 5 Cys residues from the \( N \)-terminal are conserved among family 27 \( /C_{11}-\text{Gals} \). Based on structural information on rice \( /C_{11}-\text{Gal I} \), 4 Cys residues are thought to be important for maintaining the tertiary structure of the \( N \)-terminal catalytic domain by forming two S–S bridges (C24–C56 and C106–C137).16)

Gene expression and purification
\( /C_{11}-\text{Gal II} \) cDNA was expressed in \( E. \text{coli} \). Recombinant \( /C_{11}-\text{Gal II} \) was purified 23.5-fold by HisTrap HP with a yield of 82.9%. The specific activity against \( p\text{NP-} /C_{11}-\text{Gal} \) was determined to be 52.8 unit/mg protein. Recombinant \( /C_{11}-\text{Gal III} \) was purified with a yield of 20.9%. The specific activity against \( p\text{NP-} /C_{11}-\text{Gal} \) was determined to be 9.13 unit/mg protein (Table 1). SDS–PAGE of the purified \( /C_{11}-\text{Gal II} \) and \( /C_{11}-\text{Gal III} \) enzymes revealed a single protein band (Fig. 2) with an estimated molecular mass of 42 kDa. This value agreed almost exactly with the molecular weight calculated from the amino acid sequences (viz., 41,021 for \( /C_{11}-\text{Gal II} \) and 41,521 for \( /C_{11}-\text{Gal III} \)). The N-terminal amino acid sequences of purified \( /C_{11}-\text{Gal II} \) and \( /C_{11}-\text{Gal III} \) were determined to be A-M-A-L-D-N-G-R and A-M-A-L-N-N-G-L respectively, indicating that these enzymes have
three additional amino acids (A-M-A-) at their N-terminals and are processed correctly by recombinant enterokinase.

**Enzyme properties of α-Gal II and α-Gal III**

The effects of pH and temperature on the activity and stability of α-Gal II and α-Gal III were investigated with pNP-α-Gal as substrate. α-Gals II and III were most active at pH 5.0 at 45°C. α-Gal II is stable in a wide pH range (3.5–8.5 at 25°C and for 1 h up to 80°C at pH 5.0). α-Gal III is less stable than α-Gal II (specifically, it is stable at pH 4.0–7.0 at 20°C and for 1 h up to 20°C at pH 5.0).

Some α-Gals have been reported to be inhibited by SH reagents such as p-chloromercuribenzoic acid (pCMB). Rice α-Gal II and α-Gal III were also completely inactivated (less than 6% and 8% of the control respectively) by treatment with 0.1 mM pCMB at 20°C for 1 h. Metal ions (1 mM) including Ca²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Ni²⁺, and Zn²⁺, did not affect the activity of α-Gal II or α-Gal III. But Ag²⁺ and Hg²⁺ significantly decreased α-Gal II activity, by 67% and 93%, and α-Gal III activity by 84% and 87% respectively. Rice α-Gal I was completely inhibited by pCMB and strongly inhibited by Ag²⁺ and Hg²⁺. Because Cys-162 in α-Gal I (Cys-210 in Fig. 1) is located in the catalytic pocket and is one of three cysteine residues in the molecule, these metal reagents probably attack cysteine and interfere with substrate binding in the catalytic site of α-Gal I. The same phenomena might occur in the active sites of α-Gal II and α-Gal III.

**Substrate specificities of α-Gal II and α-Gal III**

The substrate specificities of rice α-Gal II and α-Gal III were investigated using galacto-oligosaccharides, galactomanno-oligosaccharides, and polysaccharides as substrates. These two enzymes hydrolyzed galacto-oligosaccharide residues in the following decreasing order of reactivity: raffinose > melibiose > stachyose (Table 2). α-Gal III showed higher relative activity against galacto-oligosaccharides than α-Gal II did, because, of the two enzymes, α-Gal III had lower specific activity against pNP-α-Gal. Although α-Gal II hydrolyzed GM₂, GM₃, and GGM₅ to almost equal extents, α-Gal III hydrolyzed GM₂ and GM₃ much faster than it hydrolyzed GGM₅, indicating that the activity of α-Gal II against GGM₅ is higher than that of α-Gal III.

These enzymes also differed in their ability to hydrolyze polymeric substrates. In a 24-h reaction, more of the total α-galactosyl residues were liberated by α-Gal II than by α-Gal III (71° and 78% versus 22° and 33%) from guar gum and locust bean gum respectively, and galactose was the sole product of the reaction (data not shown). The time course of degala-

<table>
<thead>
<tr>
<th>Total Specific</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>activity (unit)</td>
<td>activity (unit/mg)</td>
<td>(fold)</td>
</tr>
<tr>
<td>Crude enzyme</td>
<td>388.7</td>
<td>172.8</td>
</tr>
<tr>
<td>His Trap</td>
<td>322.1</td>
<td>6.10</td>
</tr>
<tr>
<td>α-Gal II</td>
<td>191.9</td>
<td>618.1</td>
</tr>
<tr>
<td>His Trap</td>
<td>40.1</td>
<td>4.39</td>
</tr>
</tbody>
</table>

Table 1. Purification of Rice α-Gal II and α-Gal III

Fig. 2. SDS-PAGE of Purified Recombinant Rice α-Gal II and α-Gal III.

Lane 1, molecular mass markers; lane 2, purified recombinant α-Gal II; lane 3, purified recombinant α-Gal III.
cosylation by these enzymes is shown in Fig. 3. Guar gum and locust bean gum were insolubilized by removal of $\alpha$-1-C11-galactosyl residues at the times indicated by the arrowheads for $\alpha$-C11-Gal II. At the beginning of the reaction, the galactose-to-mannose ratio of guar gum was 1/2.1 and that of locust bean gum was 1/3.6. Precipitation of guar gum and locust bean gum was observed when the galactose content of the polysaccharides decreased to approximately 7%–10%. Locust bean gum was precipitated faster than guar gum, probably owing to its lower galactose content.

Some properties of rice $\alpha$-C11-Gal I, $\alpha$-C11-Gal II, and $\alpha$-C11-Gal III are compared in Table 3. They had similar molecular masses, and pH and temperature optima. $\alpha$-C11-Gal III was less stable than $\alpha$-Gal I or $\alpha$-Gal II. All rice $\alpha$-Gals hydrolyzed oligosaccharides in the order raffinose > melibiose > stachyose. In addition, they also hydrolyzed galactomannan-oligosaccharides. As previously described, $\alpha$-Gals can be classified into three groups depending on their galactomannan-oligosaccharide specificity (Gal$_3$Man$_3$ and Gal$_3$Man$_4$), and rice $\alpha$-Gal I was in the third $\alpha$-Gal category. The galactomannan-oligosaccharides degrade in the order raffinose $>$ melibiose $>$ stachyose. In addition, they also hydrolyzed galactomannan-oligosaccharides.

### Table 2. Substrate Specificity of Rice $\alpha$-Gal II and $\alpha$-Gal III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final conc.</th>
<th>Specific activity</th>
<th>Relative activity</th>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$NP-$\alpha$-Gal</td>
<td>$3 \text{ mM}$</td>
<td>52.8</td>
<td>100</td>
<td>9.13</td>
<td>100</td>
</tr>
<tr>
<td>Melibiose</td>
<td>$20 \text{ mM}$</td>
<td>0.65</td>
<td>1.2</td>
<td>0.63</td>
<td>6.9</td>
</tr>
<tr>
<td>Raffinose</td>
<td>$20 \text{ mM}$</td>
<td>1.89</td>
<td>3.6</td>
<td>0.92</td>
<td>10.1</td>
</tr>
<tr>
<td>Stachyose</td>
<td>$20 \text{ mM}$</td>
<td>0.47</td>
<td>0.9</td>
<td>0.17</td>
<td>1.9</td>
</tr>
<tr>
<td>GM2</td>
<td>$20 \text{ mM}$</td>
<td>3.58</td>
<td>6.8</td>
<td>0.24</td>
<td>2.6</td>
</tr>
<tr>
<td>GM3</td>
<td>$20 \text{ mM}$</td>
<td>3.62</td>
<td>6.9</td>
<td>0.25</td>
<td>2.7</td>
</tr>
<tr>
<td>GGM5</td>
<td>$20 \text{ mM}$</td>
<td>3.86</td>
<td>7.3</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Locust bean gum</td>
<td>0.5%</td>
<td>0.72</td>
<td>1.4</td>
<td>0.04</td>
<td>0.4</td>
</tr>
<tr>
<td>Guar gum</td>
<td>0.5%</td>
<td>0.90</td>
<td>1.7</td>
<td>0.04</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The experimental conditions are described in "Materials and Methods."

### Table 3. Some Properties of Rice $\alpha$-Gal I, $\alpha$-Gal II, and $\alpha$-Gal III

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-Gal I</th>
<th>$\alpha$-Gal II</th>
<th>$\alpha$-Gal III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>41 kDa</td>
<td>42 kDa</td>
<td>42 kDa</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>$45 \degree C$</td>
<td>$45 \degree C$</td>
<td>$45 \degree C$</td>
</tr>
<tr>
<td>pH stability</td>
<td>3–8</td>
<td>3.5–8.5</td>
<td>4–7</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>~40 °C</td>
<td>~35 °C</td>
<td>~20 °C</td>
</tr>
<tr>
<td>Specific activity ($p$NP-$\alpha$-Gal)</td>
<td>119.7 unit/mg</td>
<td>52.8 unit/mg</td>
<td>9.13 unit/mg</td>
</tr>
<tr>
<td>Guar gum</td>
<td>24%*</td>
<td>71%*</td>
<td>22%*</td>
</tr>
<tr>
<td>Locust bean gum</td>
<td>33%*</td>
<td>78%*</td>
<td>33%*</td>
</tr>
</tbody>
</table>

*The percentage of removal of galactose after 24 h of reaction.

Fig. 3. Actions of $\alpha$-Gal II and $\alpha$-Gal III on Galactomannans.

Time course of action of $\alpha$-Gal II on locust bean gum (open circle) and guar gum (open square), and $\alpha$-Gal III on locust bean gum (closed circle) and guar gum (closed square). Times at which precipitation occurred are indicated by arrows.
charide specificities of α-Gal II and α-Gal III were investigated using GM2, GM3, and GGM2, GM2, and GM3 are similar in structure to GalMan, which has a terminal α-galactosyl residue, and GGM2 is similar to GalMan, which has side-chain α-galactosyl residues. On the basis of their galactomannano-oligosaccharide specificities, α-Gal II and α-Gal III are included in the third α-Gal category, enzymes that can act on galactomannans. Of the α-Gals tested, α-Gal I was the most active against pNP-α-Gal, and α-Gal II was the most active against galactomannans. The degree of degalactosylation of galactomannans by rice α-Gal II was found to be similar to that by Thermomyces lanuginosus,19) Lucerne,20) and guar21) enzymes.

Because of their high viscosity, guar gum and locust bean gum are extensively used in the food industry. Galactomannans are also good promoters of gelling when mixed with some acidic polysaccharides, such as κ-carrageenan and xanthan gum. Although locust bean gum interacts more strongly with gelling polysaccharides, guar gum is more readily and stably available, and is much cheaper. Efficient degalactosylation of galactomannans by α-Gal II might be used to generate modified galactomannan with excellent functional properties.

Though their enzymatic properties (including pH and temperature dependencies and galacto-oligosaccharide specificities) were similar, rice α-Gals had significantly different galactomannano-oligosaccharides and galactomannan specificities. The identical amino acid residues between α-Gal I and α-Gal II make up more than 60%, and 11 amino acid residues among them are involved in the galactose binding.16) These results indicate that subtle differences in tertiary structure around the active site residues are important for their hydrolysis of galactomannan, and that further investigation of the enzyme’s structure-function is necessary.

References