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α-Galactosidase from cultured rice (*Oryza sativa* L. var. Nipponbare) cells

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Abstract

The α-galactosidase from rice cell suspension cultures was purified to homogeneity by different techniques including affinity chromatography using *N*-ϵ-aminocaproyl-α-D-galactopyranosylamine as the ligand. From 11 l of culture filtrate, 28.7 mg of purified enzyme was obtained with an overall yield of 51.9%. The cDNA coding for the α-galactosidase was cloned and sequenced. The enzyme was found to contain 417 amino acid residues composed of a 55 amino acid signal sequence and 362 amino acid mature α-galactosidase; the molecular weight of the mature enzyme was thus calculated to be 39,950. Seven cysteine residues were also found but no putative *N*-glycosylation sites were present. The observed homology between the deduced amino acid sequences of the mature enzyme and α-galactosidases from coffee (*Coffea arabica*), guar (*Cyamopsis tetragonolooba*), and *Mortierella vinacea* α-galactosidase II were over 73, 72, and 45%, respectively. The enzyme displayed maximum activity at 45 °C when *p*-nitrophenyl-α-D-galactopyranoside was used as substrate. The rice α-galactosidase and *Mortierella vinacea* α-galactosidase II acted on both the terminal α-galactosyl residue and the side-chain α-galactosyl residue of the galactomanno-oligosaccharides.

Keywords: α-Galactosidase; Galactomanno-oligosaccharides; Rice; *Oryza sativa* L. var. Nipponbare; Grammimeae; Substrate specificity

1. Introduction

α-Galactosidases (αGals, α-D-galactoside galactohydrolase, EC 3.2.1.22) widely occur in microorganisms, plants, and animals, and some of them have been purified and characterized (Dey and Campillo, 1984). αGals catalyze the hydrolysis of 1,6-linked α-galactosyl residues from oligosaccharides and polymeric galactomannans (Margolles-Clark et al., 1996; Shibuya et al., 1995a, 1997).

We have studied the substrate specificity of αGals using galactomanno-oligosaccharides such as $Ga1^3Man_3$ (*O*-α-D-galactopyranosyl-(1->6)-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose] and $Ga1^3Man_4$ (*O*-β-D-mannopyranosyl-(1->4)-*O*-[α-D-galactopyranosyl-(1->6)]-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl - (1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose] as the substrates. The structures of these galactomanno-oligosaccharides are shown in Fig. 1. *Mortierella vinacea* αGal I (Kaneko et al., 1990) and yeast αGals (Yoshida et al., 1997) are specific for the $Ga1^3Man_3$ having an α-galactosyl residue (designated the terminal α-galactosyl residue) attached to the O-6 position of the nonreducing end mannose of β-1,4-mannotriose. On the other hand, *Aspergillus niger* 5–16 αGal (Kaneko et al., 1991) and *Penicillium purpurogenum* αGal (Shibuya et al., 1995a) show preference for the $Ga1^3Man_4$ **2** having an α-galactosyl residue (designated the side-chain

Abbreviations: *p*NP-α-Gal, *p*-nitrophenyl-α-D-galactopyranoside; Gal^3Man_3, *O*-α-D-galactopyranosyl-(1->6)-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose; Gal^3Man_4, *O*-β-D-mannopyranosyl-(1->4)-*O*-[α-D-galactopyranosyl-(1->6)]-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose; PCMB, para-chloromercuribenzoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends

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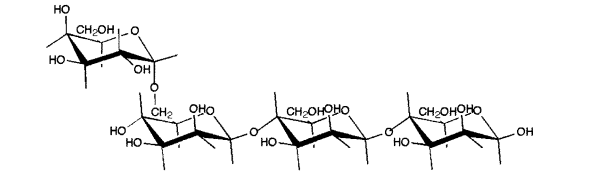
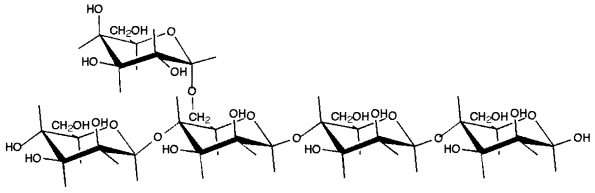


Gal^3Man_3



Gal^3Man_4

Fig. 1. Structures of galactomanno-oligosaccharides.Gal^3Man_3, *O*-α-D-galactopyranosyl-(1->6)-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose; Gal^3Man_4, *O*-β-D-annopyranosyl-(1->4)-*O*-[α-D-galactopyranosyl-(1->6)]-*O*-β-D-mannopyranosyl-(1->4)-D-mannopyranosyl-(1->4)-D-mannopyranose.

α-galactosyl residue) attached to the O-6 position of the third mannose from the reducing end of β-1,4-mannotetraose. The *M. vinacea* αGal II (Shibuya et al., 1997) acts on both substrates almost equally. Based on these observations, microbial αGals were classified into three groups based on their substrate specificity with galactomanno-oligosaccharides as substrates. In addition, *M. vinacea* αGal II and plant αGals were active on the polymeric substrates, galactomannans, with high efficiency (Courtois and Petek, 1966; McCleary, 1983; Kontos and Spyropoulos, 1995; Shibuya et al., 1997).

Genes encoding αGals have been cloned from various sources, including human. (Bishop et al., 1986), plants (Overbeeke et al., 1989; Zhu and Goldstein, 1994), yeasts (Sumner-Smith et al., 1985), filamentous fungi (den Herder et al., 1992; Margolles-Clark et al., 1996; Shibuya et al., 1997), and bacteria (Aduse-Opoku et al., 1991; Aslanidis et al., 1989; Liljestrom and Liljestrom, 1987). αGals from eukaryotes show a considerable degree of similarity and were grouped into the glycosyl hydrolase family 27, while bacterial enzymes were mainly grouped into family 36 (Henrissat, 1991).

Here we describe the purification and characterization of αGal from the cultured rice cells, and the cloning and expression of the αGal cDNA in the *Eschericia coli* and its characterization.

2. Results and discussion

2.1. Purification and enzymatic properties of α-Gal

Table 1 summarizes the purification procedure for αGal from the culture filtrate of rice cells. The specific activity of the enzyme increased from an average of 3.81 nkat/mg of protein in culture filtrate to 119.5 nkat/mg after Sephacryl S-200. Thus, the resultant purification is 524 fold with 51.9% overall recovery and the yield of the enzyme after the final step is 28.7 mg. αGal obtained after Sephacryl S-200 was found to be homogeneous by SDS-PAGE when visualized by CBB R-250 staining (Fig. 2a). The molecular weight of the αGal was estimated to be 41,000 by SDS-PAGE and 40,000 by gel filtration on Sephacryl S-200 column (Fig. 2c), indicating that the enzyme exists in a monomeric form in solution. Although some plant αGals exist in different forms, e.g., monomeric and oligomeric forms, which are interconvertible (Del Compillo et al., 1981), other plant αGals, such as coffee αGal (Barham et al., 1971) and guar αGal II (McCleary, 1983), are monomeric. In fungi, *M. vinacea* αGal I (Shibuya et al., 1995b) and *S. cerevisiae* αGal (Ruohola et al., 1986) exist in oligomeric form, but *P. purpurogenum* αGal (Shibuya et al., 1998) and *M. vinacea* αGal II (Shibuya et al., 1997) are both monomeric in solution. The pI for the rice αGal was estimated to be 7.4.

The N-terminal amino acid sequence of the rice αGal was determined to be F-E-N-G-L-G-R-T-P-Q-M-G-W-N-S-W-N-H-F-Y-X-G-I-N-E-Q-I-I-R-E-T-A-D-A-L-V-N-T-G-L. This sequence shared high similarity with plant and yeast αGals which belong to the glycoside hydrolase family 27 when compared with the sequences available in the protein and nucleic acid database using BLAST (on a web-site of the National Center for Biotechnology Information, URL: http://www.ncbi.nlm.nih.gov/BLAST/). The N-terminal amino acid sequence of the rice αGal shared similarity with the following αGals, in the decreasing order: αGal from coffee bean (*Coffea arabica*, 72%, L27992); αGal from kidney bean (*Phaseolus vulgaris*, 71%, U12927); αGal from guar (*C. tetragonoloba,* 71%, X14619); αGal from soybean (*Glycine max*, 70%, U12926); αGal II from *M. vinacea* (47%, AB018691), αGal from *S. cerevisiae* (45%, M10604), αGal I from *M. vinacea* (45%, S79440), αGal from *P. purpurogenum* (44%, AB008367), αGal from human (44%, U78027), and αGal from *A. niger* (37%, X63348).

The enzyme showed maximal activity at pH 5.0 and was slowly inactivated above pH 8 and below pH 3. The maximum activity for αGal occurred at a temperature of 45 °C; however, inactivation was observed at temperatures above 40 °C.

Some metal ions at 1 mM concentration did not affect αGal activity, but Hg^{2+} and Ag^{2+} significantly decreased αGal activity by 98 and 96%, respectively (Table 2). *p*-Chloromercuribenzoic acid (PCMB) also completely inhibited the enzymatic activity (Table 2), indicating that at least one essential Cys residue is modified by the reagent. The addition of Ca^{2+} to enzyme solution suggested a stabilizing effect and hence Ca^{2+} was added to buffers during different steps of purification like dialysis, ion exchange chromatography, and gel filtration.

Table 1
Summary of the purification of α-galactosidase from rice

	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
Culture filtrate	11,000	110,354	28,947	3.81	100	1
Ammonium sulfate ppt.	1600	97,912	5692	17.20	88.7	4.5
SP Sepharose FF	240	71,476	1251	57.14	64.8	15
Affinity gel[a]	130	65,030	42.4	1534	58.9	403
Sephacryl S-200	218	57,264	28.7	1995	51.9	524

[a] *N*-ε-Aminocaproyl-α-D-galactopyranosylamine CH Sepharose.

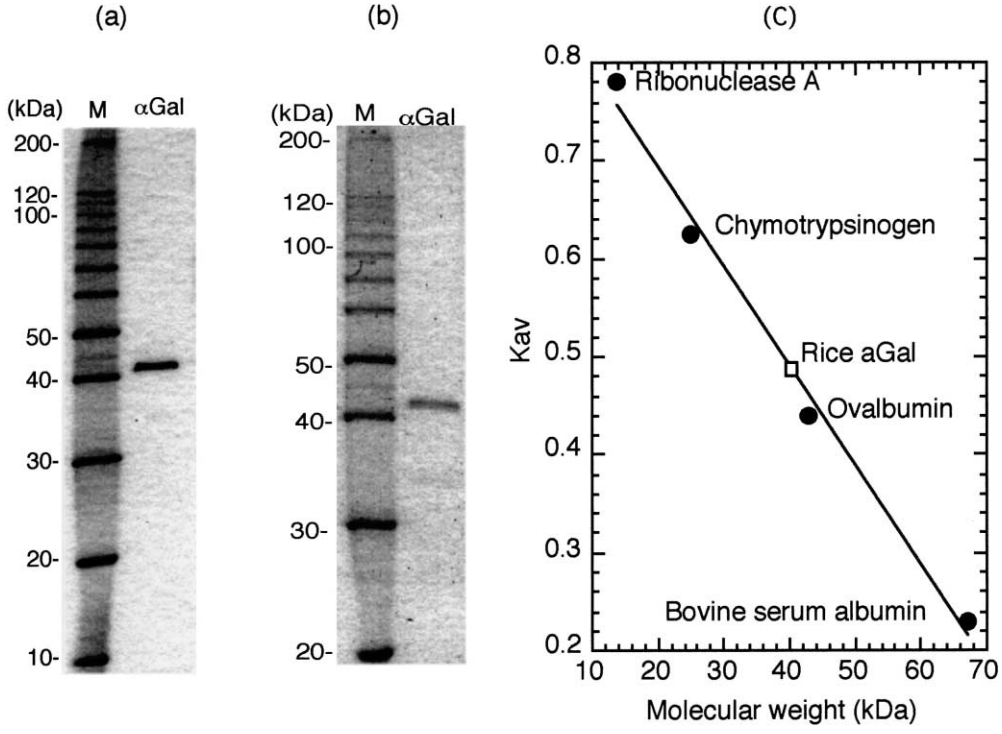


Fig. 2. Molecular weight determination of purified (a) and recombinant (b) rice αGal by SDS-PAGE, and by gel filtration on Hiprep Sephacryl S-200 column (c). Kav = (Ve-Vo)/(Vt-Vo), where Ve = elution volume, Vo = void volume and Vt = total bed volume.

Table 2
Effects of metal ions and chemicals on the a-galactosidase activity

Chemicals	Zn^{2+}	Mg^{2+}	Ca^{2+}	Cu^{2+}	Mn^{2+}	Ag^{2+}	Hg^{2+}	Fe^{2+}	Co^{2+}	EDTA	EGTA	PCMB[a]	b-ME[b]	None
α-Galactosidase activity (nkat)	1.52×10^{-1}	1.72×10^{-1}	1.64×10^{-1}	1.51×10^{-1}	1.68×10^{-1}	0.86×10^{-1}	0.03×10^{-1}	1.70×10^{-1}	1.55×10^{-1}	1.66×10^{-1}	1.68×10^{-1}	0.03×10^{-1}	1.73×10^{-1}	1.62×10^{-1}
Relative activity (%)	94	106	101	93	104	54	2	105	96	99	104	2	107	100

[a] *p*-Chloromercuribenzoic acid.
[b] β-Mercaptoethanol. Metal ions were added in the assay mixture at a concentration of 1 mM.

2.2. Substrate specificities of αGal

The substrate specificities of rice αGal were investigated by using galactose-containing oligosaccharides and polysaccharides. The enzyme hydrolyzed melibiose, raffinose and stachyose in the following decreasing order of reactivity: raffinose > melibiose > stachyose. In addition to this, Gal^3Man_3 and Gal^3Man_4 were also hydrolyzed by rice αGal (Fig. 3) and *Mortierella vinacea* α-galactosidase II as well (Shibuya et al., 1997). As previously described, αGals can be classified into three groups depending on their specificity to galactomanno-oligosaccharides and

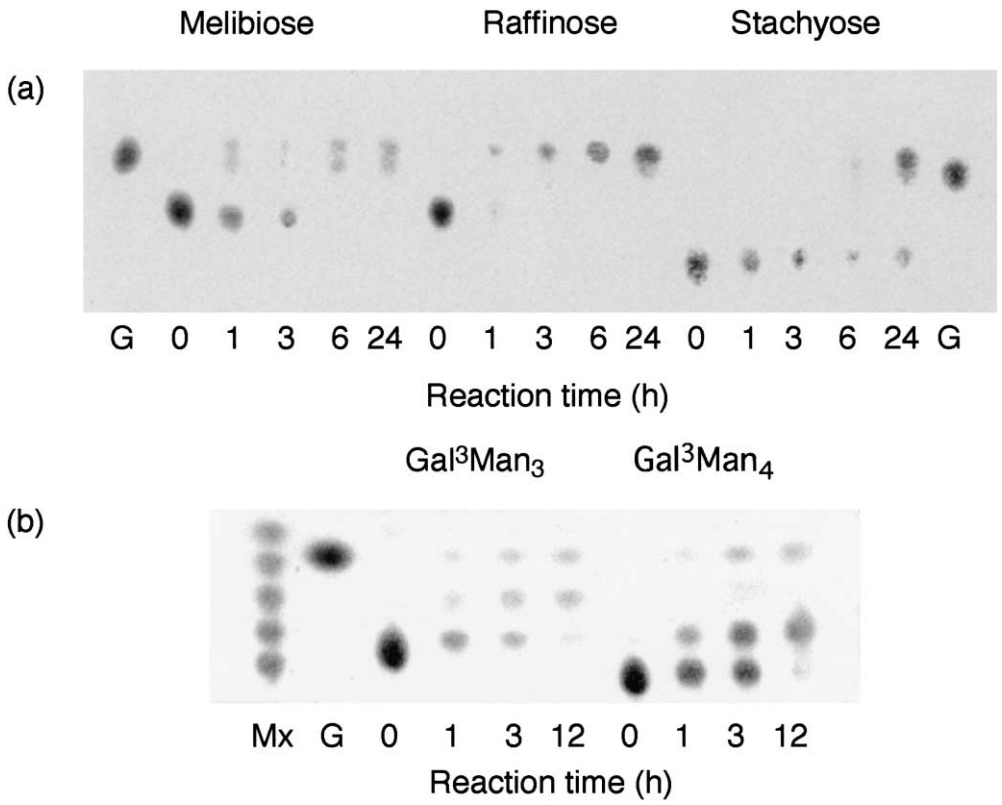


Fig. 3. Action of rice αGal on galacto-oligosaccharides (a) and on galactomanno-oligosaccharides (b). The reaction mixture contained 1% substrate, (40 μl) acetate buffer, pH 5.0, (40 μl) and enzyme solution (20 μl) (3.3 nkat), which was incubated at 30 °C. After 0, 1, 3, 6, 24 h, each of the reaction mixture was boiled for 5 min to stop the reaction. Three μl of the mixture was used for TLC to characterize the hydrolysis products. G, authentic galactose; Mx, authentic mannose to β-1,4-mannopentaose from top to bottom.

the results obtained on the galactomanno-oligosaccharides indicate that the rice αGal is grouped into the third category of αGal. The enzymes belonging to this category acted on polymeric substrates but the enzymes in the other categories did not (Shibuya et al., 1995a, 1998). The time course of the hydrolysis of the galactomannans by αGal, followed by the determination of the releasing galactose residues, is shown in Fig. 4. The upper limits for removing galactose from locust bean gum and guar gum by αGal were 32 and 24%, respectively. Some αGals including the plant αGals were reported to act on the galactomannans to various extents. Guar αGal (McCleary, 1983), coffee αGal (Courtois and Petek, 1966), *Penicillium ochrochloron* αGal (Dey et al., 1993), *M. vinacea* αGal II (Shibuya et al., 1997) effectively acted on galactomannans relative to other enzymes. Rice αGal acted on locust bean gum faster than guar gum and this same effect has been reported for other microbial and plant enzymes (Puchart et al., 2000).

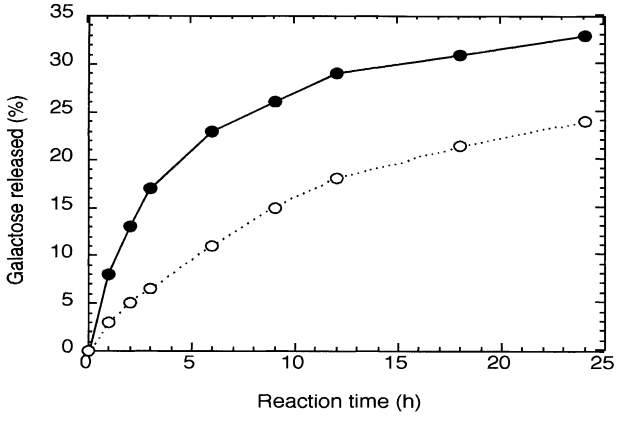


Fig. 4. Action of rice αGal on galactomannans. ●, locust bean gum; ○, guar gum.

2.3. Cloning and nucleotide sequencing of the cDNA coding for αGal

In an attempt to elucidate the structure-function relationships of rice αGal, we have cloned the cDNA coding for this enzyme. The N-terminal amino acid sequence shared high similarity with other plant αGals. Therefore, a similarity search for rice genome database using the cDNA sequence of guar and coffee bean was performed. The clone R2303 (D24643) showed high similarity with the plant αGal sequences so the complete sequencing of the clone was performed. The N-terminal sequences of the V8 protease digested fragments of rice αGal such as V-G-N-G-G-M-S-E-A-E-Y-R-S-H-F-S-I-

W-A-L and A-E-Y-R-S-H-F-S-I-W-A-L-A-K-A-P-L-L-I-G were found in the deduced amino acid sequence of R2303. RT-PCR, using a set of primers, derived from the N-terminal amino acid sequence of rice αGal and from the R2303 sequence, was successful, indicating that R2303 partially encodes the rice αGal. The first Met was found in 5′-RACE product. A consensus translation initiation signal (Lutcke et al., 1987) in the plant, GCCATGGC, was found at the first Met. The full-length cDNA of rice αGal (1410 bp) was then amplified and the complete cDNA sequence of rice αGal was determined.

The ORF of the αGal encoded 417 amino acid residues. The mature protein consisted of 362 amino acids and the calculated molecular weight was 39,950. The N-glycosylation motif "Asn-X-Thr/Ser" was not found in the sequence. This coincided with the fact that purified rice αGal migrated as a discrete single band in SDS-PAGE and showed no cross reactivity with Con A (data not shown).

2.4. Similarities between αGals and other enzymes in terms of their amino acid sequences

The deduced amino acid sequence of the rice αGal was compared with the available sequences in the protein and nucleic acid database using BLAST. The αGal with the highest similarity (*C. arabica* αGal) was 73% homologous to rice αGal over the entire amino acid sequence. There are some homologous regions from the N-terminal to the central part, but the C-terminal part shows low similarity among these enzymes (Fig. 5). A similarity search of the sequence indicated that rice αGal consists only of a catalytic domain belonging to the glycoside hydrolase family 27. There are seven Cys residues in the rice αGal and five Cys residues from the N-terminal region that are conserved among family 27 αGals. Some αGals are reported to be inhibited by SH reagents, such as PCMB. Rice αGal was also completely inactivated (less than 2% of the control) after the treatment with 1 mM PCMB at 30 °C for 30 min.

Some amino acid residues that are critical for enzymatic activity were identified by studies on mammals (humans) (Bernstein et al., 1987; Koide et al., 1990; Ishii et al., 1995; Okumiya et al., 1995) and coffee (Zhu and Goldstein, 1995; Maranville and Zhu, 2000) αGals.

An empirical identification of the aspartic acid residue as being the catalytic nucleophile in the αGal from *Phanerochaete chrysosporium* (belonging to the glycoside hydrolase family 27) has been reported. (Hart et al., 2000) This aspartic acid is located within the sequence YLKYDNC, which is highly conserved in all known family 27 glycosyl hydrolases.

Recently the tertiary structure of chicken α-*N*-acetylgalactosaminidase (αNAGAL) has been reported (Garman et al., 2002) and a model of αGal has been presented. Both of these enzymes belong to the glycoside hydrolase family 27 and two aspartic acid residues, D140 and D201, according to αNAGAL numbering, are identified as the catalytic nucleophile and proton donor, respectively. The mechanism for this class of enzymes is a double-displacement mechanism (McCarter and Withers, 1994) and each of the enzymes contains two carboxylic acids located on opposite sides of the active site.

2.5. Purification of recombinant αGal and its molecular mass

The rice αGal cDNA was expressed in *E. coli* BL21 with IPTG induction. After sonication, insoluble materials were removed by centrifugation and the supernatant was designated as the crude enzyme solution. Purification was carried out using a Hitrap Chelating HP column and ϵ-aminocaproyl-α-D-galactopyranosylamine CH-Sepharose column. Hitrap Chelating can specifically bind histidine-tagged protein and *N*-ϵ-aminocaproyl-α-D-galactopyranosylamine is a competitive inhibitor of αGals (Harpaz et al., 1974). A 176-fold purification was obtained with overall 60% recovery of the activity from the crude enzyme solution (Table 3). SDS-PAGE of the fraction corresponding to the peak of activity revealed a single protein band with a molecular weight of 42,000 (Fig. 2b), which agrees with the sum of the molecular weight of αGal (39,950), calculated from the nucleotide sequence, and His Tag sequence (1400).

2.6. Enzymatic properties of recombinant rice αGal

The enzymatic properties of the recombinant rice αGal was investigated and compared with the native enzyme produced by rice cells. The recombinant enzyme was stable up to 40 °C and between pH range 3 and 8, similar to the native enzyme. The enzymatic properties including pH, temperature dependence and substrate specificities in the galacto-oligosaccharides and galactomanno-oligosaccharide were exactly the same, indicating that the His tag, which is added to the N-terminal position of the protein, did not affect the enzymatic properties and the cDNA which has been cloned in this study encodes the rice αGal.

3. Experimental

3.1. Materials

Melibiose, raffinose, stachyose, *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal), other *p*-nitrophenyl glycosides, guar gum, locust bean gum and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

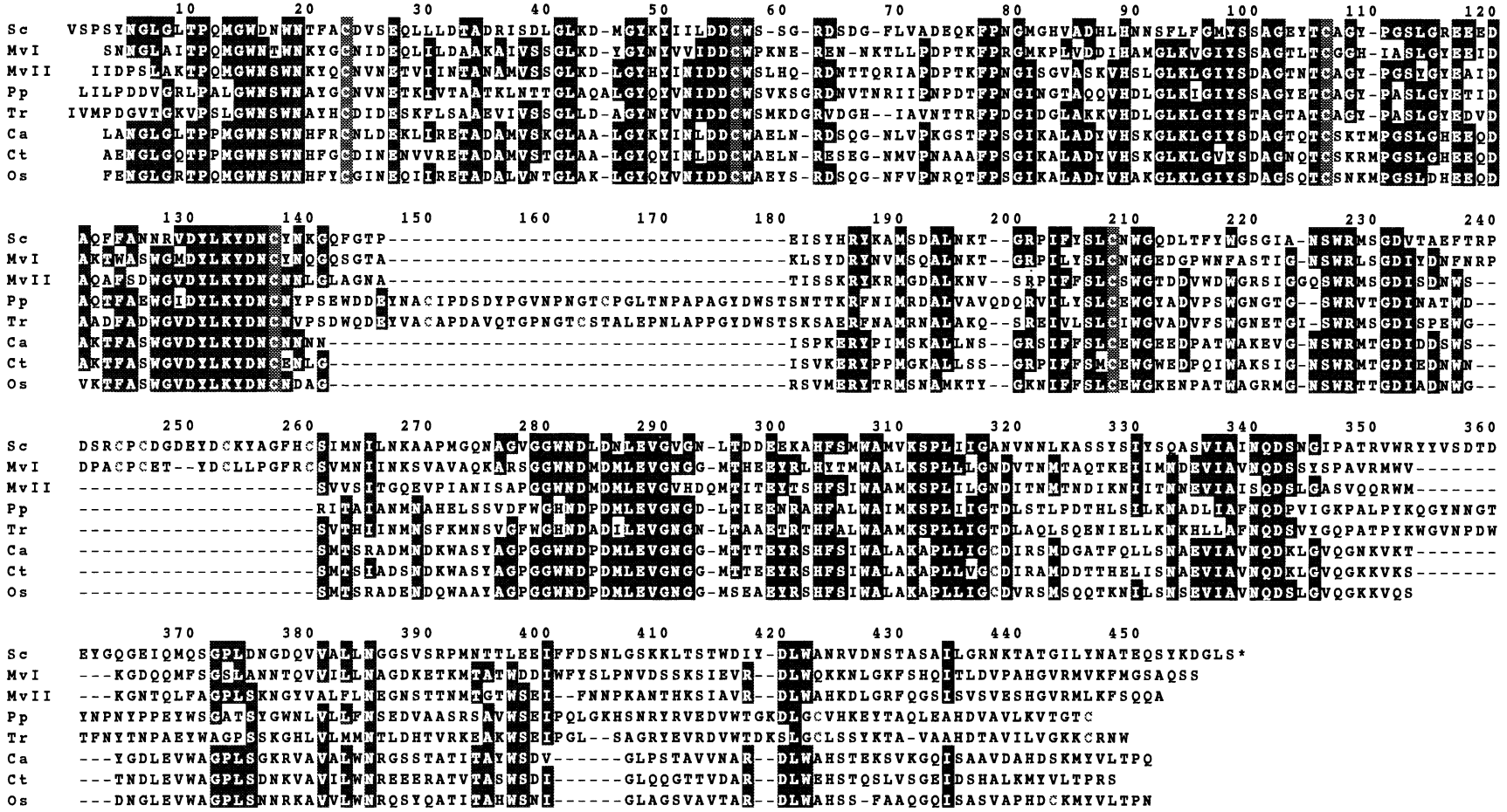


Fig. 5. Sequence homology of αGals from different sources. The sequences were aligned for optimal sequence similarity by MultAlin (www. toulouse.inra.fr/multalin.html). The numbers above sequence indicate the relative position of each amino acid sequence. Identical amino acid residues five out of eight or more at the same position, are in the black background. Sc, *Saccharomyces cerevisiae* αGal (M10604), MvI, *Mortierella vinacea* αGal I (S79440), MvII, *M. vinac*ea αGal II (AB019691), Pp, *Penicillium purpurogenum* αGal (AB008367), Tr, *Trichoderma reesei* αGal (Z69253), Ca, *Coffea arabica* αGal (L27992), Ct, *Cyamopsis tetragonoloba* αGal (X14619), Os, *Oryza sativa* αGal (AB039671).

Table 3
Summary of purification steps of recombinant αGal

	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
Crude enzyme	12,336	1280	9.64	100	1
Hitrap chelating HP	11,702	14.0	836	95	87
Affinity gel[a]	7418	4.37	1697	60	176

[a] N-ϵ-Aminocaproyl-α-D-galactopyranosylamine CH Sepharose.

3.2. Enzymatic assay and measurement of protein concentration

The αGal standard assays were performed with 1 mM *p*NP-α-Gal at 37 °C in 50 mM sodium acetate, pH 5.0. After 10 min, an equal volume of 0.2 M Na_2CO_3 was added to stop the reaction and the absorbance at 408 nm was measured. One nkat of α-galactosidase activity was defined as the amount of enzyme which release 1 nmol of *p*-nitrophenol per second under the conditions described above.

The protein concentrations of the enzyme preparations were measured with a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, USA) with bovine serum albumin as the standard.

3.3. Rice cell culture and preparation of the crude enzyme

The rice (*Oryza sativa* L. Nipponbare) cells were cultured in 500 ml shaking flasks containing 100 ml of medium (Kasamo, 1988) and were cultivated on a rotary shaker (125 r.p.m.) at 25 °C for 21 days. The culture broth was then filtered through No. 2 filter paper (Toyo Roshi Co., Ltd., Tokyo, Japan). Ammonium sulfate was added to 70% saturation level and the resulting mixture was kept at 4 °C for 16 h. The precipitate was collected by centrifugation (10,000×g, 20 min) and dissolved in a small amount of distilled water followed by dialysis against water containing 2 mM $CaCl_2$. After removal of the insoluble materials by centrifugation (12,000×g, 30 min), the obtained solution was used as the crude enzyme preparation.

3.4. Preparation of galactomanno-oligosaccharides

Galactomanno-oligosaccharide having an α-1,6-galactosyl side chain on the β-1,4-mannotetraose, Gal^3Man_4 was prepared from a hydrolysate of copra galactomannan using *Streptomyces* β-mananase (Kaneko et al., 1990). In addition, the galactomanno-oligosaccharide with a terminal galactose at the non-reducing end of β-1,4-mannotriose, Gal^3Man_3 was prepared from Gal^3Man_4 by excising the non-reducing end mannosyl residue of the saccharide with *A. niger* β-mannosidase (Kusakabe et al., 1990).

3.5. Bacterial strains, plasmids, and growth conditions

E. coli JM109, BL21 and pET28 were used for the cloning and gene expression. *E. coli* cells were cultured in Luria-Bertani (LB) broth at 30 °C with ampicillin (100 μg/ml) or kanamycin (100 μg/ml).

3.6. Enzyme purification

All purification procedures were performed at 4 °C.

- *Step 1*. The crude enzyme was dialyzed against 10 mM sodium acetate buffer, pH 4.5, containing 2 mM $CaCl_2$ and the dialyzed enzyme was loaded on a column of SP-Sepharose FF (2.5×30 cm, Pharmacia, Uppsala, Sweden) which was equilibrated with the same buffer. After washing with the same buffer, the enzyme was eluted from the column with a NaCl linear gradient from 0 to 0.5 M at a flow rate of 0.5 ml/min. The active fractions were collected and dialyzed against 0.1 M sodium acetate, pH 4.5.
- *Step 2*. The enzyme fraction was then applied to an affinity column (*N*-ϵ-aminocaproyl-α-D-galactopyranosylamine CH Sepharose, (Harpaz et al., 1974) (1.5×10 cm) which was equilibrated with 0.1 M sodium acetate, pH 4.5. The column was thoroughly washed with the same buffer, and the enzyme was eluted with 0.1 M sodium acetate, pH 4.5, containing 0.1 M galactose. After dialysis, the active fractions were collected and tested for activity, then concentrated by ultrafiltration (Centriprep 3, Amicon Inc.).
- *Step 3*. The concentrated enzyme was applied to a Hiprep Sephacryl S-200 column (1.6×60 cm) which was equilibrated with 0.1 M sodium acetate, pH 4.5, containing 10 mM $CaCl_2$. The eluate was collected in 1.0 ml fractions. The active fractions were collected and stored at -20 °C before use.

3.7. Electrophoretic analysis

SDS-PAGE was performed using a 10–20% gradient gel following the method as described by Laemmli (1970) and the protein bands were visualized by Coomassie brilliant blue R-250. The molecular weight of the enzyme was measured by SDS-PAGE using molecular weight markers (10 kDa Protein Ladder, Gibco-BRL). Isoelectric focusing was carried out with Phast System (Pharmacia) and PhastGel IEF 3–9. The protein was stained with CBB R-250 and an isoelectric focusing calibration kit (pH 3–10, Pharmacia) was used for the calibration.

3.8. Terminal amino acid sequencing of purified rice αGal

After the protein in the gel was blotted on a PVDF membrane, the membrane was stained with CBB R-250 to detect the protein. The protein band was cut out and placed on a protein sequencer (G1005A; Hewlett-Packard Co.) to determine the N-terminal amino acid sequence.

3.9. Enzymatic properties

The effects of pH on the activity and stability of αGal were investigated using a series of 0.1 M KCl–HCl buffers from pH 1.0 to 2.0, 0.1 M Glycine–HCl buffers from pH 2.5 to 3.5, McIlvaine buffers from pH 3.0 to 7.5 and 0.1 M Tris–HCl buffers from pH 7.0 to 8.5. The activities of the αGal were assayed using the conditions described for the standard method. For the determination of the pH stability of the αGal, the enzymes were preincubated in the absence of a substrate at 40 °C for 60 min and the residual activity was then assayed using the standard method. The effects of temperature on the activity of αGal were determined from 30 to 70 °C. For the temperature–stability measurement of the αGal, the enzymes were preincubated at various temperatures at pH 5.0 for 60 min and the residual activity was then determined by the standard method.

3.10. Substrate specificity

For hydrolysis of galactose-containing oligosaccharides, reaction mixtures individually contained αGal (20 ml, 3.3 nkat), 0.1 M sodium acetate buffer pH 5.0, (40 ml) and 1% (w/v) substrate (40 ml, of either melibiose, raffinose, stachyose, Gal^3Man_3 or Gal^3Man_4). After 0, 1, 3, 6, and 24 h incubation at 37 °C, the sugar samples obtained after the enzyme reactions were analyzed by HPTLC Silica gel 60 (Merck, Darmstadt, Germany) for characterization of the hydrolysis products. The reaction products were developed with 1-propanol-nitromethane-water (5:2:3, v/v), with sugars on the plate being detected by heating at 140 °C for 5 min after spraying with sulfuric acid.

For galactomannan hydrolysis, a solution was prepared containing αGal solution (0.16 ml, 26.7 nkat), 0.1 M sodium acetate buffer pH 5.0 (0.24 ml), and 1% substrate (0.4 ml, locust bean gum or guar gum). After 0, 1, 2, 3, 6, 9, 12, 18 and 24 h incubation at 37 °C, the reaction was stopped by boiling for 5 min. The amount of released D-galactose was determined by the method of Somogyi (1952) using galactose as standard and the released galactose was confirmed by TLC.

3.11. Molecular cloning and sequencing procedures

Recombinant DNA techniques were performed by conventional protocols. The N-terminal amino acid sequence of rice αGal was determined as F-E-N-G-L-G-R-T-P-Q-M-G-W-N-S-W-N-H-F-Y-X-G-I-N-E-Q-I-I-R-E-T-A-D-A-L-V-N-T-G-L. This sequence showed high similarity with the N-terminal amino acid sequence of the plant αGals. Using the full-length cDNA sequences of the αGals from coffee bean (GenBank accession number L27992) and guar (S14619), the cDNA clone, R2303, from the rice genome project (D24643) with high similarity toward these cDNAs was found by a database search using BLAST. After obtaining the cDNA clone R2303 from the rice genome project, the Institute of Agrobiological Resources, the complete sequence of R2303 was determined and it was found that the clone contained a C-terminal region of the rice αGal sequence and termination codon. The purified αGal from rice was digested with V8 protease, and the N-terminal amino acid sequences of the digested fragments were determined to be V-G-N-G-G-M-S-E-A-E-Y-R-S-H-F-S-I-W-A-L and A-E-Y-R-S-H-F-S-I-W-A-L-A-K-A-P-L-L-I-G. These sequences were found in the amino acid sequence deduced from the nucleotide sequence of R2303.

Total RNA was prepared from rice cells and poly(A)$^+$ RNA was purified with an oligo(dT)-cellulose column as previously described (Sakakibara et al., 1996). The reverse transcription method and a LA PCR Kit Ver. 2 (Takara, Tokyo, Japan) was used to amplify the partial fragment of αGal cDNA. RT-PCR was performed using the set of primers which are derived from the N-terminal amino acid sequence (sense primer, 5′-GGG GTG GAA TAG CTG GAA TC-3′) and from R2303 (antisense primer, 5′-TTC TTC GTC TGC TGG CTC AT-3′). The partial fragment of the galactosidase gene of rice was also amplified with a Perkin-Elmer Thermal Cycler (GeneAmp PCR System 2400). Each of the 30 amplification cycles consisted of a denaturation step at 94 °C for 1 min followed by annealing at 56 °C for 1 min and primer extension steps at 72 °C for 1 min. A 740 bp fragment was amplified, which was ligated into the pCR2.1 vector, and the sequence was then determined. We designed primers for 5′-RACE of αGal according to the sequence of this fragment. For 5′-RACE PCR, the Marathon ™ cDNA Amplification Kit (Clontech, Palo Alto, USA) was employed and cDNA for the PCR was synthesized by reverse transcription using the αGal specific primer (5′-CTA GTT TGG TGT CAA CAC ATA CAT CTT GCA GTC ATG-3′). Using a set of primers (sense primer, 5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′ and antisense primer, 5′-TGA TGG TTG CCT GGT ATG ACT GCC TGT T-3′), the first PCR was performed. The PCR product was diluted 20-fold and then subsequently the second PCR was performed using another set of primers (sense primer, 5′-ACT CAC TAT AGG GCT CGA GCG GC-3′ and antisense primer, 5′-GAC ATC CCA CCA TTT CCC ACT TCA A-3′). Each of

the 30 amplification cycles consisted of a denaturation step at 98 °C for 1 min followed by annealing at 56 °C for 1 min and primer extension steps at 72 °C for 1 min. The sequence of the amplified DNA fragment was determined by the dideoxy chain termination method (Sanger et al., 1977). The full-length rice αGal cDNA was amplified using the primers (sense primer, 5′-AAC GCG GAC GAA CTC AGA GCA GAG C-3′ and antisense primer, 5′-GCA TCC CAT TTC CAG CTC TAC CAA T-3′), and the amplified fragment was ligated into the pCR2.1 vector (PCRII/rice) and the complete cDNA sequence of the rice αGal was determined.

3.12. Construction of expression system and purification of recombinant enzyme

PCR amplification of the gene was performed with 2.5 U of the Taq DNA polymerase, 10 ng of plasmid PCRII/rice, a 0.2 mM concentration of each synthetic primer, a 200 mM concentration of each deoxynucleoside triphosphate, and 2 mM $MgCl_2$ in the buffer recommended by the manufacturer. Amplification was achieved with 30 cycles of 1 min of denaturation at 98 °C, 1 min of annealing at 55 °C, and 1 min of polymerase extension at 72 °C, plus an additional extension at 72 °C for 10 min. The synthetic oligonucleotide primers used for the PCR amplification were P1 (5′-CCA TGG TTY GAR AAY GGN CTN GGN CGN can CCN CAR ATG-3′) and P2 (5′-AAG CTT CTA GTT TGG TGT CAA CAC ATA CAT CTT GCA GTC ATG-3′) (The *Nco*I and *Hin*dIII restriction sites are underlined). The obtained PCR product cloned in PCRII was digested with *Nco*I and *Hin*dIII and ligated with pET-28(b) between the *Nco*I and *Hin*dIII sites. The plasmid was transferred into *E. coli* BL21.

E. coli having the rice αGal gene was cultured at 20 °C and the gene was expressed by addition of IPTG (1 mM). The *E. coli* cells were harvested and sonicated in 0.15 M NaCl/0.02 M phosphate, pH 7.2. After centrifugation, the supernatant was used as the crude enzyme solution.

The crude enzyme was applied to a Hitrap Chelating HP column (Amersham Bioscience, Little Chalfont, UK), which was equilibrated with 0.02 M phosphate, pH 7.2, containing 10 mM imidazole. After washing with the buffer, the αGal fraction was eluted with a linear gradient from 10 mM to 200 mM imidazole in 0.02 M phosphate buffer, pH 7.2. The active fraction was collected and then purified by affinity chromatography using N-ϵ-aminocaproyl-α-D-galactopyranosylamine as a ligand. The fractions rich in αGal activity were dialyzed against 2 mM $CaCl_2$ and used for further characterization.

3.13. Nucleotide sequence accession number.

The rice αGal cDNA sequence is available in the DDBJ, EMBL, and GenBank databases under accession no. AB039671.

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