Running title: Characterization of Rice β-Glucosidase

Identification of Rice β-Glucosidase with High Hydrolytic Activity towards Salicylic Acid β-D-Glucoside

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Abbreviations: JA, jasmonic acid; TA, tuberonic acid; TAG, tuberonic acid β-D-glucoside; rOsTAGG2E, recombinant OsTAGG2 produced in *Escherichia coli*; SAG, salicylic acid β-D-glucoside; pNP, *p*-nitrophenyl; oNPG, *o*-nitrophenyl β-D-glucoside; pNPG, pNP β-glucoside; *m*-SAG, *m*-β-D-glucopyranosyloxybenzoic acid; *p*-SAG, *p*-β-D-glucopyranosyloxybenzoic acid; EDTA, ethylenediaminetetraacetic acid; rOsTAGG2P, recombinant OsTAGG2 produced in *Pichia pastoris*; PVDF, polyvinylidene difluoride; SA, salicylic acid

β-Glucosidases (EC 3.2.1.21) split β-glucosidic linkages at the non-reducing end of glucosides and oligosaccharides to release β-D-glucose. One of the important functions of plant β-glucosidase is deglucosylation of inactive glucosides of phytohormones to regulate levels of active hormones. Tuberonic acid is a jasmonate-related compound that shows tuber-inducing activity in the potato. We have identified two enzymes, OsTAGG1 and OsTAGG2, that have hydrolytic activity towards tuberonic acid β-D-glucoside in rice (*Oryza sativa* L.). The expression of *OsTAGG2* is upregulated by wounding and by methyl jasmonate, suggesting that this isozyme is involved in responses to biotic stresses and wounding, but the physiological substrate of OsTAGG2 remains ambiguous. In this study, we produced recombinant OsTAGG2 in *Pichia pastoris* (rOsTAGG2P), and investigated its substrate specificity in detail. From 1 L of culture medium, 2.1 mg of purified recombinant enzyme was obtained by ammonium sulfate precipitation and Ni-chelating column chromatography. The specific activity of rOsTAGG2P (182 U/mg) was close to that of the native enzyme (171 U/mg), unlike recombinant OsTAGG2 produced in *Escherichia coli*, which had approximately 3-fold lower specific activity than the native enzyme. The optimum pH and temperature for rOsTAGG2P were pH 3.4 and 60°C. After pH and heat treatments, the enzyme retained its original activity in a pH range of 3.4-9.8 and below 55°C. Native OsTAGG2 and rOsTAGG2P showed 4.5-4.7-fold higher activities towards salicylic acid β-D-glucoside, an inactive storage-form of salicylic acid, than towards tuberonic acid β-D-glucoside (TAG), although OsTAGG2 was originally isolated from rice based on TAG-hydrolytic activity.

Key words: β-glucosidase; rice; tuberonic acid; salicylic acid; substrate specificity

β-Glucosidases (EC 3.2.1.21) split β-glucosidic linkages at the non-reducing end of glucosides and oligosaccharides with net retention of anomeric configuration. They are ubiquitous enzymes, found in all domains of living organisms, Eukaryota, Eubacteria, and Archaea, and they have a variety of functions, including degradation of biomass, hydrolysis of glycolipids, catabolism of cell-wall oligosaccharides, and activation of phytohormones.[1)] They were categorized into glycoside hydrolase (GH) families 1, 3, 5, 9, 30, and 116 on the basis of a sequence-based classification of glycoside hydrolases.[2)] Of these families, GH family 1 has the greatest variety of activities, including β-galactosidase (EC 3.2.1.23), β-mannosidase (EC 3.2.1.25), β-glucuronidase (EC 3.2.1.31), β-fucosidase (EC 3.2.1.38), in addition to β-glucosidase. Plant β-glucosidases, belonging to this family, are thought to be responsible for regulation of the physiological activity of phytohormones, which are stored in inactive glucosylated forms. It is believed that a specific enzyme hydrolyzes a phytohormone glucoside to release an active phytohormone, although only a few enzymes have been demonstrated to play this physiological role.[3-6)] Maize β-glucosidase (Zm-p60.1) and *Arabidopsis* enzymes (BG1 and BG2) have been found to have central roles in the hydrolysis of cytokinin β-glucoside and absicisic acid 1-*O*-β-D-glucosyl ester, respectively, *in vivo*.[4-6)]

We have identified two rice (*Oryza sativa* L.) β-glucosidases (OsTAGG1 and OsTAGG2),[7,8)] hydrolyzing tuberonic acid β-glucoside, a glucosylated form of tuberonic acid, a derivative of jasmonic acid regulating stress responses as to insect herbivory and pathogen attack,[9,10)] and plant growth.[11-13)] OsTAGG1 and OsTAGG2 are encoded by Os4BGlu13 (Rice Genome Project locus *Os04g0474900*) and Os4BGlu12 (*Os04g0474800*), respectively, and their sequences fall into a phylogenetic cluster of defense-related β-glucosidases, including white clover and cassava linamarinases.[14)] OsTAGG2 was found to be identical to cell-wall bound β-glucosidase purified from germinating rice seeds.[15)] Expression of *OsTAGG2* is induced by wounding, methyl jasmonate, and ethephon in 10-d-old rice seedlings.[8,16)] Subtractive hybridization cDNA

library screening also revealed that the transcript levels of *OsTAGG2* increased in response to brown hopper feeding.[17] These findings suggest that OsTAGG2 is involved in responses to wounding and biotic stress.

Since OsTAGG2 is phylogenetically close to defense β-glucosidases, it might be involved in the hydrolysis of glucosides of bioactive compounds other than TAG. In this study, we produced recombinant OsTAGG2 in the methylotrophic yeast *Pichia pastoris*, and investigated its aglycone specificity for various β-D-glucosides, including salicylic acid β-D-glucoside (SAG), an inactive form of salicylic acid activating disease resistance,[18] and its derivatives.

Materials and Methods

Materials. The structures of the β-D-glucosides analyzed in this study, other than *p*-nitrophenyl (pNP) β-D-glycosides, *o*-nitrophenyl β-D-glucoside (oNPG), and oligosaccharides, are shown in Fig. 1. From Sigma (St. Louis, MO), pNP β-D-glucoside (pNPG), oNPG, pNP β-D-fucoside, pNP β-D-galactoside, pNP β-D-xyloside, and pNP β-D-mannoside were purchased. Cellobiose and gentiobiose were from Nacalai Tesque (Kyoto, Japan). Cellotriose, cellotetraose, sophorose, and laminaribiose were from Seikagaku (Tokyo). Helicin was from Tokyo Chemical Industries (Tokyo). TAG, SAG, *m*-β-D-glucopyranosyloxybenzoic acid (*m*-SAG), and *p*-β-D-glucopyranosyloxybenzoic acid (*p*-SAG) were synthesized as reported previously.[19,20] Native OsTAGG2 and rOsTAGG2E were prepared as reported previously.[8,21]

Fig. 1

Construction of an expression plasmid for OsTAGG2. Total RNA was prepared from leaf sheath of the rice plant (*O. sativa* L. cv. Kitaake) with an RNAqueous kit (Applied Biosystems, Foster City, CA), and cDNA was synthesized with *Bca*BEST RNA PCR kit Ver. 1.1 (Takara Bio, Otsu, Japan). *OsTAGG2* cDNA was amplified by PCR with this cDNA as template, primers 5'-ATGGCGGCAGCAGGGGCAATG-3' (sense orientation) and 5'-TTTAACTGGATTACTTCCATCTCTTGTACC-3' (antisense

orientation), and Primestar HS DNA polymerase (Takara Bio). The PCR product was cloned into the *Eco*RV site of pBluescript II SK (+)(Stratagene, La Lolla, CA) and sequenced with an ABI Prism 310 Genetic Analyzer DNA sequencer (Applied Biosystems) and a Big Dye Terminator 1.1 Sequencing Kit (Applied Biosystems). The resulting plasmid was used as template in PCR to construct an expression plasmid of OsTAGG2. The PCR product, amplified with primers 5'-CGGCGGTACCATGGGCGCCGGC (sense, *Kpn*I site underlined) and 5'-TTTCCGCGGTCAGGAGGAACTTC (antisense, *Sac*II site underlined), and pPICZαA (Invitrogen, Carlsbad, CA) were digested with *Kpn*I (Takara Bio) and *Sac*II (Takara Bio), and ligated with DNA Ligation Kit Mighty Mix (Takara Bio). The DNA sequence of *OsTAGG2* in the expression plasmid was sequenced as described above.

Optimization of production of recombinant OsTAGG2 in P. pastoris. The expression plasmid of OsTAGG2, linearized by *Sac*I digestion, was introduced into *P. pastoris* strain X-33 by electroporation in a Gene Pulser (Bio-Rad, Richmond, CA) following the manufacturer's instructions. Nine colonies grown on a YPDSZ plate (10 mg/mL of yeast extract, 20 mg/mL of peptone, 20 mg/mL of D-glucose, 1 M sorbitol, 20 mg/mL of agar, and 100 μg/mL of zeocin) were selected and incubated in 10 mL of BMGY medium (10 mg/mL of yeast extract, 20 mg/mL of peptone, 13.4 mg/mL of yeast nitrogen base, 4 μg/mL of D-biotin, 10 mg/mL of glycerol, and 0.1 M potassium phosphate buffer, pH 6.0) at 30°C with vigorous shaking until A_{600} reached 2.0. Cells were collected by centrifugation and suspended in 10 mL of BMMY medium (10 mg/mL of glycerol in BMGY was changed to 0.5% v/v methanol). The cell suspension was further incubated at 30°C for 96 h. Protein production was maintained by the addition of methanol at a final concentration of 0.5% v/v every 24 h. The enzyme activity of the culture supernatant of each transformant was measured as described below. The transformant with the highest productivity of recombinant OsTAGG2 was selected for further analysis.

This transformant was incubated in 300 mL of BMGY medium as described above. The cells collected by centrifugation were suspended in 300 mL of BMMY medium, and the cell suspension was dispensed with 25 mL to each 100-mL Erlenmeyer flask. Culture induction was carried out at 20°C or 30°C in the presence of 20 mg/mL of casamino acids and/or 5 mM ethylenediaminetetraacetic acid (EDTA). The activity of recombinant OsTAGG2 (rOsTAGG2P) in the culture supernatant was measured, and the recombinant protein was detected by SDS-PAGE, in which the protein was visualized with Sil-Best-Stain One (Nacalai Tesque). The His_6-tag at the C-terminal of the recombinant protein was confirmed by Western blotting.

Purification of rOsTAGG2P. The transformant of *P. pastoris* harboring the expression cassette for *OsTAGG2* was cultured in 1 L of BMGY medium (500 mL per 2-L Erlenmeyer flask), as described above. The medium was exchanged to 1 L of BMMY medium supplemented with 5 mM EDTA, and induction culture was done at 20°C for 96 h. The culture supernatant was recovered by centrifugation, and solid ammonium sulfate was added up to 80% saturation. After incubation at 4°C overnight, the protein precipitated was collected by centrifugation and dissolved in 100 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl. The resulting sample was loaded onto a Ni-chelating Sepharose column (ϕ1.5 x 9 cm, GE Healthcare, Uppsala, Sweden). After thorough washing of the column with 50 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl, the adsorbed protein was eluted with 50 mM sodium acetate buffer (pH 5.0) containing 300 mM NaCl. Fractions of the adsorbed protein with high purity were collected and dialyzed against 50 mM sodium acetate buffer (pH 5.0). The enzyme solution was concentrated by ultrafiltration using Vivaspin 20 filters (nominal molecular weight limit 30,000; Sartorius, Göttingen, Germany) up to approximately 0.4 mg/mL, and stored at 4°C. The protein concentration of the enzyme purified was determined based on the concentration of each amino acid liberated by complete hydrolysis of the protein with 6 *N* HCl. Amino acids were measured by the ninhydrin

colorimetric method[22] with an JLC-500/V automatic amino acid analyzer (Jeol, Tokyo).

Western blotting. One μg of protein was separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA) by electroblotting in a semi-dry blotting apparatus.[23] The membrane was incubated with anti His-Tag antibody (0.4 μg, Novagen, Darmstadt, Germany) in 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mg/mL skimmed milk, and 1 mg/mL Tween 20 at room temperature for 1 h. Proteins binding the antibody were detected by incubating the membrane for 1 h with alkaline phosphatase anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA) and BCIP/NBT phosphatase substrate (1-component) (KPL, Gaithersburg, MD).

N-Terminal sequence analysis. Ten μg of protein was separated by SDS-PAGE and transferred to a PVDF membrane as described above. The bands of recombinant TAGG2, detected by staining with Coomassie Brilliant Blue R-250, were cut off from the membrane and subjected to N-terminal sequence analysis with a Procise 492 protein sequencer (Perkin Elmer, Waltham, MA).

Enzyme assay. For the standard assay, pNPG hydrolyzing activity was measured. A reaction mixture (100 μL) consisting of an appropriate concentration of the enzyme, 20 mM sodium acetate buffer (pH 5.0), and 2 mM pNPG was incubated at 37°C for 10 min. The reaction was terminated by the addition of 200 μL of 1 M sodium carbonate, and A_{405} was measured to determine the *p*-nitrophenol released. One U of β-glucosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of pNPG per min.

The substrate specificity of rOsTAGG2P was investigated by the measuring velocity of hydrolysis of the following substrates (2 mM): pNPG, oNPG, pNP β-D-fucoside, pNP β-D-galactoside, pNP β-D-xyloside, pNP β-D-mannoside, TAG, helicin, SAG, m-SAG, p-SAG, cellobiose, cellotriose, cellotetraose, sophorose, laminaribiose, and gentiobiose.

Reaction velocities for the hydrolysis of pNP β-D-glycosides and oNPG were measured as for pNPG. Liberated *o*-nitrophenol was measured based on A_{405}. In the reactions to β-glucosides other than pNPG and oNPG, the enzyme reactions were stopped by adding 200 μL of 2 M Tris-HCl buffer (pH 7.0), and D-glucose liberated was measured by the glucose oxidase-peroxidase method[24] by Glucose CII Test (Wako Pure Chemical Industries). The kinetic parameters of various substrates were determined by fitting the initial velocities at various substrate concentrations to the Michaelis-Menten equation with the Grafit version 7.0.2 computer program (Erithacus Software, West Sussex, UK).

Effects of pH and temperature on the activity and stability of rOsTAGG2P. Optimum pH and temperature were determined by measuring enzyme activities at given pH values and temperatures. The reaction pH was adjusted with 40 mM Britton-Robinson buffer (pH 2.2-11.0), composed of a mixture of 40 mM acetate, phosphate, and glycine, the pH of which was adjusted with 5 *N* NaOH. pH stability was determined on the basis of residual activity after incubation of 7.8 μg/ml of enzyme solution in 90 mM Britton-Robinson buffer at various pH values at 4°C for 24 h. Temperature stability was determined by measuring residual activity after incubation of the enzyme in 20 mM sodium acetate buffer (pH 5.0) at given temperatures for 20 min. The ranges of pH and temperature at which the enzyme retained more than 90% of its original activity were considered stable.

Results and Discussion

Optimization of the production of rOsTAGG2P

The culture medium of the transformant of *P. pastoris* carrying the expression cassette for *OsTAGG2* showed 0.5 U/mL after induction at 30°C for 96 h, but the enzyme produced barely adsorbed onto a Ni-chelating Sepharose column (data not shown). His_6-tag antibodies detected no band in the non-adsorbed fraction, suggesting

that the His_6-tag has been removed or destroyed during culture. Hence we tried to reduce the deficiency of the tag by changing the induction conditions (Fig. 2). Regardless of additive substances, more rOsTAGG2P was produced at 20°C than at 30°C. Western blotting revealed that the His_6-tag was protected only in the culture with EDTA at 20°C, although the level of production of rOsTAGG2P in the presence of EDTA was similar to that without EDTA. The addition of EDTA is thought to prevent the His-tag of the recombinant protein from cleavage. Loss of the His_6-tag occurred at 30°C even in the presence of EDTA. The addition of casamino acid decreased the production of rOsTAGG2P.

Fig. 2

Production and purification of recombinant OsTAGG2

On a 1-litter-scale of the production of rOsTAGG2P, 774 U of the enzyme was obtained. The protein in the culture supernatant was collected by precipitation with 80% saturation ammonium sulfate, and Ni-chelating Sepharose column chromatography was carried out. No enzyme activity was detected in the non-adsorbed fraction. The enzyme was recovered from the adsorbed fraction at high yield (37% of the starting material), and 2.1 mg of rOsTAGG2P (182 U/mg) was obtained. The specific activity of rOsTAGG2P was close to that of the native enzyme purified from rice (171 U/mg). On the other hand, rOsTAGG2E had approximately 3-fold lower specific activity (60.7 U/mg) than the native enzyme and rOsTAGG2P. This difference in activity between rOsTAGG2E and the others was presumably due to structural differences, as discussed below.

On SDS-PAGE, rOsTAGG2P showed two bands of similar molecular masses (Fig. 3). Both of these proteins reacted with His_6-tag specific antibody. The N-terminal sequences of the proteins of high and low molecular masses were XFTWPSR and GXXEPPVSXR, respectively. These sequences correspond to the N-terminal sequences of the mature proteins when the precursor is cleaved at the Ste13 protease cleavage site of the α-factor from the plasmid and at the signal sequence cleavage site of OsTAGG2,[8)]

respectively. These two derivatives could not be separated by other column chromatographic procedures such as cation exchange and gel filtration column chromatography (data not shown). Production of rOsTAGG2P with an expression plasmid in which *OsTAGG2* was inserted into the *Eco*RI and *Sac*II sites of the vector to delete the N-terminal extra sequence, was unsuccessful, because, unaccountably, the recombinant enzyme was digested during production. The N-terminal of OsTAGG2 is apart from the catalytic site,[25] and small difference in N-terminal sequence is not thought to cause a large functional difference, thus this preparation was used in further analysis. rOsTAGG2P is single peptide as rOsTAGG2E, as judged by the molecular mass measured by SDS-PAGE. In contrast, the native enzyme purified from rice plants is composed by two peptides, 40 and 26 kDa, encoded by a single gene, the N-terminals of which are Gly28 and Gly359, respectively.[8] This cleavage might occur through proteolysis catalyzed by a selective protease, because 11 amino acid residues around the cleavage sites of OsTAGG1[7] and OsTAGG2[8] (LPPSNGLNNSY; peptide bond between N and G is cleaved) are completely conserved.

Fig. 3

Effects of pH and temperature on the activity and stability of rOsTAGG2P

The optimum pH and temperature for rOsTAGG2P were pH 3.4 and 60°C. The optimum pH of rOsTAGG2P was lower than those of the native enzyme (pH 4.5) and rOsTAGG2E (pH 5.0).[8,16] After the pH and heat treatments, rOsTAGG2P retained its original activity in a pH range of 3.4-9.8 and below 55°C. The stable ranges for pH and temperature of rOsTAGG2P were similar to those for the native enzyme.[8]

Substrate specificities

The substrate specificities of rOsTAGG2P, rOsTAGG2E, and the native enzyme were investigated with pNP β-D-glycosides, oligosaccharides, and various β-D-glucosides, including TAG and SAG at a concentration of 2 mM, and compared (Table 1). Previously we measured reaction velocities towards various substrates at a low

concentration (50 μM) with a UPLC-MS/MS system,[8] but in this study we measured them at a higher substrate concentration by the spectrophotometric method to determine accurate reaction velocities. The substrate specificities of the native enzyme investigated by the two methods were different from each other. The reaction velocity at low concentration (close to the endogenous concentration) appears to be important physiologically, but it is too difficult to quantify exactly very low amounts of reaction product released from low concentrations of substrates. Hence the data produced in this study are presumably more reliable than previous results.

All the enzymes showed similar substrate specificities. They showed high activities towards pNPG and pNP β-D-fucoside, and low activities towards pNP β-D-mannoside and pNP β-D-xyloside. The preferences of all the OsTAGG2 derivatives for oligosaccharides were also similar to each other, although the hydrolytic velocities towards oligosaccharides of rOsTAGG2P relative to that towards pNPG were lower than the native enzyme and rOsTAGG2E. Laminaribiose was the best substrate for all the enzymes among the β-glucobioses, followed by sophorose. Gentiobiose and cellobiose were poor substrates for all the OsTAGG2 derivatives. Cellooligosaccharides longer than cellobiose were much more rapidly hydrolyzed than cellobiose. Thus +2 subsite appears to contribute to the binding of oligosaccharides linked by the β-1,4-glucosidic linkage. Table 1

OsTAGG2 derivatives showed high hydrolytic activities towards β-glucosides with various aglycon structures. The aglycon binding site of OsTAGG2 is formed mainly by hydrophobic residues, including Trp181, Phe193, and Trp365,[25] and this enzyme is thought to recognize the aglycon parts of substrates loosely. Interestingly, rOsTAGG2P and the native enzyme showed 4.5- and 4.7-fold higher activity towards SAG, respectively, and rOsTAGG2P had a 2.8-fold higher k_{cat}/K_m value for SAG than for TAG (Table 2), although OsTAGG2 was originally isolated from rice based on TAG-hydrolytic activity. Among the SAG derivatives, rOsTAGG2P showed the highest k_{cat}/K_m for *m*-SAG. SAG is thought to be an inactive storage form of salicylic acid (SA), Table 2

which regulates various aspects of growth and development and serves as a critical signal activating disease resistance, the induction of pathogenesis-related proteins, and systemic acquired resistance in various plant species.[26] SAG is synthesized in the cytosol by glucosylation of SA by UDP-glucosyltransferases, which have been identified in *Arabidopsis*[27-29] and rice,[30] and is transported into the vacuoles. Hydrolysis of SAG in the tobacco apoplast has been observed, and this reaction might be catalyzed by cell-wall-associated β-glucosidase.[31] OsTAGG2 is identical to a cell wall-bound β-glucosidase from germinated rice.[15] This suggests that OsTAGG2 is responsible for the hydrolysis of SAG in the apoplasts. Vacuolar and plasma membranes have been observed to fuse, releasing signal compounds that accumulate in the vacuoles to the apoplasts when pathogenic bacteria proliferate.[32] This process presumably contributes to the transportation of SAG from the vacuoles to the apoplasts. In plants, salicylic acid is not generally induced by mechanical wounding, but in the case of rice, an increase in SA is observed at the initial stage of wounding stress (30 min after wounding), along with a decrease in endogenous SAG.[33] An increase in the expression of *OsTAGG2* was observed 1 h after wounding, but OsTAGG2 was obtained from non-wounded rice plants, and it could contribute to the hydrolysis of SAG.[8] Mechanical wounding also induces TA and TAG in rice, as reported elsewhere.[8] In this stress response, OsTAGG2 might also hydrolyze TAG to produce TA, but the physiological functions of TA in wounding stress are controversial, and further analysis is needed, because repression of wound-inducible genes by TA has been demonstrated at much higher concentrations than the physiological.[34]

As described above, all OsTAGG2 derivatives showed higher hydrolytic activity towards SAG and its derivatives than towards TAG, and hence we have concluded that OsTAGG2 has high preference for SAG over TAG. But in the reactions to certain substrates, including oNP β-D-glucoside, pNP β-D-galactoside, and cellooligosaccharide, OsTAGG2 derivatives showed preferences significantly different from each other. This might have been due to structural differences in the OsTAGG2 derivatives. Native

OsTAGG2 is separated to two peptides, of 40 and 26 kDa, as described above.[8] The cleavage site is far from the active site,[25] but this cleavage might affect substrate specificity through slight changes in the overall structure. Another possibility as to changes in the structure of enzyme is the presence of *N*-glycans attached to the *N*-glycosylation sites of rOsTAGG2P and the native enzyme. Their molecular masses are higher than the theoretical mass, and they have 5 putative *N*-glycosylation sites. Removal of the *N*-glycans of rOsTAGG2P by endoglycosidase H did not reduce the enzyme activity at all (data not shown), and it appears that the *N*-glycans hardly contribute to the enzyme functions. *N*-Acetylglucosaminyl residues remained at the *N*-glycosylation sites after treatment, and they might have caused structural differences, which respect to the *N*-glycan-free OsTAGG2 like rOsTAGG2E. In the preparation of rOsTAGG2E, a thioredoxin tag fused at the N-terminal was eliminated, but an extra peptide comprised of 22 amino acid residues was attached before the mature enzyme.[21] The N-terminal of OsTAGG2 is far from the active site, as pointed out above, and this extension probably does not affect substrate specificity.

Conclusions

In this study, rice OsTAGG2 was successfully produced in *P. pastoris* and the recombinant enzyme was characterized in detail. Originally, this enzyme was purified from rice based on TAG-hydrolyzing activity, but it had significantly higher activity towards SAG than towards TAG. To our knowledge, this is the first report of an enzyme with high activity towards SAG, although the physiological significance of OsTAGG2 in the hydrolysis of SAG should be analyzed.

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Figure legends

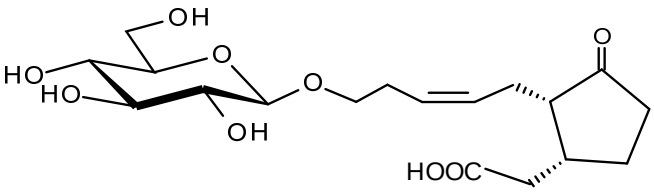
Fig. 1. Chemical Structures of the Natural Glucoside Substrates Used in This Study.

Fig. 2. Time Course of the Production of Recombinant OsTAGG2 in *P. pastoris*.

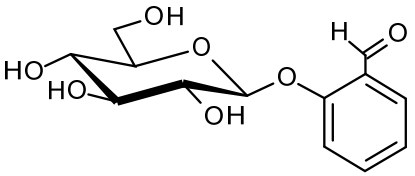
A, Enzyme activities of the culture supernatants of *P. pastoris* cultivated under various conditions. Filled and open symbols show results at 30°C and 20°C, respectively. Circles, triangles, squares, and diamonds indicate the culture in the presence of no additive, casamino acid, EDTA, and both casamino acid and EDTA, respectively. B, SDS-PAGE analysis of the culture supernatant. Induction culture was carried out for 96 h without additive (lanes 1 and 5), and in the presence of casamino acid (lanes 2 and 6), EDTA (lanes 3 and 7), and both casamino acid and EDTA (lanes 4 and 8). Lanes 1-4 and lanes 5-8 indicate the results of culture at 20°C and 30°C, respectively. Molecular masses of the standard proteins are shown on the left. C, Western blot analysis. Lane numbers correspond to those shown in panel B.

Fig. 3. SDS-PAGE and Western Blot Analyses of rOsTAGG2P.

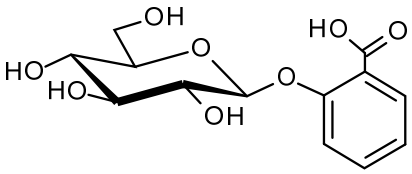
A, SDS-PAGE: N-terminal sequences of two proteins (1 and 2) of slightly different mobility are indicated on the right. Molecular masses of the standard proteins are shown on the left. B, Western blot: Triangles indicate positions corresponding to proteins 1 and 2 shown in the panel A. C, Cleavage sites of the signal peptide of rOsTAGG2P, indicating the starting positions of proteins 1 and 2 within the pre-protein sequence.



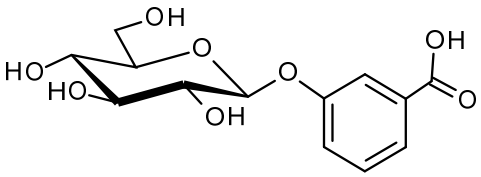
TAG



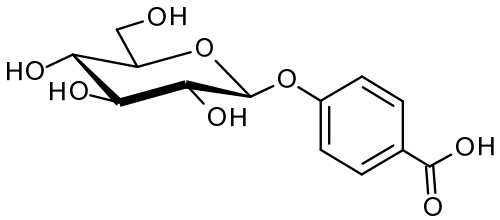
Helicin



SAG



m-SAG



p-SAG

Fig. 1, Himeno, et al.

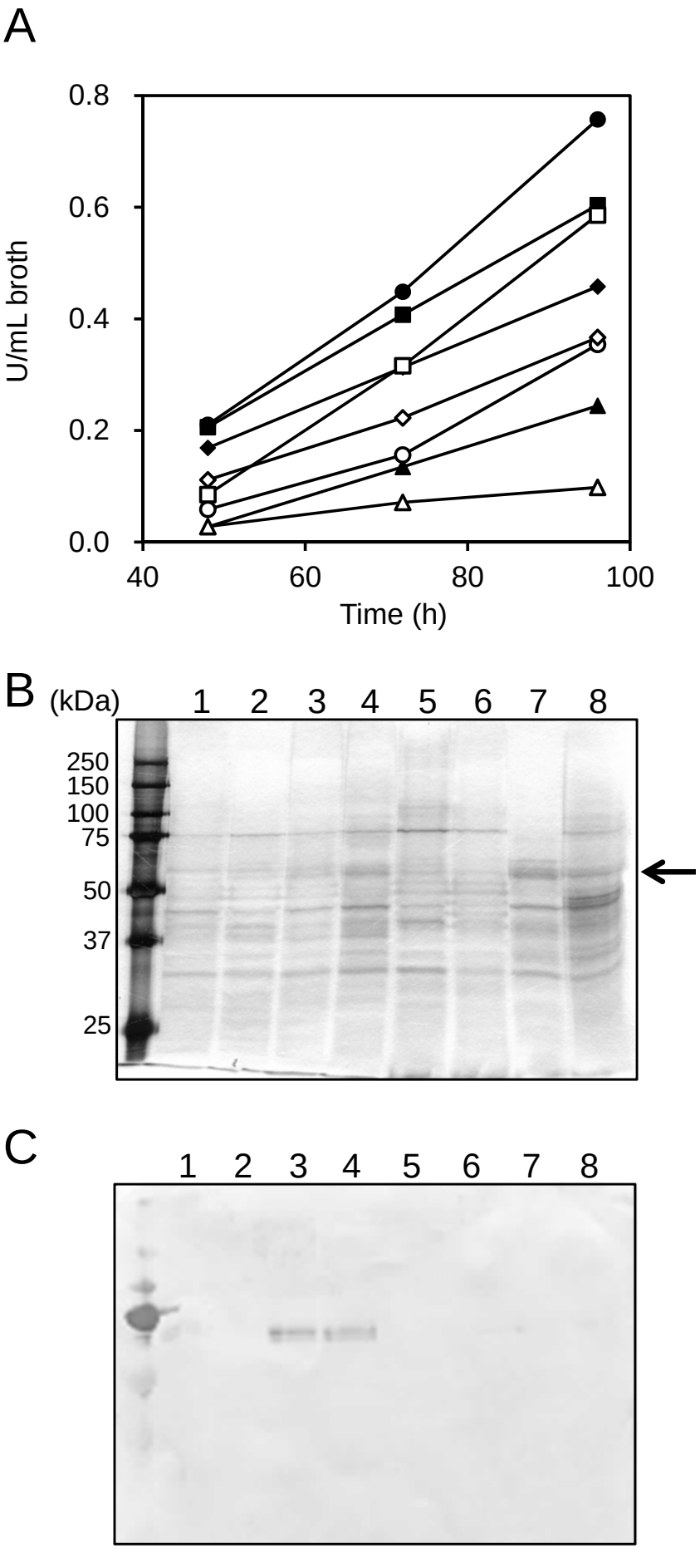


Fig. 2, Himeno et al.

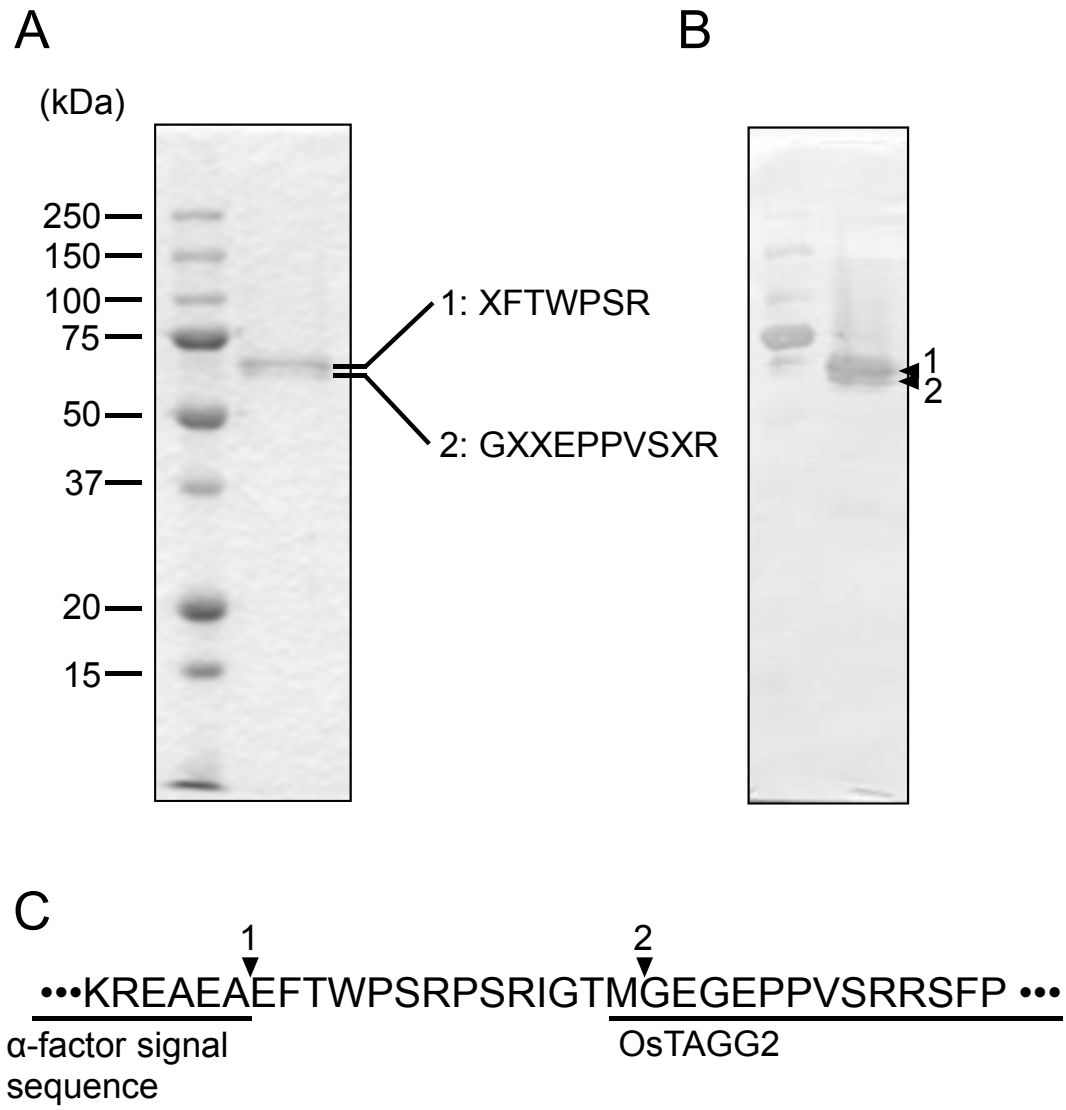


Fig. 3, Himeno et al.

Table 1. Hydrolytic Velocities of OsTAGG2 towards Various Substrates

Substrate	rOsTAGG2P		rOsTAGG2E		Native OsTAGG2	
	v (μmol/min/mg protein)	Relative *v* (%)	*v* (μmol/min/mg protein)	Relative *v* (%)	*v* (μmol/min/mg protein)	Relative *v* (%)
pNP β-D-Glucoside	182 ± 6	100	60.7 ± 1.1	100	171 ± 7	100
oNP β-D-Glucoside	182 ± 4	100	23.0 ± 1.6	34.1	300 ± 14	175
pNP β-D-Fucoside	300 ± 6	165	96.7 ± 9.9	159	138 ± 6	80.7
pNP β-D-Galactoside	45.9 ± 0.8	25.2	43.0 ± 4.5	70.8	184 ± 1	108
pNP β-D-Xyloside	6.77 ± 0.38	3.72	13.4 ± 0.32	22.1	15.3 ± 0.2	8.95
pNP β-D-Mannoside	1.55 ± 0.03	0.852	0.37 ± 0.03	0.610	1.18± 0.18	0.690
TAG	44.5 ± 1.5	24.5	50.1 ± 1.0	82.5	25.3 ± 0.3	14.8
Helicin	179 ± 4	98.4	186 ± 3	306	274 ± 5	160
SAG	201 ± 8	110	60.3 ± 1.4	99.3	118 ± 4	69.0
m-SAG	215 ± 6	118	61.0 ± 3.3	100	130 ± 10	76.0
p-SAG	164 ± 8	90.1	105 ± 5	173	157 ± 4	91.8
Cellobiose	Trace	N.D.	0.520 ± 0.003	0.857	N. H.	N. H.
Cellotriose	7.33 ± 0.07	4.02	15.5 ± 0.5	25.5	36.8 ± 3.9	21.5
Cellotetraose	13.8 ± 0.4	7.58	26.1 ± 1.2	43.0	45.6 ± 2.9	26.7
Sophorose	3.32 ± 0.37	1.82	4.28 ± 0.33	7.05	19.9 ± 0.5	11.6
Laminaribiose	11.6 ± 0.6	6.37	28.8 0.2	47.4	61.7 ± 6.2	36.1
Gentiobiose	0.117 ± 0.008	0.0643	0.450 ± 0.006	0.741	0.859 ± 0.145	0.502

Data are mean ± SD for three independent experiments. N. D., not determined. N. H., not hydrolyzed. Trace, accurate activity could not be determined due to low activity.

Table 2. Kinetic Parameters of Recombinant OsTAGG2 Produced in *P. pastoris* for the Hydrolysis of Various Substrates

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
pNP β-D-Glucoside	466 ± 4	2.71 ± 0.11	172
oNP β-D-Glucoside	359 ± 9	2.01 ± 0.16	179
pNP β-D-Fucoside	499 ± 9	1.85 ± 0.19	270
pNP β-D-Galactoside	236 ± 19	6.83 ± 1.19	34.6
TAG	72.7 ± 3.6	1.41 ± 0.14	51.6
SAG	458 ± 21	3.21 ± 0.33	143
m-SAG	563 ± 21	1.99 ± 0.15	283
p-SAG	286 ± 11	1.33 ± 0.25	215
Helicin	497 ± 48	2.65 ± 0.51	188