J. Biochem. **142,** 491–500 (2007)
doi:10.1093/jb/mvm174

Function-unknown Glycoside Hydrolase Family 31 Proteins, mRNAs of which were Expressed in Rice Ripening and Germinating Stages, are α-Glucosidase and α-Xylosidase*

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Received July 2, 2007; accepted July 18, 2007; published online September 17, 2007

In rice (*Oryza sativa* L., var Nipponbare) seeds, there were three mRNAs encoding for function-unknown hydrolase family 31 homologous proteins (ONGX-H1, ONGX-H3 and ONGX-H4): ONGX-H1 mRNA was expressed in ripening stage and mRNAs of ONGX-H3 and ONGX-H4 were found in both the ripening and germinating stages [Nakai *et al.*, (2007) *Biochimie* 89, 49–62]. This article describes that the recombinant proteins of ONGX-H1 (rONGXG-H1), ONGX-H3 (rONGXG-H3) and ONG-H4 (rONGXG-H4) were overproduced in *Pichia pastoris* as fusion protein with the α-factor signal peptide of *Saccharomyces cerevisiae*. Purified rONGXG-H1 and rONGXG-H3 efficiently hydrolysed malto-oligosaccharides, kojibiose, nigerose and soluble starch, indicating that ONGX-H1 and ONGX-H3 are α-glucosidases. Their substrate specificities were similar to that of ONG2, a main α-glucosidase in the dry and germinating seeds. The rONGXG-H1 and rONGX-H3 demonstrated the lower ability to adsorb to and degradation of starch granules than ONG2 did, suggesting that three α-glucosidases, different in action to starch granules, were expressed in ripening stage. Additionally, purified rONGXG-H4 showed the high activity towards α-xylosides, in particular, xyloglucan oligosaccharides. The enzyme hardly hydrolysed α-glucosidic linkage, so that ONGX-H4 was an α-xylosidase. α-Xylosidase encoded in rice genome was found for the first time.

Key words: α-glucosidase, α-xylosidase, glycoside hydrolase family 31, *Pichia pastoris*, starch granule-binding.

Abbreviations: GH, glycoside hydrolase family; 4-MUG, 4-methylumberyferyl α-d-glucopyranoside; ONG and ONX, *Oryza sativa* L., var Nipponbare α-glucosidase and α-xylosidase, respectively; ONGX-H, homologue of ONG or ONX; *ong*, *onx* and *ongx-h*, cDNA encoding ONG, ONX and ONGX-H, respectively; ORF, open reading frame; rONG, rONGX-H and rONX, recombinant proteins of ONG; ONGX-H and ONX, respectively; PPC, *Pichia pastoris* cells harbouring expression plasmid for rONGX-H production; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PG, phenyl α-D-glucoside; pNPG, *p*-nitrophenyl α-D-glucopyranoside; pNPX, *p*-nitrophenyl α-D-xylopyranoside; XGOS, xyloglucan oligosaccharide.

A rice species, *Oryza sativa* L., ssp. *japonica*, var Nipponbare, is the model cereal plant having a modest genome size of 430 Mb pairs. The mapping of its genome sequence was completed (*1*), and Rice Annotation Project Database predicted the functions of genes, allowing us to understand the biology of rice (*2*). Rice genome encoded seven proteins homologous to glycoside hydrolase family (GH) 31 enzymes. GH-classification was done by the sequence similarities of glycosidases (*3–5*). Members of GH 31 enzymes are α-glucosidase (EC 3.2.1.20), α-xylosidase (EC 3.2.1.-), α-glucan lyase (EC 4.2.2.13) and isomaltosyltransferase (EC 2.4.1.-). Three rice genomic genes, P0011D01.29 (located in the chromosome 1), P0701B08.7-1 and B1153E06.2 (both located in the chromosome 6) are predicted to encode GH 31 α-glucosidase. α-Glucosidase is an enzyme that catalyses the liberation of α-D-glucose from the non-reducing terminal of α-glucoside substrate. The enzyme has been found in many plants, in particular, widely in the storage tissue such as seeds (*6–11*). Its physiological function is a degradation of starch granules together with α-amylase, β-amylase and debranching enzyme (*12–15*). For a long time, it has been believed that the initial attack to starch granules was performed by α-amylase (a key enzyme) in the germination stage of plant seeds,

*ONGX-H1, ONGX-H3 and ONGX-H4 (or ONX4) in this article correspond to ONG1, ONG3 and ONG4 in our previous publication (reference 6), respectively.

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due to its ability to adsorb to and degradation of starch granules. Recently, we found that rice α-glucosidase was also able to hydrolyse starch granules by adsorption (*6*). A large amount of α-glucosidase activity was stored in the rice seeds before germination, so that α-glucosidase was a new key enzyme in the early germinating stage prior to the α-amylase expression. The annotation predicted that P0504H10.9 located in the chromosome 1 was a gene of α-xylosidase. α-Xylosidase catalyses the liberation of α-xylose from the non-reducing terminal of α-xyloside substrate. *Arabidopsis* (*16*), cabbage (*16*), nasturtium (*17*, *18*), pea (*19*), pine (*20*) and soybean (*21*) α-xylosidases are considered to involve in the metabolism of xyloglucan, a hemicellulosic polysaccharide of type I plant cell wall (*22*), cooperatively with xyloglucan-specific endo-β-glucanase (xyloglucanase), xyloglucan endotransglycosylase, β-galactosidase, β-glucosidase and α-fucosidase (*21*, *23–26*). Even though these annotation works predicted the enzyme function, the amino acid sequences of α-xylosidase and α-glucosidase are very similar to each other (*16*), so that heterogeneous gene-expression studies are essential to learn the catalytic reactions of the above three function-unknown proteins. OSJNBa0090D11.21 located in the chromosome 3 and B1123E10.103 and B1123E10.115 located in the chromosome 7 were predicted to encode α-glucosidase II, a trimming enzyme of *N*-linked sugar chain (*27*). No gene encoding α-glucan lyase or isomaltosyltransferase belonging to the GH 31 is present in the rice genome.

In the previous study (*6*), we purified and characterized a rice dry seed α-glucosidase (ONG2), encoded by *ong2* corresponding to B1153E06.2. The enzyme exhibited the ability to hydrolase malto-oligosaccharides and soluble starch effectively, and was capable of attacking the starch granules, suggesting that the α-glucosidase was involved in starch metabolism in the germinating seeds. The ONG2 mRNA was expressed in ripening stage and not in germination stage of seeds, indicating that ONG2 was produced in ripening seeds and remained in dry and germinating seeds. Three more cDNAs (*ongx-h1*, *ongx-h3* and *ongx-h4*), having the similarity to *ong2* in amino-acid sequence level, were also isolated from mRNA pool of the ripening seeds (*6*). The *ongx-h1*, *ongx-h3* and *ongx-h4* corresponded to P0701B08.7-1, B1153E06.2 and P0504H10.9, respectively. Interestingly, ONG2 and ONGX-H3 mRNAs were generated by the alternative splicing of first exon of B1153E06.2. However, two GH 31 homologous proteins, encoded by *ongx-h3* and *ongx-h4*, have not been actually identified as active enzymes yet, and ONGX-H1 was not purified completely (*6*), so that the functions of three homologues have remained unknown. The purpose of this study was to elucidate the functions of GH 31 homologous proteins. For this, it is necessary to produce the enzymes using a higher eukaryotic expression system, such as methylotrophic yeast, *Pichia pastoris*, having many advantages of protein processing, folding and post-translational modification (*28*, *29*). Recently, many foreign genes are expressed with *P. pastoris* system, by which the secretion is possible using *Saccharomyces cerevisiae* α-factor signal peptide (*30*, *31*). In addition, high expression level of gene encoding heterologous protein has been obtained by using a developed plasmid-borne alcohol oxidase 1 (*AOX1*) promoter, which is derived from *P. pastoris*, regulated tightly by methanol (*32–34*). Using this yeast system, barley high pI α-glucosidase was produced as a completely active enzyme, properties of which were similar to those of native enzyme (*35*). This article describes the production, purification and characterization of the GH 31 homologous proteins encoded by three cDNAs (*ongx-h1*, *ongx-h3* and *ongx-h4*), situated in distinct loci of rice genome.

MATERIALS AND METHODS

Substrates—Maltose, soluble starch, *p*-nitrophenyl α-D-glucopyranoside (pNPG), *p*-nitrophenyl α-D-xylopyranoside (pNPX) and 4-methylumberyferyl α-D-glucopyranoside (4-MUG) were purchased from Nacalai Tesque Chemical (Kyoto, Japan); maltotriose, kojibiose and nigerose from Wako Pure Chemical Industry (Osaka, Japan); isomaltose from Tokyo Chemical Industry (Tokyo, Japan); phenyl α-D-glucoside (PG) from Sigma (St Louis, MO, USA); 4,6-*O*-benzylidene *p*-nitrophenyl α-D-maltoheptaoside from Megazyme International Ireland (Wicklow, Ireland); malto-tetraose, -pentaose, -hexaose, -heptaose, nigerose and rice starch granules were kindly supplied by Dr Nakakuki and Dr Yamamoto, Nihon Shokuhin Kako (Tokyo, Japan). To remove possible impurities, maltose was further purified by repeated crystallizations, and soluble starch was washed with deionized water before use. The preparation of xyloglucan oligosaccharides mixture (XGOS) and isoprimeverose (6-*O*-α-xylopyranosyl-glucopyranose) was described in our previous paper (*36*).

Construction of Expression Vectors—Three expression vectors, ongxh1/pPICZαA, ongxh3/pPICZαA and ongxh4/pPICZαA, derived from pPICZαA expression vector (Invitrogen, Carlsbad, CA, USA) were made for the production of recombinant ONGX-H1, ONGX-H2 and ONGX-H3 (rONGX-H1, rONGX-H2 and rONGX-H3), respectively, fused with *S. cerevisiae* α-factor secretary peptide. For the construction of the expression vectors, each cDNA was amplified by PCR using 5′ forward primer containing a restriction site for *KpnI* (N1pα1, 5′-ATTGGTACCGCCGGCAATGGCTACCGG-3′ for *ongx-h1* and *ongx-h3*; N4pα1, 5′-ATTGGTACCAGCAATGGCGTGTTTGCC-3′ for *ongx-h4*; the restriction site and initiation codon being underlined singly and doubly, respectively), and 3′ reverse primer containing a restriction site for *XbaI* (N1pα2, 5′-AAATCTAGATTAATAGGTCATAACGAC-3′ for *ongx-h1*; N2pα2, 5′-AAATCTAGATTAATAGGTCATCACGAC-3′ for *ongx-h3*; N4pα2, 5′-AAATCTAGACTATGCTTCGATCTGCAT-3′ for *ongx-h4*; the restriction site and termination codon being underlined singly and doubly, respectively) together with the plasmid (ong1/pBluescript II SK, ong3/pBluescript II SK or ong4/pBluescript II SK) (*6*) as a template, respectively. KOD DNA polymerase (TOYOBO, Osaka, Japan) was used for all PCR amplifications. The amplified fragments were digested with *KpnI* and *XbaI*, and then the resultant DNA fragments were ligated with pPICZαA, prior to being digested with *KpnI* and *XbaI*, downstream the *S. cerevisiae* α-factor secretary sequence

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in the vector. Sequences of the fragments were confirmed to have no PCR error.

Transformation of P. Pastoris—All steps were performed according to the manufacturer's instructions (EasySelect^{TM} Pichia Expression Kit, Invitrogen). All plasmids (ongx-h1/pPICZαA, ongx-h3/pPICZαA and ongx-h4/pPICZαA) were digested and linearized by *Bgl*II digestion, and then were transformed into *P. pastoris* strain GS115 by electroporation to facilitate integration into the host genome. The transformed cells were spread on YPDS agar plate (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol and 2% agar) containing 100 μg/ml zeocin, and incubated for 3 days at 30°C. Single colonies were streaked onto both MDH agar plate (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% glucose and 1.5% agar), followed by screening for methanol utilization plus (Mut$^+$) with MMH agar plate (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol and 1.5% agar) for 3 days at 30°C.

Cell Density for Methanol Induction—For the determination of cell density appropriate for the overproduction of recombinant enzyme, each transformed *P. pastoris* cells (PPC1, PPC3 and PPC4: transformants harbouring ongx-h1/pPICZαA, ongx-h3/pPICZαA and ongx-h4/pPICZαA, respectively) was grown in BMGY medium along with shaking at 30°C for 24 h. The cells harvested by centrifugation at 3,000*g* for 5 min were resuspended to BMMY medium by adjusting cell density (A_{600}) to 1–50 (A_{600} 10, 20 and 50 for PPC1 and PPC3; A_{600} 1, 10, 20, 40 for PPC4), and then the production of rONGX-H1, 3 or 4 was induced with methanol in BMMY medium for 96 h at 30°C. The α-glucosidase and α-xylosidase activities towards maltose and XGOS, respectively, were monitored in the culture supernatant of PPC1, PPC2 and PPC4. Cell density was also measured by A_{600} of the culture.

Production and Purification of Recombinant Enzymes—Three transformants (PPC1, PPC3 and PPC4) were grown in 5 ml of BMGY medium along with shaking at 30°C for 20 h, and then 1 ml of each culture was inoculated into 1,000 ml of BMGY medium. After growth for 24 h at 30°C, the cells harvested by centrifugation at 3,000*g* for 5 min were resuspended to 550 ml of BMMY medium with A_{600} 20 (PPC1 and PPC3) to induce the expression. In case of PPC4, the same culture using 1,000 ml of BMGY medium was repeated four times, and then 1,360 ml of BMMY medium was prepared by adjustment of A_{600} to 40. Every 24 h, methanol was added to a final concentration of 0.5% to maintain induction. After induction for 96 h at 30°C, each culture was centrifuged at 15,000*g* for 10 min at 4°C, and the supernatant was used for the purification of rONGX-H1, 3 and 4. All purification procedures were performed at 4°C.

Ammonium sulfate was added to the culture supernatant for PPC1 (550 ml) up to 80% saturation, and the suspensions were maintained at 4°C for 16 h. The resulting precipitate collected by centrifugation at 15,000*g* for 10 min was dissolved in 10 mM sodium phosphate buffer (pH 6.0, buffer-A), and dialysed against same buffer. The dialysed solution was applied to DEAE-TOYOPEARL 650M column ($3.3 \times 33\,cm^2$, 230 ml) equilibrated with the 10 mM buffer-A. The adsorbed protein was eluted with a linear gradient of 0–1.0 M NaCl. Active fraction was concentrated with Centriprep YM-30 (Millipore, Billerica, MA, USA), dialysed against 20 mM buffer-A containing 50 mM NaCl, and applied to Sephacryl S-100 column ($3.0 \times 119\,cm^2$, 841 ml) equilibrated with the same buffer. Collected active fraction was dialysed against 10 mM buffer-A containing 40% saturation of ammonium sulfate. The dialysed solution was applied to Butyl-TOYOPEARL 650M column ($3.0 \times 33\,cm^2$, 230 ml) equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 40–0% saturation of ammonium sulfate, and active fractions (purified rONGX-H1) were used for analysis of enzyme properties.

Ammonium sulfate was added to the culture supernatant of PPC3 (550 ml) up to 40% saturation. The supernatant obtained by centrifugation at 15,000*g* for 10 min was passed through columns of Butyl-TOYOPEARL 650M and Sephacryl S-100 by this order with the same procedure as described earlier in the article. Active fraction was dialysed against 20 mM sodium phosphate buffer (pH 7.0), and was applied to CM-TOYOPEARL 650M column ($1.6 \times 20\,cm^2$, 40 ml). The adsorbed proteins were eluted with a linear gradient of 0–1.0 M NaCl. Active fractions were concentrated with Centriprep YM-30, and dialysed against 20 mM sodium acetate buffer (pH 6.0, buffer-B) containing 50 mM NaCl. The dialysed solution was applied to Sephacryl S-100 column ($1.7 \times 53\,cm^2$, 120 ml) equilibrated with the same buffer, and active fractions (purified rONGX-H3) were pooled.

Ammonium sulfate was added to the culture supernatant for PPC4 (1,360 ml) up to 80% saturation. The precipitate collected by centrifugation at 15,000*g* for 10 min was dissolved in 10 mM buffer-A, and then ammonium sulfate was added up to 40% saturation. The solution was applied to Butyl-TOYOPEARL 650M column as described earlier in the article. Active fractions were collected, dialysed against 20 mM buffer-B, and subjected to a DEAE-TOYOPEARL 650M column ($1.6 \times 20\,cm^2$, 40 ml) equilibrated with 20 mM buffer-B. The adsorbed proteins were eluted with a linear gradient of 0–1.0 M NaCl. Active fractions were concentrated with Centriprep YM-30, and dialysed against 20 mM buffer-B containing 50 mM NaCl. The dialysed solution was applied to Sephacryl S-100 column ($3.0 \times 119\,cm^2$, 841 ml), and active fractions (purified rONGX-H4) were collected.

Enzyme Activity Assays—The activities for α-glucosidase and α-xylosidase were measured by release of glucose and xylose from maltose and XGOS, respectively. One unit of α-glucosidase activity (or α-xylosidase activity) was defined as the amount of enzyme hydrolyzing 1 μmol substrate (or liberating 1 μmol xylose from substrate) per min. The reaction mixture consisted of 40 μl of substrate, 20 μl of enzyme solution and 40 μl of 0.1 M sodium acetate (pH 4.5). The mixture with α-glucosidase was incubated for 10 min at 37°C, and the reaction was terminated by addition of 200 μl of 2 M Tris–HCl (pH 7.0). The liberated glucose was assayed by Tris–glucose oxidase–peroxidase method with our modification (*37*) by Glucose AR-II Test (Wako Pure

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Chemical Industry). The reaction mixture with α-xylosidase was incubated for 10 min at 35°C. The reaction was terminated by boiling for 3 min, and the released xylose was assayed by the *p*-bromoaniline method of Roe and Rice (*38*) as modified by Edward *et al*. (*39*).

Measurement of Protein Concentration—Protein concentration of culture supernatant was measured by the method of Bradford (*40*), with bovine serum albumin as standard. Protein concentration in purification procedure was estimated spectrophotometrically using $E^{1\%}_{1cm}$ at 280 nm of 10.0. The purified enzyme was calculated from amino acid contents of protein hydrolysate (6 M HCl at 110°C for 24 h) determined using JEOL JLC/500V (Tokyo, Japan) equipped with ninhydrin-detection system.

Gel Electrophoresis—Molecular weights of purified enzymes were estimated based on a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the Laemmli's method (*41*) using the gel prepared with 8% (w/v) acrylamide gel for separation with Mini-Protean III (Bio-Rad Laboratories, CA, USA). Deglycosylation of purified enzymes was performed under the conditions as follows: the reaction mixture (100 µl) consisting of enzyme (10 µg of each protein), 50 mM sodium acetate buffer (pH 4.5) and Endoglycosidase H (2 U, Roche Diagnostics, Indianapolis, IN, USA) was incubated at 37°C for 24 h, and then 2 µl of the mixture was subjected on SDS–PAGE. Mark12™ MW Standard (Invitrogen) was used as protein marker. Proteins were stained with Rapid CBB KANTO (Kanto Chemical, Tokyo, Japan).

Effects of pH and Temperature—The optimum pHs of recombinant α-glucosidases were examined at 37°C for 10 min using maltose as substrate. The reaction mixtures consisted of 40 µl of 0.5% maltose, 20 µl of enzyme solution (0.022 µg for rONGX-H1 and 0.037 µg for rONGX-H3) and 40 µl of 40 mM Britton-Robinson buffer modified by us (*8*) at various pHs from 2.5 to 8.0. For the pH-stability, reaction mixtures consisted of 10 µl of enzyme solution (0.073 µg for rONGX-H1 and 0.093 µg for rONGX-H3) and 90 µl of 40 mM modified Britton-Robinson buffer (pH 2.3–12.5) were incubated at 4°C for 24 h. To 20 µl of the pH-treated mixtures, 40 µl of 1 M sodium acetate (pH 4.5) and 40 µl of 0.5% maltose were added. The reaction mixtures were incubated at 37°C for 10 min to measure the residual activity. For the thermal stability, reaction mixture consisted of 20 µl of enzyme solution (0.718 µg rONGX-H1 or 0.880 µg rONGX-H3) and 40 µl of 0.1 M sodium acetate (pH 4.5) was kept at 30–70°C for 15 min, and then was immediately cooled at 0°C. The residual activity was measured at 37°C for 10 min by the addition of 40 µl of 0.5% maltose to the mixture.

The optimum pH of recombinant α-xylosidase were examined at 35°C for 10 min with 0.2% XGOS and 0.94 µM enzyme solution in 16 mM Britton-Robinson buffer (pH 2.5–8.0). For the pH stability, 0.94 µM enzyme solution in 36 mM Britton-Robinson buffer (pH 2.3–12.5) was kept at 4°C for 24 h. To the mixtures, 0.5% XGOS and 1 M sodium acetate (pH 4.5) were added. The reaction mixtures were incubated at 35°C for 10 min, and the residual activity was measured. For the thermal stability, 1.6 µM enzyme solution in 0.1 M sodium acetate (pH 4.5) was kept at 30–70°C for 15 min, and then the residual activity was measured at 35°C for 10 min by addition of 40 µl of 0.5% XGOS.

Substrate Specificity—Maltose (1.5–10 mM), maltotriose (0.9–3.75 mM), -tetraose (1.05–3.75 mM), -pentaose (0.6–2.5 mM), -hexaose (0.64–2.4 mM) and -heptaose (0.6–3 mM), soluble starch (rONGX-H1, 0.61–2.7 mM; rONGX-H3, 0.61–2.2 mM), isomaltose (rONGX-H1, 3–14 mM; rONGX-H3, 10–40 mM), kojibiose (0.4–1.5 mM), nigerose (1.125–5 mM) and PG (2.1–12 mM) were used as substrate for analysis of substrate specificities for recombinant α-glucosidases (*i.e*. rONGX-H1 and rONGX-H3). Michaelis constant (K_m) and molecular activity (k_0) were estimated by Lineweaver–Burk plots (1/*s*-1/*v* plots) with the experimental error <2%. In case of analysis for substrate specificity of recombinant α-xylosidase (*i.e*. rONGX-H4), we used isoprimeverose, pNPX, maltose, isomaltose, kojibiose, nigerose, pNPG and 4-MUG (2 mM for each substrate) and XGOS (2 mg/ml). The liberated xylose and glucose were measured for α-xyloside substrates (pNPX and XGOS) and for isoprimeverose and α-glucoside substrates (the other), respectively.

Starch Granules Binding and Degrading Assays—Prior to experiments, rice starch granules were washed five times by 0.1 M sodium acetate buffer (pH 4.5) to remove the remaining soluble sugar. For binding ability assay, reaction mixture (500 µl) consisting of 0.35 µM enzyme and 5 mg starch granules was incubated at 4°C in 0.1 M sodium acetate buffer (pH 4.5) containing 0.05% bovine serum albumin and 5% ammonium sulfate. After sedimentation of starch granules by centrifugation at 10,000*g* for 1 min, the α-glucosidase activity remaining in supernatant was measured to evaluate the amount of enzyme bound to starch granules. For degrading ability assay, reaction mixture (500 µl) consisting of 0.35 µM enzyme and 5 mg starch granules was incubated at 37°C in 0.1 M sodium acetate buffer (pH 4.5) containing 0.05% bovine serum albumin. After centrifugation at 10,000*g* for 1 min, glucose liberated in supernatant was measured by the modified Tris–glucose oxidase–peroxidase method.

RESULTS

Plasmid Construction—We substituted α-factor signal peptide of *S. cerevisiae*, derived from pPICZαA expression vector, for a signal peptide of ONGX-H1, 3 or 4 to facilitate secretion of recombinant enzymes by *P. pastoris*. An N-terminal amino-acid of ONGX-H1 (Ala39) was analysed using its partially purified protein (*6*), allowing us to know a signal sequence (Met1 to Thr38), but those of ONGX-H3 and ONGX-H4 were unknown, since both proteins have not been actually obtained as active enzymes from rice seeds. Therefore, the signal peptides of ONGX-H3 and ONGX-H4 were analysed by SignalP 3.0 server (*42*), predicting a signal peptidase-cleavage site between Ala30 and Ala31 for ONGX-H3 and between Ser30 and Ser31 for ONGX-H4. The algorithm for ONGX-H3 predicted a signal peptide

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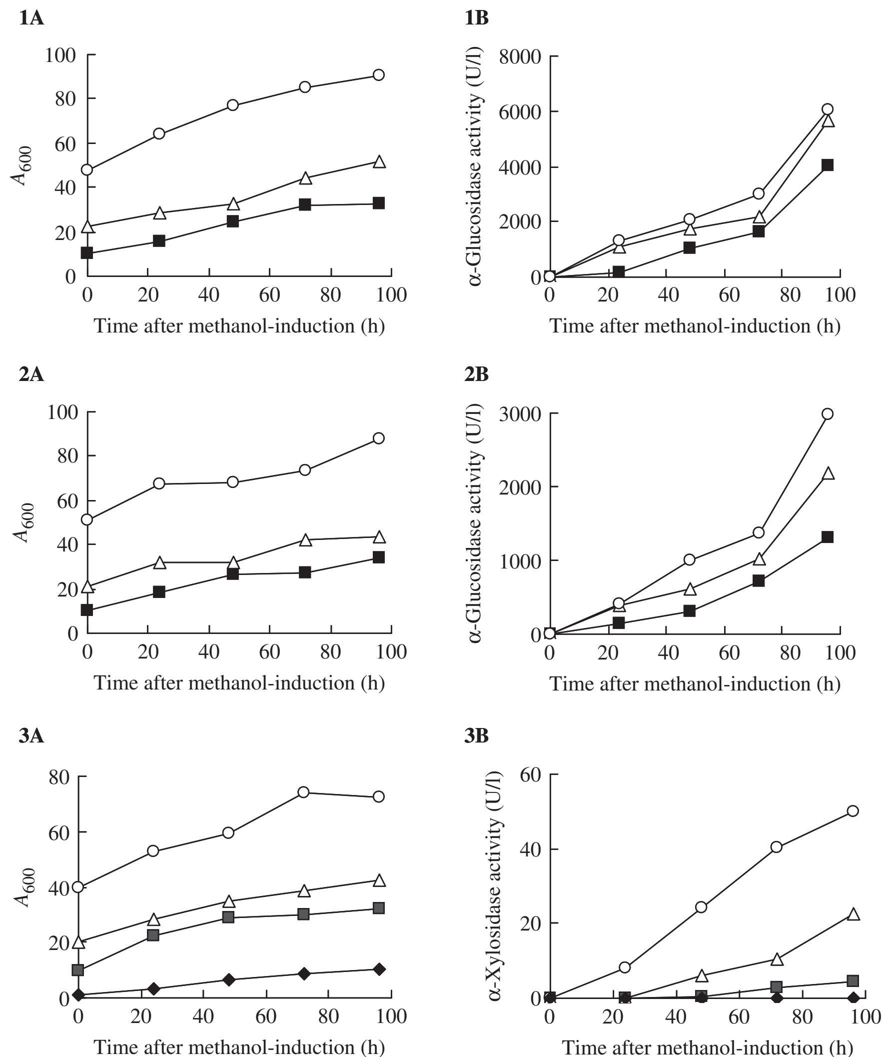

Fig. 1. **Time-courses of cell growth and enzyme activity in culture of PPC1, PPC3 and PPC4.** PPC1, PPC3 or PPC4 (carrying expression vector for ONGX-H1, ONGX-H3 or ONGX-H4, respectively) was grown at 30°C for 96 h in 5 ml BMMY medium with methanol induction. 1A, 2A and 3A show the cell growth (A_{600}) of PPC1, PPC3 and PPC4, respectively. α-Glucosidase activities of PPC1and PPC3 are displayed in 1B and 2B, respectively, and 3B shows the α-xylosidase activity of PPC4. Panels 1 and 2: at the initial stage of culture, cell density (A_{600}) was adjusted to 10 (solid square), 20 (open circle) and 50 (open triangle); panel 3: those of this panel are 1 (solid rhombus), 10 (solid square), 20 (open triangle), 40 (open circle).

probability of 1.0 and a cleavage site with a probability of 0.31 between 30 and 31. The algorithm for ONGX-H4 predicted a signal peptide probability of 1.0 and a cleavage site with a probability of 0.849 between 30 and 31. Each ORF without a signal sequence-coding region was amplified by PCR and were placed under control of *P. pastoris* AOX1 promoter in pPICZαA vector to be inducted by methanol in very high level.

Production of rONGX-H1 and rONGX-H3 by P. Pastoris—Figure 1 shows the α-glucosidase activities from the recombinant *P. pastoris*, PPC1 and PPC3 harbouring an expression vector for ONGX-H1 or ONGX-H3, by varying cell concentrations in the initial stage of culture using 5 ml BMMY medium. All the culture media tested exhibited α-glucosidase activities, which increased gradually by further induction with methanol in comparison with the control *P. pastoris* not carrying ONGX-H1/pPICZαA and ONGX-H3/pPICZαA. α-Xylosidase activity was not found in the culture supernatant of PPC1 and PPC3. The α-glucosidase activities increased remarkably by the high level of cell density (A_{600} 20) at the starting stage of induction, while more cell density of initiation (A_{600} 50) hardly affects enzyme production. After 96 h, the culture media of PPC1 and PPC3, initiated with A_{600} 20, exhibited 5.6 and 2.2 U/ml of α-glucosidase activities, respectively. For purification of rONGX-H1 and rONGX-H3, PPC1 and PPC3 were cultivated in a large-scale of BMMY medium (550 ml of A_{600} 20), giving α-glucosidase activities of 3,900 and 1,100 U after 96 h induction at 30°C, respectively.

Purification and Characterization of rONGX-H1 and rONGX-H3—By ion-exchange, hydrophobic and gel-filtration chromatography, rONGX-H1 and rONGX-H3 were purified up to 35 and 27 U/mg protein, respectively. The purification procedures of rONGX-H1

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Table 1. **Summary of purification for rONGX-H1, rONGX-H3 and rONGX-H4 produced in *P. pastoris*.**

Procedure	Total protein (mg)	Total activity[a] (U)	Specific activity[a] (U/mg)	Purification (-fold)	Recovery (%)
rONGX-H1					
Culture supernatant	149[b]	3,900	26	1.0	100
DEAE-TOYOPEARL650M (pH 6.5)	70[c]	1,100	15	0.58	27
Sephacryl S-100	44[c]	980	22	0.85	25
Butyl-TOYOPEARL 650M	17[d]	600	35	1.3	16
rONGX-H3					
Culture supernatant	108[b]	1,100	10	1.0	100
DEAE-TOYOPEARL650M (pH 6.5)	65[c]	730	11	1.1	83
Sephacryl S-100	31[c]	510	17	1.7	27
Butyl-TOYOPEARL 650M	16[c]	400	25	2.5	25
Sephacryl S-100	5.2[d]	140	27	2.7	16
rONGX-H4					
Culture supernatant	120[b]	78	0.64	1.0	100
DEAE-TOYOPEARL650M (pH 6.5)	190[c]	48	0.25	0.17	62
Sephacryl S-100	93[c]	39	0.42	0.65	50
Butyl-TOYOPEARL 650M	5.2[d]	18	3.5	5.5	23

[a]α-Glucosidase activity for rONGX-H1 and rONGX-H3; α-xylosidase activity for rONGX-H4. [b]These values were estimated by Bradford method using BSA as standard. [c]These values were calculated under the assumption that $E^{1\%}_{1\mathrm{cm}}$ at 280 nm was 10.0. [d]These values were calculated on the basis of the fact that $E^{1\%}_{1\mathrm{cm}}$ at 280 nm was = 15.2 for rONGX-H1, 17.2 for rONGX-H3 and 14.9 for rONGX-H4.

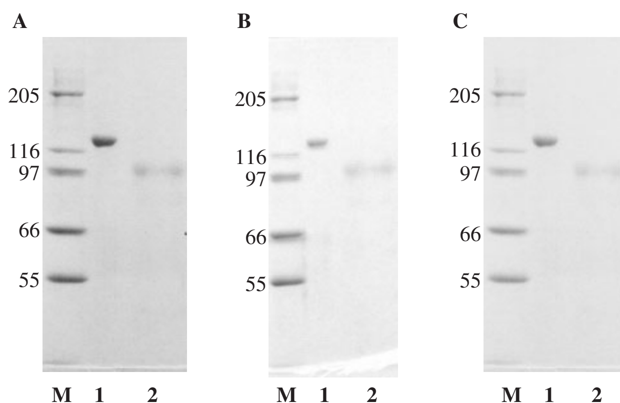

Fig. 2. **SDS–PAGE of purified recombinant enzymes.** Lane M shows molecular marker proteins: myosin (M_r, 205,000), β-galactosidase (116,000), phosphorylase *b* (97,000), bovine serum albumin (66,000), glutamic dehydrogenase (55,000), molecular weights of which (divided by 1,000) are displayed to the left side of each panel. Lane 1 in A, B, C indicates purified rONGX-H1, rONGX-H3 and rONGX-H4, respectively. Lane 2 shows Endoglycosidase H-treated enzymes.

and rONGX-H3 are summarized in Table 1. Purity of isolated protein was confirmed by SDS–PAGE, of which molecular weights were also estimated to be 124,000 (Fig. 2). The molecular weights (98,000) of their deglycosylated forms with Endoglycosidase H were smaller than those of intact enzymes. These results indicate that rONGX-H1 and rONGX-H3 were glycoproteins. The effects of pH and temperature on activity were examined. Both of rONGX-H1 and rONGX-H3 had the same optimum pH at 4.5, and were stable in the pH range of 3.0–10.5 after incubation at 4°C for 24 h. They were stable up to 42°C for 15 min, and lost the activity completely by incubation at 70°C. Both of rONGX-H1 and rONGX-H3 did not attack any α-xyloside substrates, but exhibited α-glucoside-hydrolyzing activity, proving that they are α-glucosidases (*here ONGX-H1 and ONGX-H3 are renamed ONG1 and ONG3, respectively*). Substrate specificities of rONG1 and rONG3 were investigated by hydrolytic velocities on substrates of various concentrations. The kinetic parameters of both enzymes were similar except for those towards isomaltose. They showed higher activity towards homogeneous substrates such as glucobiose than heteroside substrate like PG (Table 2). The k_0/K_m values for various glucobioses decreased in the following order: nigerose (composed of α-1,3-glucosidic linkage) > maltose (α-1,4) > kojibiose (α-1,2) > isomaltose (α-1,6). The k_0 and k_0/K_m values for nigerose were higher than those for maltose. The K_m for kojibiose was lower than that for maltose. The parameters for isomaltose were obviously distinct between rONG1 and rONG3: k_0/K_m values were about 23 and 1.3% of those for maltose, respectively. In comparison with the hydrolysis of malto-oligosaccharides, two enzymes exhibited a high activity towards soluble starch. Additionally, rONG1 and rONG3 adsorbed to and degraded of rice starch granules (Fig. 3). Purified enzyme preparations did not hydrolyse 4,6-*O*-benzylidene *p*-nitrophenyl α-D-maltoheptaoside (reducing- and non-reducing-terminal glucose residues of maltoheptaose are linked to *p*-nitrophenyl and 4,6-*O*-benzylidene groups, respectively; a specific substrate for α-amylase), indicating that the rONG1 and rONG3 preparations did not contain any α-amylase activity.

Production of rONGX-H4 by P. pastoris—The α-glucosidase and α-xylosidase activities of produced rONGX-H4 were monitored in 5 ml BMMY medium of PPC4, the methanol-induced cultivation of which was initiated with various cell densities. As shown in Figs 1–3, α-xylosidase activity was observed in the culture media, where there was a small maltose-hydrolysing activity less than 1/1,000. As in the case of rONG1 and rONG3 production, the initial cell density was an important factor. In the cultivation of PPC4

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Table 2. **Kinetic parameters for hydrolysis of various substrates by rONG1 and rONG3[a].**

	rONG1			rONG3		
Substrate	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($s^{-1}\,mM^{-1}$)	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($s^{-1}\,mM^{-1}$)
Maltose	2.4	42.8	17.7	2.8	33.4	12.1
Maltotriose	1.2	50.5	41.0	1.3	41.1	31.6
Maltotetraose	1.5	50.2	33.9	1.7	43.2	25.4
Maltopentaose	1.3	53.0	40.6	1.4	41.2	29.7
Maltohexaose	1.1	55.8	52.1	1.2	43.0	37.2
Maltoheptaose	0.80	57.8	72.0	0.79	41.3	52.2
Isomaltose	7.0	28.4	4.06	26	4.12	0.159
Nigerose	1.8	61.5	34.5	2.5	55.0	22.1
Kojibiose	1.0	12.8	12.3	0.97	9.40	9.71
Phenyl α-glucoside	3.7	4.59	1.25	3.6	3.53	0.98
Soluble starch	0.85[b]	84.2	98.6	2.0[b]	122	61.3

[a]Errors in estimation of kinetic parameters were less than 2.0%. [b]Concentration of non-reducing terminal.

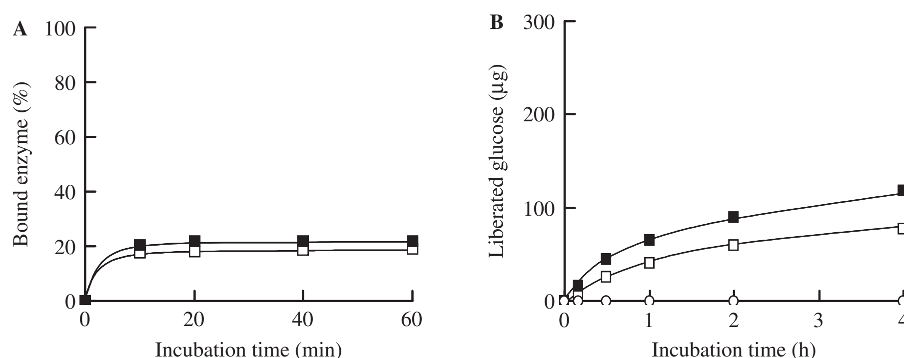

Fig. 3. **Adsorbing and degrading abilities towards rice starch granules by rONG1 and rONG3.** (A) Adsorbing ability: open and solid squares show the percentages of rONG1 and rONG3 bound to rice starch granules (5 mg/0.5 ml of reaction mixture), respectively. (B) Degrading ability: open and solid squares show the amount of glucose liberated from rice starch granules (5 mg/0.5 ml of reaction mixture) by rONG1 and rONG3 (0.35 μM each enzyme), respectively. Open circles show soluble sugar liberated from starch granules without enzyme (control).

as well as those of PPC1 and PPC3, α-xylosidase activity of high level was produced by the initial cell density of more than 20. After 96 h induction, the culture medium of PPC4 (initial cell density being 40) exhibited 0.05 U/ml of α-xylosidase activity. For purification of rONGX-H4, we cultured PPC4 in 1,360 ml BMMY medium by adjusting initial cell density to A_{600} 40. The 96 h cultivation with continuous methanol induction gave us the α-xylosidase activity of 78 U.

Purification and Characterization of rONGX-H4—The purification procedure of rONGX-H4 is summarized in Table 1. By SDS–PAGE analysis, rONGX-H4 isolated with a final chromatography of gel-filtration was confirmed to be a homogenous protein having the molecular weight of 127,000 (Fig. 2). Treatment by Endoglycosidase H generated a deglycosylated form, the molecular weight of which was 99,000, indicative of a glycoprotein. The effects of pH and temperature on the hydrolytic activity towards XGOS were examined. The optimum pH was 4.5, and the enzyme was stable from pH 4.0 to 9.0. The enzyme was stable up to 40°C, and lost the activity completely at 70°C. We investigated substrate specificity of rONGX-H4 towards α-xyloside and α-glucoside substrates. Table 3 summarizes the initial velocities on 2 mM substrates and 2 mg/ml XGOS. rONGX-H4 showed the substrate specificity only against α-xyloside, in particular, XGOS (2.1 μmol xylose/mg protein/min). The hydrolytic velocity for isoprimeverose and pNPX were about 5.4 and 3.5% of those for XGOS, respectively. The enzyme hardly hydrolysed α-glucosidic linkage of maltose, isomaltose, kojibiose, nigerose, pNPG and 4-MUG. Based on these substrate specificities, ONGX-H4 was found to be an α-xylosidase (*here rONGX-H4 is renamed ONX4*).

Table 3. **Substrate specificity of rONGX4.**

Substrate[a]	Initial velocity (μmol/mg protein/min)	Relative value (%)
XGOS	2.1	100
Isoprimeverose	0.11	5.4
pNPX	0.073	3.5
Maltose	0.0018	0.088
Isomaltose	0.0055	0.26
Kojibiose	0.0028	0.13
Nigerose	0	0
pNPG	0.037	1.8
4-MUG	0.021	1.0

[a]Substrate concentration is 2.0 mM, except for XGOS (2 mg/ml).

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DISCUSSION

In the present study, based on the substrate specificities it becomes apparent that *ong1* and *ong3* encode α-glucosidase, meaning that three α-glucosidase genes are active in the ripening stage of rice seeds (*6*). The mRNAs of ONG2 and ONG3 were generated by alternative splicing from B1153E06.2 (*6*). Expression patterns of these two alternative splicing products were different in the ripening seeds. ONG2 mRNA was detected at 12 days after flowering, and increased during further maturation process. In contrast, ONG3 mRNA, appearing at 12 days after flowering, was increased temporarily (around 16 days after flowering), and then decreased during further maturation (*6*). There is no evidence that ONG3 mRNA is translated to the protein of ONG3 (for this, it is essential to perform further biochemical study, a western blot analysis using a highly specific antibody to discriminate each α-glucosidase, for example), but we consider that ONG3 might be formed in the ripening seeds by the control of alternative splicing process. ONG2 was a main α-glucosidase in dry seeds, synthesized at the ripening stage and stored in the dry seeds. Also in the dry seeds, we found very small amounts of ONG1 (1% activity of ONG2) (*6*), the mRNA expression pattern of which was identical to ONG3 mRNA, implying that the possible protease-catalysed degradation of ONG1 (and ONG3, if being produced) occurs at the last stage of ripening after the transcription is terminated. Our previous (*6*) and current studies suggest that (i) there are two types of α-glucosidases in rice ripening seeds: the one is produced in the ripening stage and disappears [initial germination stage-inactive α-glucosidase: ONG1 and ONG3] and the other is produced in the ripening stage and remains as an active enzyme at the initial germination stage [initial germination stage-active α-glucosidase: ONG2]; (ii) alternative splicing regulates the gene expression (and also protein production, probably) of the aforementioned two α-glucosidases (ONG3 and ONG2); and (iii) the regulation of ONG1 production is different from those of ONG2 and ONG3: ONG1 was synthesized in the germinating stage after 48 h of imbibition as well as in the ripening stage, while there is no appearance of mRNA of ONG2 or ONG3 in germination (*6*).

Substrate specificities of rONG1 and rONG3 were almost identical to ONG2 (*6*), except for small k_0 values of two recombinant enzymes (1/5–1/8 of ONG2), also reducing k_0/K_m values in the same grade. One more exception is a high specificity of rONG1 towards isomaltose: in k_0/K_m levels, rONG1 attacks 10-fold to 25-fold quicker than ONG2 and rONG3 do, respectively, implying that isomaltose in rice ripening seeds is degraded mainly by ONG1, if this sugar occurs in ripening stage (α-glucosidases different in the substrate specificities existed in the ripening seeds). It was found that rONG1 and rONG3 (this study) as well as ONG2 (*6*) hydrolysed rice starch granules directly by adsorption. Degrading and adsorbing abilities of three α-glucosidases is distinct, in the order of ONG2-I > ONG2-II > rONG1 > rONG3, especially, where the starch-adsorbed ONG2-I (an isoform of ONG2 is described subsequently) attained 60% of whole proteins tested (*6*). ONG2 is a key enzyme in the starch degradation metabolism in the initial stage of germination (*6*), since α-amylase, another key enzyme, is produced at 1.5 day after imbibition (Nakai, *et al*., unpublished data), and there was a large α-glucosidase activity before germination (0.15 U per one dry seed) (*6*). ONG2 may contribute to energy production before α-amylase-catalysed starch degradation. Interestingly, ONG2 received the post-translational limited proteolysis in its N-terminal region to form two isoforms, ONG2-I (removal of Val97-Leu122) and ONG2-II (removal of Val97-Ala112). This post-translational proteolysis did not affect the specificity towards soluble substrates, but decreased the adsorbing ability to starch granules, implying that a small region of Ala113-Leu122 contributes to the adsorption. Amino acid sequences around three cleavage sites of ONG2 are conserved in those of ONG1 and ONG3, so that there is a possibility that the same post-translational limited proteolysis occurs in ONG1 and ONG3, controlling their digestion of starch granules. The physiological roles of three α-glucosidases in rice seeds are considered to be that: (i) initial germination stage-inactive α-glucosidase [ONG1 (and ONG3, probably)] may contribute to the degradation of starch granules at the early stage of ripening by being regulated with the post-translational proteolysis; (ii) ONG2 hydrolyses starch granules directly at the ripening and at the initial germination stage; (iii) ONG1 attacks starch granules at the germination stage together with α-amylase, since ONG1 mRNA was generated at 24–48 h after imbibition (*6*); and (iv) ONG1 is a candidate to hydrolyse isomaltose in rice seeds.

The expression level of rONX4 (16 mg/1l culture) was one order of magnitude smaller than those of rONG1 (172 mg/1l culture) and rONG3 (110 mg/1l culture). It is considered that the low production-level is due to difference in codon usage between rice (*O. sativa* L., var Nipponbare) and *P. pastoris*. ONX4 mRNA contains many rare codons [37 CCG (Pro), 36 GCG (Ala), 18 GGG (Gly), 10 UGC (Cys)] for the translation by *P. pastoris*, compared with those of ONG1 and ONG3, causing inefficient production in transformant cells.

It was decided that *onx4*, corresponding to P0504H10.9, encoded α-xylosidase by analysing substrate specificity of rONX4, which hydrolysed α-xylosides but not α-glucosides (Table 3). In our previous study (*6*), the temporal change of α-xylosidase activity in the ripening and the germinating seeds was well synchronized with that of *onx4* mRNA. Additionally, the transcriptional signal of *ong4* was strongly found in the ripening seeds of 8 days after flowering, during which obvious α-glucosidase activity was not detected. These data support that *onx4*-cording protein is an α-xylosidase. α-Xylosidase encoded in rice genome was found for the first time.

The rONX4 exhibited the extremely high activity towards XGOS (Table 3). Nasturtium α-xylosidase, which displayed the substrate specificity similar to rONX4, specifically catalysed the liberation of xylosyl residue, attached to the backbone glucosyl residue situated farthest from the reducing end of XGOS (*17*). Cabbage (*16*), pea (*19*) and soybean (*21*) α-xylosidases were also reported to exhibit the high specificity towards XGOG, suggesting that plant α-xylosidases are important

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enzymes to remove xylose unit from XGOS in relation to the xyloglucan metabolism of type I primary cell wall. Xyloglucan consists of β-1,4-glucan backbone that carries α-Xyl*p*-, β-Gal*p*-(1→2)-α-Xyl*p*- and α-Fuc*p*-(1→2)-β-Gal*p*-(1→2)-α-Xyl*p*- side chains attached to the OH-6 of β-glucosyl residues of the main chain (*23*). In the metabolism of xyloglucan, endo-enzymes attack this polysaccharide to form XGOS, followed by digestion of XGOS with several exo-enzymes. But, β-glucosidase cannot hydrolyse XGOS having Xyl residue at its non-reducing end, unless α-xylosidase removes this Xyl moiety. ONX4 may play an essential role in cell wall degradation at the ripening and germinating stages of rice seeds, both of which are the important growing duration for rice.

ONX4 mRNA and α-xylosidase activity appeared at 3 h after imbibition (*6*), meaning that the germination is active, even before the expression of α-amylase. High energy is required at this stage. This also supports that ONG2 is a key enzyme physiologically to degrade of starch granules in the initial germinating stage, that is, ONG2 may supply the energy (glucose) by the one-step reaction, a direct hydrolysis of the storage polysaccharide (starch granules) at that stage.

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