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# Pullulanase from rice endosperm

Yoshiki Yamasaki✉, Susumu Nakashima and Haruyoshi Konno

*Research Institute for Bioresources, Okayama University, Kurashiki-shi, Okayama, Japan*

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**Pullulanase (EC 3.2.1.41) in non-germinating seeds was compared with that in germinating seeds. Moreover, pullulanase from the endosperm of rice (*Oryza sativa* L., cv. *Hinohikari*) seeds was isolated and its properties investigated. The pI value of pullulanase from seeds after 8 days of germination was almost equal to that from non-germinating seeds, which shows that these two enzymes are the same protein. Therefore, the same pullulanase may play roles in both starch synthesis during ripening and starch degradation during germination in rice seeds. The enzyme was isolated by a procedure that included ammonium sulfate fractionation, DEAE-cellulofine column chromatography, preparative isoelectric focusing, and preparative disc gel electrophoresis. The enzyme was homogeneous by SDS/PAGE. The molecular weight of the enzyme was estimated to be 100 000 based on its mobility on SDS/PAGE and 105 000 based on gel filtration with TSKgel super SW 3000, which showed that it was composed of a single unit. The isoelectric point of the enzyme was 4.7. The enzyme was strongly inhibited by β-cyclodextrin. The enzyme was not activated by thiol reagents such as dithiothreitol, 2-mercaptoethanol or glutathione. The enzyme most preferably hydrolyzed pullulan and liberated only maltotriose. The pullulan hydrolysis was strongly inhibited by the substrate at a concentration higher than 0.1%. The degree of inhibition increased with an increase in the concentration of pullulan. However, the enzyme hydrolyzed amylopectin, soluble starch and β-limit dextrin more rapidly as their concentrations increased. The enzyme exhibited α-glucosyltransfer activity and produced an α-1,6-linked compound of two maltotriose molecules from pullulan.**

**Keywords**: endosperm, glucosyl transfer, pullulanase, rice (*Oryza sativa* L. cv. *Hinohikari*), thiol reagent

## INTRODUCTION

Pullulanase (EC 3.2.1.41) is distributed in plants and bacteria. The enzyme in plants readily cleaves the α-1,6-glucosidic linkage in pullulan, β-limit dextrin, and α-limit dextrin, while it can debranch amylopectin to a lesser extent than polyglucans (Drummond *et al.*, 1970; Lee *et al.*, 1971; Dunn *et al.*, 1973; Gordon *et al.*, 1975; Yamada & Izawa 1979; Okita & Preiss, 1980; Li *et al.*, 1992). Therefore, it has been suggested that pullulanase in plants is necessary for starch degradation in conjunction with α-β-amylases. However, it has also been suggested that pullulanase may be necessary for starch synthesis, where it may play an essential role in determining the fine structure of the amylopectin molecule (Pan & Nelson, 1984; James *et al.*, 1995; Mouille *et al.*, 1996; Nakamura *et al.*, 1996; 1997; Kubo *et al.*, 1999).

On the other hand, remarkable pullulanase activity is detected in the endosperm of rice seeds. This enzyme may play a role in starch synthesis during ripening of starch and remain in the seed. However, pullulanase activity increased during germination of the rice seeds, while the starch content of seeds was markedly decreased.

We attempted to examine the difference between the pullulanase in non-germinating seeds and that in germinating seeds.

✉Corresponding author: Yoshiki Yamasaki, Research Institute for Bioresources, Okayama University, Kurashiki-shi, Okayama 710-0046, Japan; e-mail: yosikiy@rib.okayama-u.ac.jp

**Abbreviations**: DEAE, diethylaminoethyl; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Moreover, we isolated pullulanase from the endosperm of rice seeds and investigated its properties.

## EXPERIMENTAL PROCEDURE

**Enzyme assay.** Pullulanase activity was determined as follows. The reaction mixture containing 1 mg of pullulan and enzyme solution in 0.5 ml of 50 mM NaOAc buffer, pH 6.0, was incubated at 37°C for 30 min. After incubation, the reaction was stopped by boiling the mixture for 5 min. The amount of maltotriose formed was measured according to the method of Somogyi (1952).

**Determination of protein.** Protein was determined by the method of Warburg and Christian (1942). The protein profiles from column chromatography were determined by measuring the absorbance of the eluates at 280 nm.

**Electrophoresis.** SDS/PAGE was performed on 7.5% gels according to the method of Laemmli (1970). After the run, the gels were stained for protein with Phast Blue R (R-350).

**Estimation of molecular weight.** The $M_r$ of the purified enzyme was estimated by SDS/PAGE (Laemmli, 1970) and gel filtration using HPLC. Gel filtration was performed on a column (0.46×60 cm) of TSKgel Super SW 3000: mobile phase, 50 mM phosphate buffer, pH 7.0, flow rate, 0.35 ml/min, temperature, 25°C, detection, UV (280 nm).

**Estimation of the isoelectric point.** The isoelectric point of the enzyme was determined by electrofocusing (Yamasaki *et al.*, 2007).

**High-performance liquid chromatography.** The products formed from substrates treated with pullulanase were determined by HPLC using a Tosoh HPLC System 8020 (Tosoh Co., Tokyo, Japan). Chromatography was performed on a column (0.46×15 cm) of Cosmosil 5NH2-MS eluted with 60% acetonitrile: flow rate, 1.0 ml/min, temperature, 30°C, detection, RI (Cosmosil Chromatogram Index 2003; Nacalai Tesque Inc., Kyoto, Japan).

## RESULTS AND DISCUSSION

### Pullulanase activity of endosperm rice seeds

Rice seeds showed remarkable pullulan- and soluble starch-hydrolyzing activities. Both of these activities in seeds were also found in the polished rice that had been threshed by a threshing machine (not shown). After 8 days of germination, the activity of pullulanase increased to three times that in non-germinating seeds, which shows that the enzyme will play a role in starch digestion (Fig. 1). The pI value of pullulanase in seeds after 8 days of germination was almost equal to that in non-germinating seeds (Fig. 1). This shows that the two enzymes are the same protein. Therefore, pullulanase will play a role in starch synthesis during the ripening of starch and remain in the seed. The same enzyme will play a role in starch degradation during germination.

### Isolation of pullulanase

Rice (*Oryza sativa* L., cv. *Hinohikari*) seeds were homogenized in a homogenizer (Nissei Excel Auto-Homogenizer; Nihonseiki Co., Tokyo, Japan) with 25 mM NaOAc buffer, pH 5.3, containing 0.5 M NaCl and 5 mM mercaptoethanol. The supernatant was brought to 0.9 satn. with $(NH_4)_2SO_4$. After dissolving the precipitate in 25 mM NaOAc buffer, pH 5.3, the enzyme solution was dialysed against 25 mM Tris/HCl buffer, pH 8.0, and the dialysate was applied to a DEAE-cellulofine column (2×18 cm). After the column was washed with 25 mM Tris/HCl buffer, pH 8.0, a linear gradient of 0–1 M NaCl in the buffer was applied. The fraction that exhibited pullulanase activity was subjected to preparative

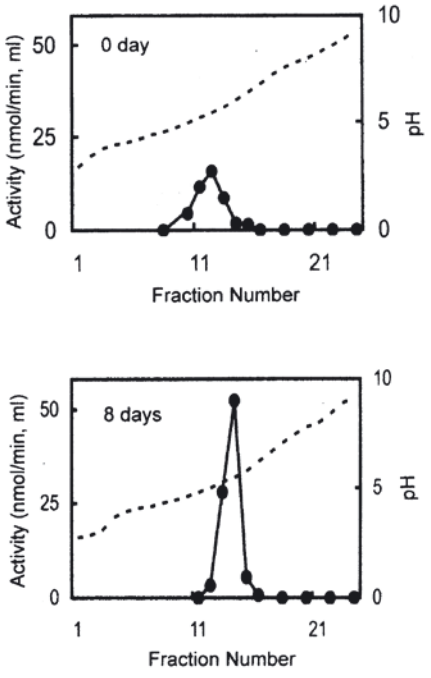


**Figure 1. Isoelectric focusing of crude extracts of non-germinating seeds (upper figure) and germinating seeds (lower figure).**
Crude extracts of non-germinating seeds and germinating seeds after 8 days were subjected to isoelectric focusing (pH 3.5–10). After 24 h at 80 V, the two gels were cut into 3-mm sections and each section was crushed in 2 ml of deionized water. The fractions were measured to determine pullulan-hydrolyzing activities and pH. ●, Pullulan-hydrolyzing activity, which maltotriose liberated from pullulan was measured by the method of Somogyi (1952); - - -, pH.

**Table 1. Substrate specificity of the purified pullulanase.**

Substrates, 1 mg in 0.5 ml of 50 mM NaOAc buffer, pH 6.0, were incubated with the purified enzymes at 37°C for 30 min.Malto-oligosaccharide liberated was determined by the method of Somogyi (1952) as described in Experimental Procedure.

| | Relative rate of hydrolysis (%) | $K_m$ value ($mg \times ml^{-1}$) | $V_{max}$ ($nmol \times ml^{-1} \times sec^{-1}$) | $k_{cat} \times s^{-1}$ | $k_{cat}/K_m \times s^{-1}$ |
|---|---|---|---|---|---|
| Pullulan | 100.00 | 0.625 | 0.328 | 23.1 | 70.4 |
| Amylopectin | 34.1 | 1.538 | 0.031 | 2.18 | 1.42 |
| Soluble starch | 15.8 | 1.538 | 0.041 | 2.89 | 1.88 |
| Glycogen | 6.3 | | | | |
| β-Limit dextrin | 19.5 | 0.909 | 0.047 | 3.31 | 3.64 |

isoelectric focusing (IEF) (pH range, 3.5–10) using an HSI GT Tube Gel Electrophoresis Unit (Hoefer Scientific Instruments, CA, USA). Electrophoresis was performed at 100 V for 20 h. The enzyme solution was further purified by preparative disc gel electrophoresis, pH 9.0, using an HSI GT Tube Gel Electrophoresis Unit. Electrophoresis was performed at a constant current of 25 mA for 4 h. The enzyme solution was applied to a Vivapure Maxi D/Q spin column and the column was eluted with 25 mM Tris/HCl buffer, pH 8.0, containing 1 M NaCl. These procedures led to 37-fold purification with 10.1% recovery. The purified enzyme was homogenous by SDS/PAGE (Fig. 2).

**General properties**

The molecular weight of the enzyme was estimated to be 100000 based on its mobility on SDS/PAGE and 105000 based on gel filtration with TSKgel super SW 3000, which showed that it was composed of a single unit. Pullulanases from rice (Iwaki & Fuwa, 1981), sugar-beet (Li *et al.*, 1992), spinach (Ludwig *et al.*, 1984; Schindler *et al.*, 2001) and millet (Yamasaki *et al.*, 2007) are also single peptides. The isoelectric point of the enzyme was determined to be 4.7 by analytical IEF.

The optimum pH of the enzyme was determined to be 6.0. After 20 h of preincubation at 30°C with 50 mM McIlvaine's buffer, the enzyme was stable in a pH range of 5.5–8.0. The optimum temperature for the enzyme activity was 55°C after 15 min of incubation. After 15 min of preincubation with 50 mM NaOAc buffer, pH 6.0, at various temperatures, the enzyme was found to be stable at temperatures up to 50°C.

When the enzyme was preincubated in 50 mM NaOAc buffer, pH 6.0, containing 5 mM metal ions at 37°C for 30 min, $Cu^{2+}$, $Hg^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Mn^{2+}$ reduced enzyme activity by 60% or more.

β-Cyclodextrin strongly inhibited the enzyme activity in a non-competitive fashion. This inhibition of the enzyme is similar to those of rice (Iwaki & Fuwa, 1981), spinach (Ludwig *et al.*, 1984) and sugar-beet (Li *et al.*, 1992). The enzyme was not activated by thiol reagents such as dithiothreitol, 2-mer-

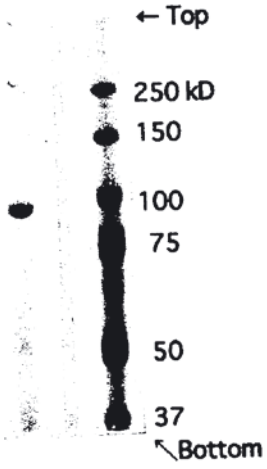


**Figure 2. SDS/PAGE of purified rice pullulanase.**
SDS/PAGE was carried out as described in the Experimental Procedure. Left, pullulanase; right, Precision Plus Protein Standards (Dual Color, Bio-Rad).

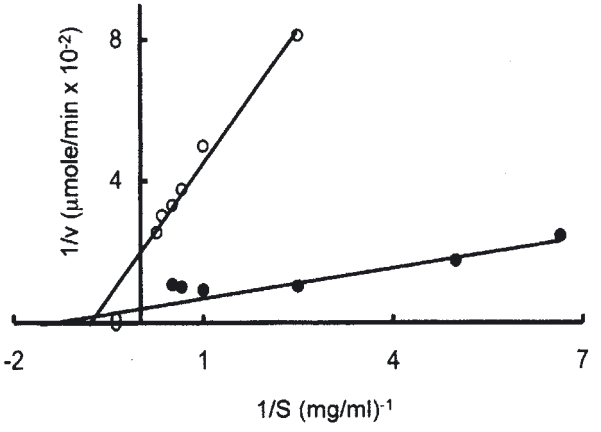


**Figure 3. Lineweaver-Burk plots for hydrolysis of pullulan and amylopectin by rice pullulanase.**
The reaction mixture containing pullulan, amylopectin and enzyme solution in 0.5 ml of 50 mM NaOAc buffer, pH 6.0, was incubated at 37°C for 30 min. (●), Pullulan; (○), amylopectin.

captoethanol and glutathione (not shown), although their thiols activated pullulanases from leaves of sugar-beet (Li *et al.*, 1992) and spinach (Schindler *et al.*, 2001).

**Substrate specificity**

Various substrates were incubated with the enzyme under standard conditions (Table 1). The enzyme most preferably hydrolyzed pullulan and liberated only maltotriose. However, pullulan hydrolysis was inhibited at a concentration higher than 0.1% (Fig. 3). The degree of inhibition increased with an increase in the concentration of pullulan. On the other hand, the enzyme also readily hydrolyzed β-limit dextrin, soluble starch and amylopectin, and hydrolyzed glycogen weakly. When the enzyme was incubated with amylopectin, the enzyme hydrolyzed amylopectin more rapidly as the concentration increased (Fig. 3). The same results were also obtained with soluble starch and β-limit dextrin (not shown).

***α*-Glucosyl transfer action**

When the enzyme reacted with pullulan, unknown substances were produced. The reaction mixture was lyophilized and dissolved in a small amount of water. The solution was applied to a column (1.6×93 cm) of Bio-Gel P2 and eluted with 20 mM EDTA. The main substance (X) was eluted between maltoheptaose and maltopentaose. X was eluted between maltohexaose and maltoheptaose by HPLC and completely hydrolyzed by purified pullulanase to liberate only maltotriose. Therefore, we concluded that X should be Glcp α1→4 Glcp α1→4 Glcp α1→6 Glcp α1→4 Glcp α1→4 Glcp, which is an α-1,6-linked compound of two maltotriose molecules.

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