Purification and Characterization of Polygalacturonase from Polished Rice

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Two isoforms of polygalacturonase (PG, E. C. 3. 2. 1. 15), namely, PG-1 and PG-2, were purified from the endosperm of polished rice, (*Oryza sativa* cv. Koshihikari, the most extensively produced rice variety in Japan). PBE 94 chromatofocusing indicated *p*Is of 5.6 and 5.1 for PG-1 and PG-2, respectively. These enzymes had same molecular weights approx. 67 kDa and consisted of a monomer. PG-1 and PG-2 exhibited optimum activities at pHs 5.0 and 4.5, respectively, and optimum temperatures of 60°C. Furthermore, these were stable at near-neutral pHs and retained 40% of their activities at 70°C. These results suggest that PG-1 and PG-2 are activated when rice is being cooked, and that the amount of pectin that combined with cells decreased by approximately 40%. Moreover, purified polygalacturonases decomposed pectin during rice cooking. It is surmised that the two PGs in the rice endosperms, decompose the pectin during rice cooking, thereby loosening the intercellular cement that gives firmness to plant tissues, and affecting the texture of cooked rice.

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Pectin and related substances are components of the cell membrane or cell filling substances that protect cells in higher plants, such as fruit and vegetables. Pectin acts as an intercellular cement that gives firmness to plant tissues¹⁾. The role of pectin is binding cell and holding water in tissue, etc²⁾. Pectin decomposition effects on the viscosity and texture of perishable foods or processed products, such as fruit and vegetables³⁾. At the cell wall level, this is of great significance leading to textural softening and the loosening of cell structures in fruit and vegetables.

Pectin is an acidic polysaccharide mainly composed of polygalacturonic acid in which D-galacturonic acids are connected in a straight chain by α -1, 4 combinations. The carboxyl group of galacturonic acid is partially etherified by a methyl group. It exists as a matrix component around and among the fibrillar components of the cell wall^{4),5)}.

Thus, it is expected that polygalacturonase (PG, E. C. 3. 2. 1. 15) and pectinesterase (E. C. 3. 1. 1. 11) mainly participate in pectin decomposition⁶⁾. PG, an important pectolytic glycanase, is a primary enzyme that plays a significant role in pectin dissolution *in*

vivo. It catalyzes the hydrolytic decomposition of α (1→4) galacturonan linkages of pectin. PE hydrolyzes the methyl ester bond of pectin to give pectic acid and methanol. PG has been reported in avocados⁷⁾, bananas⁸⁾, peaches⁹⁾, pears¹⁰⁾, rice shoots¹¹⁾, strawberries¹²⁾, tomatoes¹³⁾, mangoes¹⁴⁾ and other fruits. However, no polygalacturonase has been reported in cereals.

The role of PG in fruit ripening has been studied most extensively in tomatoes¹⁵⁾. The PG antisense gene was introduced into the tomato, and a tomato variety with controlled pectin decomposition has been developed^{15),16)}. PG gene expression has been studied in many other fruits including peaches¹⁷⁾, melons¹⁸⁾, apples¹⁹⁾, avocados²⁰⁾, kiwifruit²¹⁾, strawberries²²⁾, and bananas²³⁾.

As a cereal food, it is thought that the hardness of rice is affected by reduced degree of combination between cells. That is, rice texture is influenced by the decomposition and solubilization of pectin, which combines the cells during rice cooking. Therefore, when endogenous polygalacturonase decomposes pectin during rice cooking, it is considered that rice texture changes.

In this study, we purified and characterized

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polygalacturonase from polished nonglutinous rice and clarify its effect on rice hardness.

Materials and Methods

Unpolished rice grains of the Koshihikari variety produced in Chiba, Japan were used in this study. This variety is of the Japonica type. Its production is approximately 3,361,000 t (approx. 40% of rates of the total national crop yield) in Japan in 2006²⁴⁾.

1. Pectin extraction

Polished rice and cooked rice were homogenized in two volumes of 0.5% ammonium oxalate. After continuous shaking for 1 hr, the precipitate was removed by centrifugation at $18,000 \times g$ for 30 min. Precipitation was carried out again. The supernatant was collected. Alpha-amylase (10,000U, Sigma-Aldrich Co.) was mixed with the supernatant and incubated at 37°C for 12 hr. The mixture was added to 4 volumes of 99.5% ethanol and the resulting mixture was centrifuged at $18,000 \times g$ for 30 min. The precipitate was then collected and added to 80% ethanol and the mixture was centrifuged at $18,000 \times g$ for 30 min. This step was 6 times. The fraction obtained was considered as part of the pectin fraction. Pectin was measured by the *m*-hydroxydiphenyl method²⁵⁾. What deducted the amount of decrease in the pectin content of the polished rice and cooked rice was denoted the amount of pectin reduction during cooking.

2. Enzyme assay

Polygalacturonase activity was assayed by measuring the amount of reducing sugars released. A standard assay was carried out by incubating 0.1 ml of enzyme solution with 0.25 ml of 0.5% pectic acid as a substrate and 0.15 ml of 0.2 M McIlvaine buffer (pH 4.5) at 50°C for 60 min. After incubation, decomposed pectic acids were quantified as residual sugars by the method of Somogyi-Nelson^{26), 27)}, using galacturonic acid as the standard. One unit (U) of enzyme activity was defined as the amount of 1 μ M of reducing sugars formed per minute under standard assay conditions.

3. Protein determination

Total protein content was determined following Bradford's assay²⁸⁾ using Coomassie Brilliant Blue G-250 as the reactant and bovine serum albumin as the standard protein. The protein content in the chromatographic eluent fraction was estimated from the UV absorbance at 280nm.

4. Enzyme purification

All procedures were carried out at 4 °C unless otherwise specified.

5. Preparation of crude extract

Brown rice grains were polished with a compact home rice-polishing machine (MR-D 500, Twinbird Co.) ; 10% (w/w) of the surface was removed. The polished rice grains were added to 1.8 volumes of H₂O, and the resulting mixture was incubated for 1 hr and homogenized. The homogenate was shaken in a reciprocal shaker (100 rpm) for 1 hr. Then the precipitate was removed by centrifugation at $18,000 \times g$ for 30 min. The supernatant was collected and ammonium sulfate was added to 50% saturation. The supernatant was again collected by centrifugation at $18,000 \times g$ for 30 min, and ammonium sulfate was added to 80% saturation. The precipitate obtained was resuspended in 0.02 M McIlvaine buffer (pH 4) containing 0.01 M KCl and dialyzed against the same buffer. The dialyzed sample was centrifuged at $18,000 \times g$ for 30 min to remove precipitates.

6. Ion exchange chromatography

The crude extract was loaded on a CM Sepharose fast flow column (GE Healthcare) equilibrated in 0.02 M McIlvaine buffer (pH 4.0). The column was rinsed with same buffer and bound proteins were eluted with a stepwise gradient of 0, 0.1 and 1.0 M NaCl in the same buffer. Fractions having polygalacturonase activity were pooled, dialyzed against 0.025 M histidine-HCl buffer (pH 6.2) and concentrated using Amicon Ultra-15 10,000 NMWL (Millipore Co.).

7. Polybuffer exchanger 94 (PBE 94)

The enzyme obtained after the CM Sepharose chromatography was loaded on a Polybuffer exchanger 94 (PBE 94) (GE Healthcare) equilibrated in 0.025 M histidine-HCl buffer (pH 6.2). Proteins were eluted with Polybuffer 74 (GE Healthcare)-HCl (pH 4.0). The active fractions were concentrated.

8. Superdex 200 10/300 GL

The enzyme obtained after the chromatofocusing chromatography was loaded on a Superdex 200 10/300 (GE Healthcare) equilibrated in 0.02 M McIlvaine buffer (pH 5.0) containing 0.15 M NaCl. Elution was carried out in the same buffer. The active fractions were pooled and dialyzed against 0.02 M McIlvaine buffer (pH 3.5).

9. Secondary-ion exchange chromatography

The enzyme obtained after the gel filtration was

loaded on Mono S (GE Healthcare) equilibrated in 0.02M Mcllvaine buffer (pH 3.5). The column was washed with the same buffer, and bound proteins were eluted with a gradient of 0 to 0.17M NaCl and a stepwise gradient of 1.0M NaCl in the same buffer above. The active fractions were pooled.

10. Electrophoresis

SDS-PAGE was carried out by the method of Laemmli on a 7.5% slab gel⁽²⁹⁾. Precision Plus Protein™ Standards (Bio-Rad Co.) were used as the molecular standard proteins. Gel staining was performed with silver nitrate using a Silver Stain II kit (Wako Co.).

11. Effects of temperature and pH

The optimum pH of the enzyme's activity was determined at 50°C using 0.06M Mcllvaine buffer (pHs 3 to 8).

The pH stability of the enzyme was determined by preincubating the enzyme (3 µg/ml) in 0.06M Mcllvaine buffer (from pHs 3 to 8 in 0.5 steps) at 4°C in absence of the substrate. Aliquots were drawn at regular intervals and the enzyme's activity was determined at pH 4.5.

The optimum temperature of the enzyme's activity was determined at 30 ~ 70°C in 0.06 M Mcllvaine buffer (pH 4.5).

Temperature stability determined by preincubating the enzyme (3 µg/ml) in the absence of the substrate at 30 ~ 70°C in the above buffer. Aliquots were drawn at regular intervals and assayed under optimum conditions (50°C and pH 4.5).

12. Effects of inhibitors/metal ions

The enzyme (3 µg/ml) was preincubated at 4°C for 30 min in the presence of iodoacetic acid, *p*-chloromercuribenzoate (*p*-CMB), 2-mercaptoethanol (2-Me), dithiothreitol (DTT) and ethylene diamine tetraacetic acid (EDTA), respectively.

The effect of metal ions was determined by preincubating the enzyme in the assay buffer at 4°C for 30 min in the presence of Na⁺, K⁺, Mg²⁺, Ca²⁺, Cu²⁺, Sn²⁺ and Hg²⁺. Residual enzyme activity was assayed at 50°C after the addition of the substrate.

13. Decomposition of pectin and pectic acid by polygalacturonase in rice during cooking

The enzyme (3 µg/ml) was mixed with pectin or pectic acid and incubated under the same conditions as those in cooking rice with an electric rice cooker (National SR-3100). After incubation, the amount of decomposed substrate was quantified as residual

sugars by the method of Somogyi-Nelson^{(26), (27)}.

Results and Discussion

1. Decomposition of pectin in rice during cooking

The polished rice and cooked rice had pectin contents of 239.2 ± 34.3 mg/1 kg dry weight and 145.2 ± 22.0 mg/1 kg dry weight (Table 1). The pectin content of rice decreased by about 39.3% after cooking. Therefore, it was surmised that pectin becomes soluble with heating and that endogenous polygalacturonase decomposes pectin in rice during cooking. In mangoes, with progressive ripening and textural softening, the total pectin content decreased from 2.0 to 0.7% fresh weight⁽³⁰⁾. In fruit or vegetables, when polygalacturonase degrades pectin, softening. However, in cereals like rice, such a phenomenon has not been observed yet and it remains unknown whether pectin decomposition clearly leads to change in taste or structure. Our results show that rice pectin decomposed during cooking, as observed in fruit and vegetable. Hence, the polygalacturonase action in rice, particularly during cooking was examined.

Table 1 Percent decomposition of pectin in rice during cooking

Pectin (mg/1 kg dry weight)		Decomposition (%)
Polished rice	Cooked rice	
239.2 ± 34.3	145.2 ± 22.0	39.3

2. Purification of polygalacturonase of polished rice

On CM sepharose, the bound protein was eluted with a stepwise gradient of 0, 0.1 and 1.0M NaCl and the enzyme was eluted in 0.1M NaCl. One activity peak was obtained. The peak fractions from 31 to 35 possessing higher specific activities were used for further purification. The enzyme was further purified on a PBE 94 chromatofocusing column and the PGs were divided into two active fractions, PG-1 and PG-2, with relative abundances of 79% and 21%, respectively. The elution pHs of these two PGs were approximately 5.6 (PG-1) and 5.1 (PG-2). These two active fractions were chromatographed by gel filtration on Superdex 200 10/30 GL. A comparison of the PG-1 and PG-2 elution volumes on gel filtration with standard molecular weight markers indicated molecular weights of approximate 68 kDa, similar to those

Table 2 Purification of polygalacturonase

Procedure	Total activity ($\times 10^{-3}$ U)	Total protein (mg)	*Specific activity	Activity yield(%)
Crude extract	1,958	3.14×10^3	6.2	100.0
Ammonium Sulphate Sat. 45–80%	1,400	1.09×10^3	12.8	71.5
CM Sepharose	780	2.70×10^1	286.9	39.8
PBE 94				
PG–1	336	1.32×10^0	2,554.3	17.2
PG–2	81	1.87×10^0	434.2	4.1
Superdex 200 10/300 GL				
PG–1	113	7.79×10^{-2}	14,511.2	5.8
PG–2	61	4.73×10^{-2}	12,892.6	3.1
Mono S				
PG–1	12	7.51×10^{-3}	15,978.4	0.6
PG–2	8	7.27×10^{-3}	11,009.2	0.4

*Specific activity is expressed as $\times 10^{-3}$ U per mg of protein.

(Rice : fresh weight 1.0 kg)

reported for PGs from peaches (68kDa)⁹⁾ and rice shoots (63kDa)¹¹⁾. Each active fraction was purified by secondary-ion exchange chromatography on a Mono S column. Both PG-1 and PG-2 bound to the column and were eluted using approximately 0.05 M NaCl with linear gradients.

Purified PG-1 had a maximum activity of 16.0U/mg protein, corresponding to an increase of at least 2563-fold and an overall yield (from five purification runs) of at least 0.6%, and PG-2 had a maximum activity of 11.0U/mgprotein, an increase of at least 1766 fold and a yield (from five purification runs) of at least 0.4%, compared with the crude extract (Table 2). On SDS-PAGE after treatment with SDS and 2-mercaptoethanol, both PG-1 and PG-2 resolved into a single band with a molecular weight of approximate 67kDa (Fig.1), similarly to that reported for PG from rice shoots (63kDa)¹¹⁾. This molecular weight is slightly than that of PGs from plant sources reported to date.

The purified polygalacturonase from polished rice revealed two isoforms (PG-1 and PG-2). These PG isoforms have been purified from very few fruits, such as a tomatoes^{31), 32)}, bananas³³⁾ and mangoes¹⁴⁾.

It was suggested that both PG-1 and PG-2 from polished rice are monomers of the enzyme. The PG-2 monomer has been reported in tomatoes^{31), 32)}, and rice shoots¹¹⁾ among others. In contrast, the PG-3 and PG-1 have been reported in bananas and tomatoes, respectively; these are composed of subunits of glycoproteins^{8), 31), 32)}.

Fig. 1 SDS-PAGE pattern of purified polygalacturonase

M: standard molecular weight markers

Lane: purified polygalacturonase

3. pH and temperature optima and stabilities

Purified PG-1 from polished rice showed a pH stability in the range of 4.5 ~ 7.0 (the relative activity > 80%), with a pH optimum of pH 5.0. Purified PG-2 showed a pH stability in the range of 4.0 ~ 6.5, with a pH optimum of 4.5. These enzymes resembled those in peaches, apples, potatoes, tomatoes, rice shoots and strawberries in pH optimum, which has a range of 4.0-5.5^{9), 11), 12), 34), 35)}. The pH stability of these enzymes from polished rice was comparatively a broad acidic range, similarly to those of polygalactronase from peaches and mangoes (PG-1)^{9), 14)}. The activities of PG-1 and PG-2 from polished rice observed over a wide range of pHs is important in rice cooking. This result indicates that the polygalacturonase of polished rice is activated when rice is cooked.

Both PG-1 and PG-2 showed optimum activities

at 60°C. The temperature optima of polygalacturonase from rice shoots and strawberry have been reported^{(11), (12)}. PG-1 and PG-2 retained 40% of their activities at 70°C for 30 min (Fig. 7). There is as yet no report on polygalacturonase that is stable at such a high temperature. The temperature optima of polygalacturonase from various fruits were found to be near 40°C and inactivated at 60°C^{(14), (33)}. It was thought that polygalacturonase from polished rice had thermostability comparable with other sources. Thus, it is thought that it is activated by rice cooking since this enzyme's activity remains even near 70°C. These results suggest that polygalacturonase affects rice texture by decomposing pectin in the endosperm during rice cooking.

4. Effects of inhibitors/activators

The sulfhydryl binding reagents iodoacetamide and *p*-CMB inhibited PG-1 and PG-2 in a concentration-dependent manner. *p*-CMB at 10 mM and iodoacetamide showed a percent inhibition of 86.9 for PG-2 (Table 3). On the other hand, the sulfhydryl agents DTT and 2-Me at 1 mM stimulated PG-1 activity by approximately 28.4 and 16.2%, respectively; further increase in their concentration to 10 mM resulted in activation decreases by 59.1 and 14.5%, respectively; PG-2 resulted in activation decreases at percentages higher than these. These results show that PG-1

and PG-2 concentrations depend on the presence of sulfhydryl binding reagents. The activation of the enzyme preparation with the incorporation of sulfhydryl agents lends support to the need for sulfhydryl groups in the functioning of the enzyme. PG-1 activity was influenced by the sulfhydryl agents in the assay system. Therefore, PG-1 appears to be a sulfhydryl-activated enzyme. Similarly, PG-2 is considered to harbor SH groups in its active center. This has also been observed in bananas⁽⁸⁾.

5. Effect of metal ions

The effects of metal ions and chelating reagents were determined after a 30 min incubation at 4°C (Table 3). Hg²⁺ strongly showed showing approximately 100% inhibitions. Heavy-metal ions such as Cu²⁺ and Sn²⁺ inhibited PG-1, however, PG-2 had little influence compared with PG-1. Mn²⁺ slightly inhibited only PG-1. A considerable stimulation of PG-2 activity by Ca²⁺ and Mg²⁺ at 1 mM was observed. No such stimulation of PG-1 activity was observed. Na⁺ and K⁺ at 1 mM stimulated PG-2 activity by about 34.5% and 20.7%, respectively. In PG-1, a maximum drop in activity of 50% of was observed. EDTA did not inhibit PG-1 or PG-2, suggesting that the metal ions are unnecessary for activity. The inhibition of polygalacturonase activities by Cu²⁺ may be due to the oxidation of sulfhydryl groups, indicating that the sulfhydryl groups play an important role in

Table 3 Effect of reagents and metal ions on Polygalacturonase

Reagent	PG - 1		PG - 2	
	Relative Activity(%)		Relative Activity(%)	
	1 mM	10 mM	1 m M	10mM
Control	100.0	100.0	100.0	100.0
Iodoacetic acid	103.9	82.9	92.8	86.8
<i>p</i> -CMB	85.1	20.5	91.8	13.1
2-Me	116.2	85.5	97.0	53.9
DTT	128.4	40.9	118.9	69.3
EDTA	131.5	99.6	110.0	89.8
Na ⁺	54.6	N.T.	134.5	N.T.
K ⁺	80.1	N.T.	120.7	N.T.
Ca ²⁺	117.7	N.T.	123.3	N.T.
Mg ²⁺	104.6	N.T.	156.0	N.T.
Cu ²⁺	88.2	N.T.	115.8	N.T.
Mn ²⁺	88.6	N.T.	102.2	N.T.
Sn ²⁺	59.6	N.T.	93.7	N.T.
Hg ²⁺	3.7	N.T.	9.4	N.T.

N.T. : Not Tested

their. This is possible the result of the sulfhydryl binding reagents and sulfhydryl agents. PG-1 and PG-2 in polygalacturonase isoforms from polished rice showed considerably different characteristics ; however, similar inhibitions have been reported for polygalacturonase from bananas and mangoes^{8),14)}.

6. Decomposition of pectin and pectic acid by polygalacturonase in rice during cooking

To confirm that purified polygalacturonase decomposes pectin and pectic acid during rice cooking, we conducted an experiment that entails changing the pH of standard assay to 7 and incubating the enzyme solution under the conditions as those for cooking rice with an electric rice cooker. In addition, those without enzyme addition are tested as control. The rate of decomposition of a substrate was computed from the amount of increase in the amount of reducing sugars. The curve showing the temperature change occurring in an electric rice cooker shows an increase from 20°C to 100°C for approximately 20 min. It is thought that pectin in the rice is decomposed by purified polygalacturonase 15 min after turning on the rice cooker. Consequently, 12.4% pectin and 37.7% pectic acid were decomposed. Thus, it was confirmed that purified polygalacturonase decomposed pectin, which binds the cells during cooking. The rate of decomposition of pectic acid was higher than that of pectin. The substrate specificity of the enzyme for pectin in rice may have been influenced by a short methoxyl group.

Conclusions

Generally, the taste of cooked Japanese rice is greatly influenced by the texture of the rice. Therefore, sensory evaluation has long been used in assessing rice quality. However, amylose and protein affect rice texture³⁶⁾. Furthermore, many studies of the action of the endogenous amylase of polished rice during cooking have been reported. For example, from the amylogram of milled rice flour, it was found that endogenous amylase decomposes endosperm starch and decreases the viscosity of rice paste^{37),38)}. Moreover, alpha amylase (E.C. 3. 2. 1. 1) and beta amylase (E.C. 3. 2. 1. 2) have been purified from polished rice, and these enzymes have produced oligosaccharides during rice cooking^{39)~41)}. Thus, endogenous amylase decomposes endosperm starch during rice cooking, thereby, giving rice to stickiness. Furthermore, research on the action of

endogenous enzymes and a genetic viewpoint of the rice taste has progressed⁴²⁾.

Thus, a study concerning the taste of cooked rice was carried out. In this study, the pectin content of rice decreased during cooking rice and polygalacturonase was found in the endosperm of rice. It is suggested from the characteristics of PG-1 and PG-2 that they maintain their activities even during rice cooking. Consequently, they induce the softening of the structure rice grains, thereby affecting the texture and taste of rice. PG-1 and PG-2 differ in their activities, but they both affect rice texture. By clarifying the association between rice variety and storage period, the relevance of polygalacturonase in rice to taste has been clarified. The function of polygalacturonase in fruit and vegetables ripening has been studied most extensively on plant physiology or ecology. But there are few reports on the view point of food science and this report is the significant in this field.

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精白米由来のポリガラクトクロナーゼの 精製と性状

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米胚乳に内在するポリガラクトクロナーゼ (PG) の炊飯中の作用を検討すると共に、本酵素を精製し一部の性状を明らかにした。細胞間の結着に関与するペクチン含量は、炊飯中に約40%減少した。米胚乳からPGを抽出・精製し、2つのアイソザイム (PG-1, PG-2) を確認した。分子量は約67,000, 等電点は5.6 (PG-1) と5.1 (PG-2) を示した。最適温度は60℃, 温度安定性は70℃で約40%の活性を保持していた。最適pHは5.0 (PG-1) と4.5 (PG-2) を示し、炊飯調理時のpH 7付近において約40%活性を保持していた。また、基質分解試験の結果、精製PGが炊飯条件でペクチンおよびペクチン酸を分解することを確認した。以上のことから、米胚乳中の2つのPGが炊飯過程で米のペクチンを分解し、細胞間の結着を緩め、米飯のテクスチャー形成に影響を与えることが推察される。

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