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RESEARCH ARTICLE

Presence of β-Amylase in Ramie Leaf and its Anti-staling Effect on Rice Cake

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Abstract Presence of β-amylase in ramie leaf and its anti-staling effect on starch-based foods were assessed. The ammonium sulfate fractionate (80% saturation) of the ramie leaf extracts showed a β-amylase activity, giving maltose (Glc2) as a major product, exclusively, when incubating with maltopentaose (Glc5) or soluble starch at 45°C, pH 6.0. The starch-based food product (rice cake) prepared with freeze-dried ramie leaf enzyme revealed that the linear maltooligosaccharides ranging from Glc2 to Glc6 significantly increased and the shorter branch chains (DP<15) of amylopectin increased whereas the longer branch chains (DP>16) decreased in the product. These results demonstrated that maltosyl residue was released from the non-reducing end of the longer branch chains of amylopectin by β-amylase. The ramie leaf-treatment sample significantly reduced the retrogradation rate during 48 h storage at 4°C. As an alternative plant-origin enzyme, the ramie leaf β-amylase has potential for a novel anti-staling additive.

Keywords: ramie leaf, plant-origin β-amylase, amylopectin, starch-based food, anti-staling additive

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Introduction

The β-amylases, found widely in plants and microorganisms, catalyze the hydrolysis of alpha-1, 4-linkage, releasing maltose from the non-reducing ends of glucans. The enzymes are well characterized in higher plants such as barley and wheat (1,2). In the food industry, the plant-origin β-amylase is preferred due to safety and high specificity compared to microbial sources. In particular, barley β-amylase has been most utilized in the food industry such as beer fermentation and starch conversion process. However, the plant β-amylase needs some alternative sources other than cereals or major crops which are used as staple foods for human nutrition. Recently, we found for the first time the presence of β-amylase in ramie leaves. Ramie leaves are major by-products from the textile industry and can be used for food products because of their nutritional characteristics (3,4).

Staling of starch-based foods such as bread and rice cake has been intensively investigated to reduce the high starch retrogradation rate during storage that leads to deterioration of quality and a short shelf life. To slow the retrogradation process, many attempts have led to development of various enzymes, emulsifiers, oligosaccharides, and polysaccharides. Of these, enzyme treatment is a promising way of modifying starch structure directly in which changes in molecular size, ratio of amylose to amylopectin, molecular weight and branch chain length distribution are introduced by the enzyme reaction (5).

The ramie leaf rice cake is a wellbeing food for the better health and nutritional effects that is free from coloring preservatives and other chemical additives. Recently, farmers added ramie leaves to rice cake in which the ramie leaves are blanched and ground with rice powder, made into dough (3,6). However, our preliminary study showed

the blanching process at farm-level destroyed the enzyme in the ramie leaves. To our knowledge, there was neither investigation on the enzymes present in the ramie leaf nor their utilization in food processing. Thus, the aims of this study were to characterize the ramie leaf β-amylase and to develop a novel technology using the enzyme as an anti-staling additive for starch-based foods.

Materials and Methods

Materials Rice and rice powder were purchased at a local market in Seoul, Korea. The ramie leaf was obtained from a ramie leaf farm in Hansan, Korea. Isoamylase from *Pseudomonas amyloderamosa* was purchased from Sigma-Aldrich (St. Louis, MO, USA), and all other chemicals used were of analytical grade. The ramie leaves after harvesting from the ramie farm were washed immediately to remove dust and kept at –20°C. The ramie leaves blanched by hot water (75-90°C) for 10 min were used as control. Both blanched and unblanched ramie leaves were freeze-dried, milled and kept in a desiccator for further purpose.

Characterization of β-amylase activity of ramie leaf Freeze-dried ramie leaf powder (50 g) was soaked in 50 mL of Tris-HCl 50 mM (pH 7.5) at room temperature for 1 h with shaking. The extracted solution was collected by centrifugation at 6,000×*g* for 30 min at 4°C and then precipitated by 80% ammonium sulfate saturation. The precipitate was re-dissolved in 50 mM Tris-HCl (pH 7.5). Soluble starch solution (0.5%) or maltopentaose solution (0.1%) was incubated with the precipitate in 50 mM Tris-HCl (pH 7.5) at 30°C. The sampling was carried out every 24 h for 72 h.

Preparation of rice cakes with ramie leaf Boiling water (60 mL) was slowly added to 100 g rice powder (80 mesh) and 1.5 g freeze dried ramie leaf powder were mixed with and kneaded for 5-10 min and then kept for 3 h at 45°C. Then the dough was steamed for 20 min and immediately freeze-dried or held at 4°C for further measurements.

Hardness measurements using a texture analyzer For assessment of the mechanical properties of the rice cake, we performed TPA using a Texture Analyzer (GU7 1YL; Stable Microsystem Ltd., Surrey, UK) with the aluminum probe (20×20 mm). Each rice cake was sliced (10×10×15 mm, l×d×l) and compressed to 40% of its original height. The test speed of Texture Analyzer was set to 2 mm/s.

Differential scanning calorimetry (DSC) The retrogradation of rice cake samples was measured with a differential scanning calorimeter (Seiko, Okuma, Japan) after 5-48 h storage at 4°C. The freeze-dried samples (20 mg) of each treatment were sealed in aluminum pans and then heated from 20 to 110°C at a rate of 10°C/min. The degree of retrogradation was expressed as the enthalpy calculated from the area of the endothermic peak between 40 and 80°C. For gelatinization degree analysis of the partially gelatinized dough the enthalpy was measured by DSC endotherm of dough after kneading with boiling water.

Determination of oligosaccharides, amylose and branch chain length distribution Branch chain length analysis was carried out using high performance anion exchange chromato-graphy (HPAEC) as reported previously (7). TLC analysis was conducted according to Robyt's method (8). The reaction mixture was developed on a Whatman K5F silica gel plate (Whatman, Maidstone, UK) with isopropanol/ethyl acetate/water (3:1:1, v/v/v). The amylose content of the rice cake was measured colorimetrically based on amylose-iodine complex formation, as previously described (9).

Results and Discussion

Characterization of crude β-amylase from ramie leaf extract A crude β-amylase from ramie leaf extract was obtained by precipitation with 80% saturation of $(NH_4)_2SO_4$. When the crude precipitate was reacted with Glc_5 at 45°C for 24-72 h, the reaction provided exclusively a pair product of Glc_2 (1) and Glc_3 (2) that is the typical products of β-amylase action, while Glc_5 gradually decreased with reaction time. Therefore, the action pattern appeared very similar to be β-amylase activity, but not α-amylase activity, because the hydrolysis products of Glc_5 should include a pair of Glc_1 and Glc_4 by α-amylase (10). Likewise, the reaction with soluble starch gave Glc_2 as major product and several other minor products such as Glc_3 (2) and maltodextrins (7,8) (Fig. 1A). The results revealed that the substrates, starch and Glc_5 were hydrolyzed to mainly produce maltose by β-amylase action present in ramie leaf. Additionally, minor reaction products of DPs>6 were shown in lane 1-3 (Fig. 1A). The results indicated that α-amylase was likely present in trace amount. The blanched samples barely hydrolyzed Glc_5 and soluble starch, showing that the blanching completely inactivated ramie leaf β-amylase. This also suggests that the conventional heat treatment process by farmers is not able to modify the rice starch due to a total loss of enzyme activity. The dense spot (9) at Glc_1 region appeared to include ramie leaf pigments (Fig. 1A). To obtain the reaction rate profile the ramie leaf

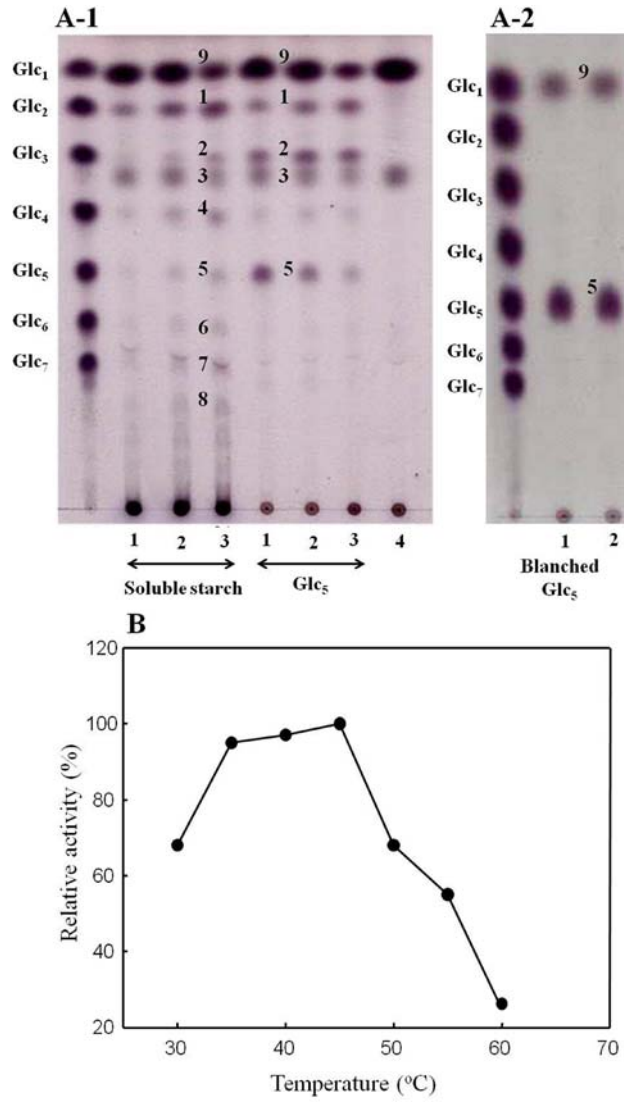

Fig. 1. (A) Thin-layer chromatographic analysis of the reaction of blanched (A-2) and unblanched ramie β-amylase with soluble starch and Glc5 (A-1). Thin-layer chromato-graphy was carried out according to Robyt's method. Lanes 1, 2, and 3 correspond to the following time points: 24, 48, and 72 h, respectively. Lane 4 shows the ramie leaf extract without substrate. **(B) The optimum temperature profile of ramie leaf β-amylase.** The ramie leaf precipitate was incubated with Glc5 for 1 h at various temperatures.

precipitate was incubated with maltopentaose for 1 h at various temperatures. The enzyme was active over a broad

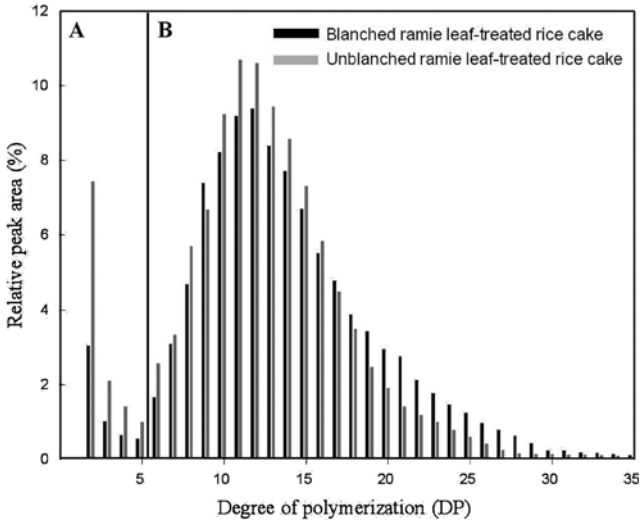

Fig. 2. Normalization of the peak area (%) of ramie leaf-treated rice cake starch. Before (A) and after (B) isoamylase treatment

range of temperature (30-55°C) showing the highest value at 45°C) (Fig. 1B).

Changes in amylose and maltooligosaccharides contents of ramie leaf-treated rice cake As shown in Table 1 and Fig. 1A, the ramie leaf treatment significantly produced more maltooligosaccharides than that of the control, providing the linear maltooligosaccharides ranging from 2 to 5 glucose units. Glc_2 produced by ramie leaf-treated rice cake was 0.39 mg/g, much more than 0.06-0.12 mg/g of Glc_3/Glc_5, demonstrating that Glc_2-Glc_5 were produced from amylose and amylopectin by the β-amylase reaction of ramie leaf during incubation (45°C for 3 h) and come-up time to the temperature of steaming. The ramie leaf treatment also lowered the amylose content. In the ramie leaf-treated rice cake the number of shorter branch chains (DP<15) increased whereas the number of longer branch chains (DP>16) decreased compared to those of control (Fig. 2). This demonstrated that maltosyl residue was released from the non-reducing end of the longer branch chains of amylopectin by ramie leaf β-amylase, resulting in the production of modified amylopectin with shorter branch chains.

Table 1. Contents of amylose and maltooligosaccharides in ramie leaf-treated rice cake

Treatment	Degree of gelatinization in dough (%)	Amylose (mg/g)	Maltooligosaccharides (mg/g)				
			Glc_1	Glc_2	Glc_3	Glc_4	Glc_5
Control							
Blanched ramie leaf	0	181	0.66	0.14	0.04	0.03	0.02
Ramie leaf-treated							
Unblanched ramie leaf	15	141	0.45	0.39	0.12	0.08	0.06

Table 2. Hardness (TPA) and retrogradation rate of ramie leaf-treated rice cake

Treatment	Storage at 4°C	
	0 h	48 h
	Hardness (g)	ΔH (mJ/mg)
Control		
Blanched ramie leaf	2,391.7	0.51
Ramie leaf-treated		
Unblanched ramie leaf	1,949.0	0.34

Textural properties and retrogradation of ramie leaf rice cake The textural properties of ramie leaf-treated rice cakes were evaluated using TPA analysis (Table 2). The hardness of the ramie leaf rice cake was lower than that of the control, demonstrating in the production of the rice cake with softer texture. The structural modification of amylopectin may attribute to the textural change. As shown in Table 2, the control sample had a higher retrogradation rate than that of the ramie leaf rice cake after 48 h storage at 45°C. The results revealed that the ramie leaf treatment effectively reduced the starch retrogradation during storage at 4°C. The essential mechanism by which starch-hydrolyzing enzymes retard starch retrogradation is not fully understood yet, because of the complexity of the process. However, many investigators suggested that the structure of the amylopectin may be an important factor in retrogradation (11,12). Additionally it is often suggested that the enzymes such as β-amylase modify amylopectin molecules in certain ways that reassociation of these molecules become less effective to increase matrix rigidity during storage (5). Juszczak *et al.* (13) showed that medium maltodextrin (DP=5) gave the strongest reduction in viscosity and maltodextrins in the range of DPs<4 and DPs>5 had a markedly smaller effect. In conclusion, the findings of this study suggest that the reduction in amylose content, rearrangement of amylopectin branch chains, and formation of maltooligosaccharides accompanied by β-amylase activity may synergistically affect the retardation of starch retrogradation.

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Disclosure The authors declare no conflict of interest.

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