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Treatment of ramie leaf β-amylase for preliminary purification

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Abstract The thermal properties of ramie leaf β-amylase (RBA) were examined to develop a novel process for enzyme purification. The thermostability of RBA extract prepared from ramie leaf powder was examined at various temperatures. RBA activity decreased slightly, whereas other carbohydrate-active enzymes, such as D-enzyme, were rapidly inactivated during 30 min incubation at 60°C. When the heat-treated extract was incubated with various substrates, maltose was produced exclusively as the major product, whereas the untreated crude extract produced maltose and other maltooligosaccharides. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, fewer protein bands were observed for the heat-treated extract than the untreated extract, indicating that the thermostable RBA was partially purified and other thermolabile enzymes were eliminated. Thus, the treatment of the RBA extract at 60°C for 30 min resulted in 5.4-fold purification with a recovery yield of 90%.

Keywords: plant β-amylase, enzyme purification, enzyme disproportionation, starch retrogradation, ramie leaf

Introduction

β-Amylase (EC 3.2.1.2, 1,4-α-D-glucan maltohydrolase) catalyzes the hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides to remove successive maltose units from the non-reducing ends of the chain. This enzyme acts on starch, glycogen, and oligosaccharides to produce β-maltose by inversion (1).

A series of carbohydrate-active enzymes in plants, including β-amylase, are involved in the sugar metabolic pathway, which is essential for plant growth (2). Among them, β-amylase plays a major role with the disproportionating enzyme (D-enzyme). The D-enzyme catalyzes transfer of maltooligosaccharides to the C-4 position of another glucan, thereby producing a series of elongated α-glucans in plant leaves, whereas β-amylase is involved in breakdown of the α-glucans and the release of maltose from the α-glucans for energy production (2-4).

β-Amylase has great potential for application in baking, brewing, and starch processing in various food and bio-industries, where it is used to produce maltose syrup and prevent retrogradation of rice cake confectionery. Plant-originating β-amylase is preferred due to its safety and high specificity compared with those of microbial sources (5,6). However, until now, the β-amylase source has been limited to several edible plants, such as barley, wheat, and soybean. Furthermore, the wide application of plant-derived enzymes requires a simple and easy purification process. However, purifying β-amylase from other enzymes and pigments in plants is usually tedious and requires a complex procedure (7-10). In contrast to conventional purification methods, heat treatment has benefits for producing industrial enzymes (11,12). Our preliminary study showed that ramie leaf β-amylase (RBA) was stable at 50-70°C, which is a higher temperature than that of other plant enzymes. In this study, we developed a heat treatment technique as a preliminary purification step to produce RBA.

Anti-staling enzymes are added to wheat flour before preparing dough or during mixing of dough in the baking industry, and they are activated at the temperature at which starch gelatinization occurs. As these enzymes are inactivated during the baking process due to the high temperature, the enzymes are not involved in excess starch hydrolysis (13). Farmers in many Asian countries add blanched ramie leaves to rice cake to stabilize the pigments. However, RBA retards the retrogradation of rice starch during storage (6). The blanching process has not been optimized or standardized based on β-amylase stability. In addition, a series of enzymes, such as chlorophyllase, polyphenol oxidase, and anthocyanin, which are degradation enzymes in plant tissues, initiate deterioration in quality during frozen storage (14-17). Thus, the quality-influencing enzymes must be inactivated to reduce these changes in foods containing vegetables. We also propose a model for farm-level heat treatment of ramie leaves to extend the application of RBA as an anti-staling reagent and for quality control.

Materials and Methods

Ramie leaves

Ramie leaves were obtained from a ramie leaf farm in South Korea. The ramie leaves were collected after harvesting, washed, stored at 20°C, and freeze-dried.

Enzyme extraction

Freeze-dried ramie leaf powder (50 g) was soaked in 1,000

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mL of 50 mM Tris-HCl (pH 7.5) at 4°C for 1-2 h with gentle shaking. The extracted solution was centrifuged at 6,000×*g* for 30 min at 4°C. The supernatant was used as the crude enzyme extract.

Heat treatment

The enzyme extract (20 mL) was heat-treated in a test tube pre-warmed in a water bath at various temperatures of 50-60°C. The heat-treated enzyme samples were removed at a particular time, transferred to an ice bath, and residual enzyme activity was determined.

Enzyme activity assay

β-Amylase activity was determined by the method of Bernfeld with slight modification (18). The β-amylase-containing sample (1.0 mL) was added to 1.5 mL of 1% (w/v) starch solution and incubated at 50°C for 5-24 h. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid solution, and the mixture was heated in boiling water for 5 min. After cooling to room temperature, 8 mL of distilled water was added, and absorbance was read at 540 nm using a spectrophotometer (Shimadzu Corp., Tokyo, Japan). The extract used as a blank control was inactivated by boiling for 10 min. The reducing sugar concentration was determined using a maltose standard curve.

Thin layer chromatography (TLC)

The TLC analysis was carried out using a Whatman K5F silica gel plate with isopropanol:ethyl acetate:water (3:1:1, v/v/v) as the solvent system to confirm maltose production by β-amylase (19). The TLC plate containing reaction products was developed twice, and the reducing sugars were detected using the naphthol-sulfuric acid (H_2SO_4) method. Each plate was thoroughly dried and dipped in a methanol solution containing 3 g of *N*-(1-naphthyl)ethylenediamine and 50 mL of concentrated H_2SO_4 per liter. The plates were dried and placed in an oven at 110°C for 10 min. Purple-black spots that appeared on a white background indicated the products.

Analysis of oligosaccharides by high-performance anion-exchange chromatography (HPAEC)

The enzyme reaction was carried out at 50°C using 0.2-2 mM of the G5-G7 substrates in a double volume of 0.1 N NaOH. The reacted samples were analyzed using an HPAEC system (Dionex-300; Dionex, Sunnyvale, CA, USA) with an electrochemical detector (ED40; Dionex), a CarboPac™ PA-1 anion-exchange column (250×4 mm; Dionex), and a guard column. The column was rst equilibrated with 150 mM NaOH. The sample was eluted with varied gradients of 0-600 mL of sodium acetate in 150 mM NaOH at a ow rate of 1 mL/min (20).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the literature by Laemmli with slight modification (21). The enzyme solution was filtered through a membrane filter (Falcon BD, Franklin Lakes, NJ, USA), mixed with an equal volume of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), boiled for 5 min, and applied to TGX acrylamide gel (Bio-Rad). Tris/glycine buffer containing 0.1% (w/v) SDS was used as the electrode buffer. Electrophoresis was carried out at 16 mA for the stacking gel and 18 mA for the separating gel.

Protein concentration

Protein concentrations were determined by the Bradford method (22) using bovine serum albumin as the standard.

Results and Discussion

Effect of temperature on RBA activity

Residual activity of the RBA extract was determined after incubation at various temperatures for different times. When the extract was incubated at 55°C for 30 or 60 min, 105 and 114% of the RBA activity was retained, respectively, demonstrating that the enzyme is extremely stable at 55°C (Table 1). Furthermore, enzyme activity was 90% of the original after 30- or 60-min treatments at 60°C. These results show that the RBA extract can be heated to 55-60°C for 30-60 min during purification without a significant loss in enzyme activity. The β-amylase activity of *Abrus precatorius* was maintained 90% at the treatment at 55°C for 60 min (9). The wheat β-amylase was decreased to 50% during the heat treatment at 50°C for 30 min (5).

SDS-PAGE

Figure 1 shows the SDS-PAGE result of the heat-treated and untreated RBA extracts. The heat-treated RBA migrated as a range of protein bands in SDS-PAGE (lane 1, Fig. 1). Most of the protein bands in the crude extract were removed by the 60°C heat treatment for 30 min, whereas the band corresponding to a molecular mass of 55 kDa (lane 1, Fig. 1) remained as the major band. The molecular mass of RBA appears to be within the range of molecular masses (42-110 kDa) of other

Table 1. Effect of heat treatment on RBA activity

Temperature (°C)	Heat treatment				
	0 min	10 min	20 min	30 min	60 min
50	100	95±5.4	99±6.7	99±7.1	106±6.8
55	100	98±6.2	99±6.9	105±7.7	114±8.1
60	100	86±4.8	93±5.5	91±4.6	89±6.2
65	100	97±5.2	88±4.8	84±4.2	77±4.9

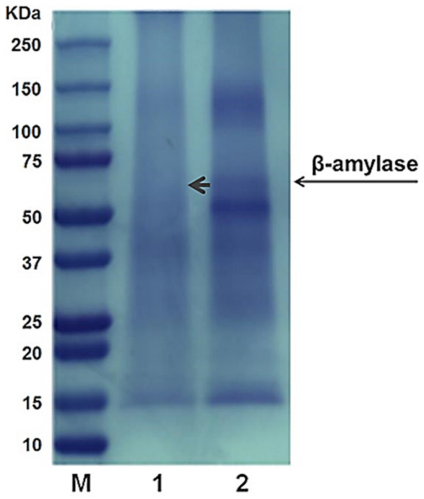

Fig. 1. SDS-PAGE of partially purified ramie leaf β-amylase. Protein molecular marker (lane M); crude extract (lane 2); partially purified enzyme after 30-min heat treatment at 60°C (lane 1).

plant β-amylases (8,23-25). Several plant β-amylases have isozymes at 44 and 55 kDa, or four isozymes at 54-59.7 kDa as in barley (8). The molecular masses of β-amylase from germinating millet seed and *Abrus precatorius* were estimated to be 58 kDa (25) and 60 kDa (9), respectively. In contrast, sweet potato β-amylase has a molecular mass of 152 kDa and exists as a tetramer (7). These results indicate that RBA was partially purified by heating the RBA extract.

Preliminary purification of RBA by heat treatment

Purification using the heat treatment is summarized in Table 2, wherein the extract was incubated at 60°C for 30 min. As shown in Table 2, protein content decreased significantly from 0.18 to 0.03 mg/mL after the heat treatment, demonstrating that most proteins were precipitated by heat. RBA specific activity increased from 2×10^{2} to 1.13×10^{1} U/mg protein, which was attributed to the reduction in protein content by precipitation. Based on specific activity, the enzyme was purified 5.4-fold and achieved a yield of 90% (Table 2). Thus, the enzyme could be purified from other enzymes, by 30-min heat treatment at 60°C that RBA activity was fully retained, but the other carbohydrate-active enzymes might be inactivated. The D-enzyme from *Arabidopsis* lost activity when incubated for 10 min at 60°C (26).

Plant tissues contain large amounts of phenolic compounds, pectic substances, and protein. Heat treatment has been used effectively to remove these unnecessary substances. The effectiveness of a heat treatment depends on whether the desired enzyme is relatively heat-stable and the unwanted proteins are denatured and precipitated from solution (12). The 30-min treatment at 60°C was the most effective for thermostability. This effect of the thermal treatment on the RBA extract suggests that the heat-treatment can be used as an alternative purification method to produce preliminary purified RBA as β-amylase for industrial applications. In particular, this heat treatment process may allow for large-scale production of plant β-amylase by selectively discriminating β-amylase from other heat-labile enzymes present in plant leaves.

TLC and HPAEC analyses of the reaction products

The reaction products of the heat-treated RBA extract were analyzed quantitatively by HPAEC and TLC after being incubated with the maltooligosaccharides, maltopentaose (Glc5) and malto-heptaose (Glc7) as well as soluble starch, respectively (Figs. 2-5). As shown in Figs. 2-4, when Glc5 and Glc7 were reacted with the heat-treated extract, the reaction exclusively produced the paired, maltose (Glc2) (**3** in Fig. 2) and maltotriose (Glc3) (**4** in Fig. 2) products from Glc5 and Glc2 (**3** in Fig. 2) and Glc5 (2 in Fig. 2) from Glc7 as the major products. In contrast, the untreated extract yielded various products, including the product pairs of glucose (Glc1) (Fig. 3) and Glc4 (**5** in Fig 2) and Glc2 (**3** in Fig. 2) and Glc3 (**4** in Fig. 2), demonstrating that other enzymes may be present in the extract. It seems that this pattern is very likely to be due to the action of the D-enzyme, as this enzyme transfers a glucose moiety from maltose to another maltose molecule, producing various maltooligosaccharides, such as Glc1 (Figs. 3 and 4) and Glc4 (**5** in Fig. 2) and Glc2 (**3** in Fig. 2) and Glc3 (**4** in Fig 2). Similarly, the Glc7 hydrolysis products from the heat-treated extract were identified as Glc2 (**3** in Fig. 2) and Glc5 (**2** in Fig. 2), whereas those of the untreated extract were the pairs Glc2 (**3** in Fig. 2) and Glc5 (**2** in Fig. 2) and Glc1 (Fig. 4) and maltohexaose (Glc6) (**7** in Fig. 2). Furthermore, the reaction mixture of the heat-treated extract containing soluble starch yielded Glc2 (**3** in Fig. 2) as the major product, whereas various maltooligosaccharides were formed from the untreated extract, demonstrating that the heat treatment isolated RBA from the D-enzyme. The dense spot (**1** in Fig. 2) at Glc1 appeared to include pigment. The untreated extract gave the reaction products, Glc2-Glc4 after 1h and 5h reaction, respectively while the heat-treated extract did not produce the products (a and b in Figs. 2 and 3). The results indicated that D-enzyme may produce the various products by the reaction with various maltodextrins (Glc2-Glc4) present in the extract, but the D-enzyme in the heat-treated extract was inactivated due to heat lability by heat treatment. Overall, the untreated sample produced various maltooligosaccharides, whereas maltose was produced exclusively by the heat-treated extract (Figs. 2-5). Based on the

Table 2. Purification of RBA by heat treatment

	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	$(3.75\pm0.14)\times10^{-3}$	0.18±0.012	$(2.08\pm0.17)\times10^{-2}$	100	1
Heat treatment	$(3.4\pm0.19)\times10^{-3}$	0.03±0.002	$(1.13\pm0.09)\times10^{-1}$	90	5.4

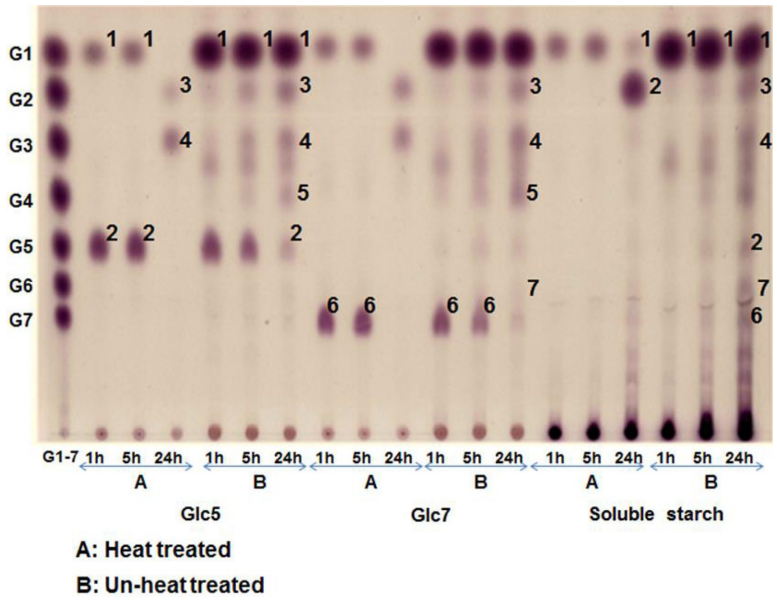

Fig. 2. TLC analysis of the reaction products from the heat-treated enzyme extract and the untreated extract with maltopentose (Glc5), maltoheptose (Glc7), and soluble starch. The enzyme samples were incubated with the substrate (1%, w/v) at 50°C. 1: glucose (Glc1) and pigments. The dense spot (1) at Glc1 may include pigments and glucose (Glc1); *2*: maltopentaose (Glc5); 3: maltose (Glc2); 4: maltotriose (Glc3); 5: maltotetraose (Glc4); 6: maltoheptaose (Glc7); 7: maltohexaose (Glc6).

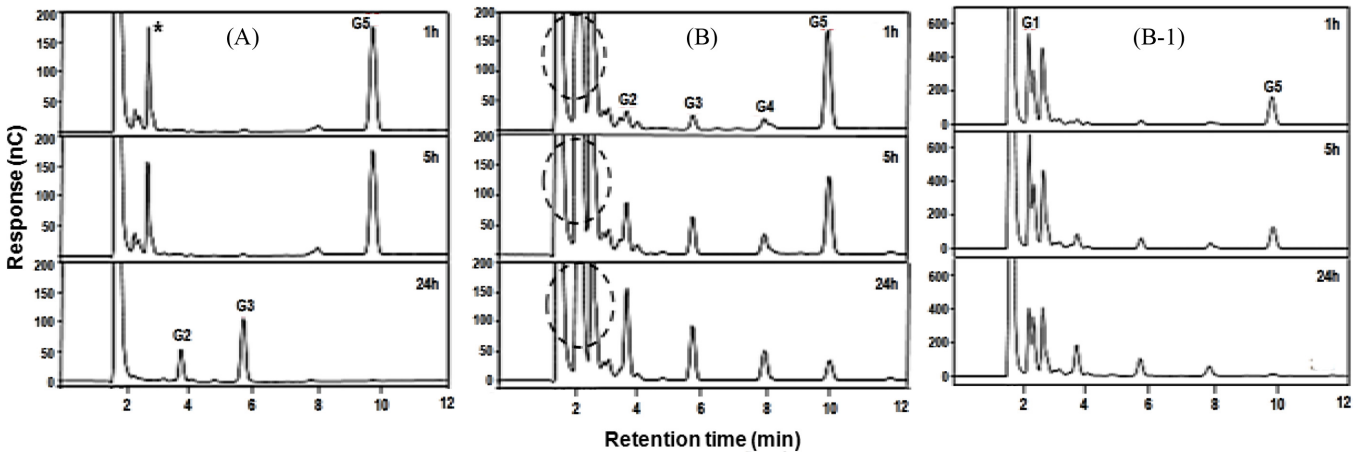

Fig. 3. HPAEC analysis of the reaction products from the heat-treated enzyme extract (A) and the untreated extract (B) with maltopentose (Glc5). The chromatogram in the dotted circle (B) was enlarged (B-1).

major paired products, the heat-treated extract included β-amylase exclusively. These findings indicate that RBA remained without loss of enzyme activity, but that the D-enzyme was likely inactivated by the 30-min heat treatment at 60°C.

Possible application for high retention of β-amylase in blanched ramie leaves

Many investigations have shown that β-amylase treatment reduces the retrogradation rate of starch-based foods by modifying the molecular structure of amylopectin (6,27-29). Thus, it is desirable to maintain intact β-amylase activity in plant tissues after heat treatment. On the other hand, thermal treatment (blanching) is needed to stabilize frozen vegetables by inactivating the quality-deteriorating enzymes that affect product quality during storage (14-17). Chlorophyllase is the key enzyme involved in chlorophyll breakdown during unfrozen and frozen storage. This enzyme is highly susceptible to inactivation and is often restricted by its lack of long-term stability. Chlorophyllase from *Brassica oleracea* is rapidly inactivated when the temperature exceeds 40°C. Enzyme activity remained <60 and 40% after 30-min incubation at 50 and 60°C, respectively (30). In contrast, residual RBA activity was 105 and 91%, respectively, after a 30-min treatment at 55 or 60°C. Comparing the thermostability of RBA and chlorophyllase may help determine the optimal heat treatment in which chlorophyllase is maximally inactivated but RBA remains intact and active. If the thermostability of ramie leaf chlorophyllase is in the range of that of *B. oleracea,* the ramie leaf blanching treatment for rice cakes could be optimized for processing at 60°C for 30 min, during which chlorophyllase would be inactivated, but RBA activity would be maximally retained, as suggested by

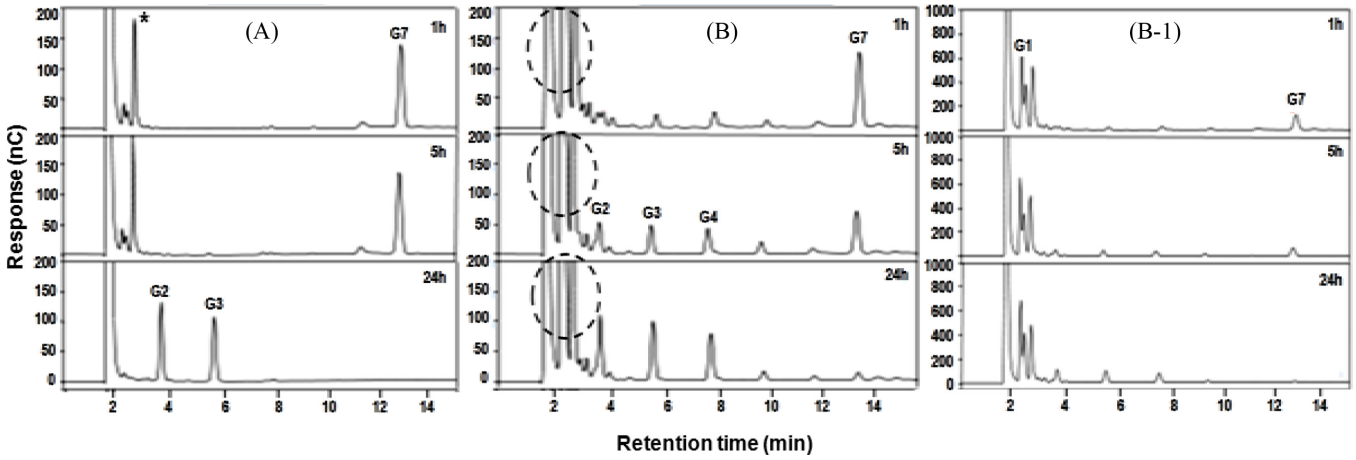

Fig. 4. HPAEC analysis of the reaction products from the heat-treated enzyme extract (A) and the untreated extract (B) with maltoheptose (Glc7). The chromatogram in the dotted circle (B) was enlarged (B-1).

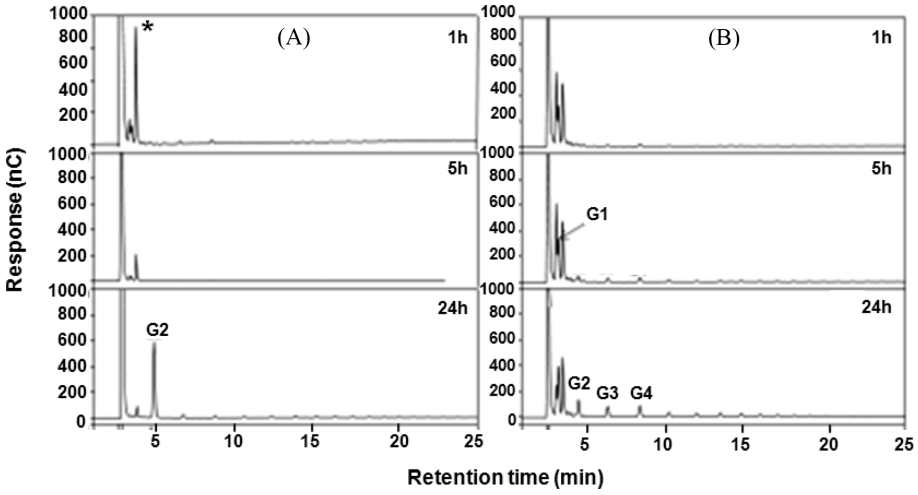

Fig. 5. HPAEC analysis of the reaction products from the heat-treated enzyme extract (A) and the untreated extract (B) with soluble starch.

Fig. 5. However, the thermostability of ramie leaf chlorophyllase should be further investigated under conditions comparable to those in the present experiment. In addition, the optimal ramie leaf thermal treatment may be slightly different from that of the extract, as enzyme stability depends on substances in the cytosol and the heat transfer media.

Conclusion

A plant β-amylase was partially purified using protein precipitation and inactivation of other heat-labile enzymes by a heat treatment procedure. Unlike the conventional method, the technique is simple, rapid, and inexpensive and resulted in excellent recovery and enzyme purity. Furthermore, the high retention of RBA activity after heat treatment makes this approach applicable to food processing to reduce the retrogradation rate of starch-based foods. However, further characterization of various quality-deterioration enzymes in ramie leaves is required to minimize undesirable changes in color, flavor, or nutritional loss and to optimize the thermal process.

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