Polymorphism of Acid and Neutral α -Glucosidases in Banana Pulp: Changes in Apparent pIs and Affinity to Con A of the Enzymes during Ripening

Yôtarô KONISHI,* Sachiko KITAZATO, Ryôko ASANO
and Nobuji NAKATANI

Department of Food and Nutrition, Faculty of Science of Living, Osaka City University,
3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558, Japan

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We observed the presence of acid and neutral α -glucosidases with optimum pHs of 4.5 and 6.5, respectively, and their multiple forms in banana pulp. By chromatofocusing analysis, each α -glucosidase from preclimacteric bananas was found to be of pI > 7.4, which disappeared upon ripening, although another form with pI 5 as a major component appeared instead. It is unknown whether this phenomenon is due to protein *de novo* synthesis and/or post-translational modifications of the enzyme. A large amount of acid α -glucosidase from preclimacteric bananas was shown to be a glycoprotein that bound to a Con A-Sepharose column. While this type of enzyme decreased during ripening, it remained a possibility that sugar components of the enzyme might be processed during ripening. The neutral α -glucosidase had no affinity to the column through ripening. These results provide preliminary information on the mechanism of appearance of α -glucosidase multiforms as well as on a useful strategy for purification of acid and neutral α -glucosidases from banana pulp at various stages of ripening.

Starch breakdown is one of the most noticeable changes in ripening of climacteric fruits. In bananas, the starch content decreases from 15–20% in the preclimacteric phase to less than 1% in the postclimacteric phase.^{1,2)} Exogenous ethylene shortens the preclimacteric period.

In most nonphotosynthetic starch storage tissues like banana pulp, α -amylase has been considered to be involved in the initiation of starch degradation to produce malto-oligosaccharides, since this enzyme is able to bind to starch granules.^{3,4)} Afterwards, the malto-oligosaccharides are degraded *via* hydrolytic and/or phosphorytic pathways.⁵⁾ There are many reports showing the close relationships between starch degradation in bananas and the increased activities of starch hydrolytic enzymes including α - and β -amylases,^{6–9)} debranching enzyme,⁹⁾ and α -glucosidase.⁹⁾ Phosphorylase activity, however, has been shown

to decrease as starch degradation proceeds.^{6,7,10)} Unfortunately, the exact mechanism or the regulation of starch degradation in bananas has not been fully explained, because of lack of characterization of the related enzymes or the concentration of intermediates in banana tissues. In addition, heterogeneity of the enzymes of starch metabolism makes the above point complex.⁴⁾

α -Glucosidase [EC 3.2.1.20] in banana tissues has attracted little attention. Garcia and Lajolo⁹⁾ observed an increased activity of α -glucosidase at the climacteric phase and heterogeneity of this enzyme by activity staining after polyacrylamide gel electrophoresis. Their report led us to attempt to purify α -glucosidase from banana pulp tissues. We have reported that there existed acid and neutral α -glucosidases and their polymorphism.¹¹⁾ In this paper, we report the changes in acid and neutral forms of α -glucosidases on

* To whom correspondence should be addressed.

chromatofocusing or a Con A affinity column during ripening of bananas.

Materials and Methods

1) *Reagents.* 4-Methylumbelliferyl- α -D-glucoside (4MU α G) was a product of Sigma. Polybuffer 74, Polybuffer exchanger (PBE) 94, CM-Sepharose CL-6B, and Con A-Sepharose were obtained from Pharmacia LKB Biotechnology. Polyvinylpyrrolidone (PVP) 25 was from Nacalai Tesque, Inc., Kyoto.

2) *Banana samples.* Green bananas (*Musa sapientum* L. cv. Giant Cavendishii) before and after treating with ethylene (approx. 1000 ppm for 24 hr at 15°C) were obtained from the Osaka Banana Center. They were stored at 22°C until use. Banana pulp (20 g), after slicing for green bananas, was homogenized with 30 ml of 0.1 M glycylglycine buffer (pH 7.0) containing 1% PVP (to bind polyphenols¹²), 20 mM cysteine (to prevent browning), and 0.1 mM EDTA using a Potter–Elvehjem teflon homogenizer. After centrifugation (20,000 $\times$ g, 30 min), a supernatant (crude extract) was obtained. Enzymes in the crude extract were precipitated by adding polyethylene glycol (PEG) 6000 (0.2 g/ml) or by ammonium sulfate (60% saturation).

3) *Enzyme assay.* α -Glucosidase activity was measured fluorimetrically using 4MU α G as a substrate.¹³ The reaction mixture (1 ml) contained 0.1 ml of enzyme, 0.6 ml of 0.1 M citrate–phosphate buffer (pH 4.5 or 6.5), and 0.3 ml of 0.33 mM 4MU α G. The reaction was done at 37°C for 30 min and stopped by adding 3 ml of 0.5 M glycine–NaOH buffer (pH 10.5). Fluorescence was measured with a Shimadzu RF-540 spectrophotometer at an emission wavelength of 450 nm and an excitation wavelength of 366 nm. One unit was defined as the amount of enzyme producing 1 μ mol 4MU/min.

4) *Measurements of CO₂.* The banana sample was placed for 1 or 2 hr in a container ($V=16$ l). The CO₂ production was measured using a Gastec CO₂ sensor (Gastec Co., Ltd., Kanazawa).

5) *CM-Sepharose CL-6B column chromatography.* Material precipitated by 60% saturation with ammonium sulfate was dissolved in 10 mM sodium acetate buffer (pH 6.2), dialyzed against the same buffer, and put on a CM-Sepharose CL-6B column (1 $\times$ 16 cm). After the column was washed with the same buffer, it was eluted with a linear gradient (10–400 mM sodium acetate, pH 6.2).

6) *Chromatofocusing.* The precipitate obtained by adding PEG to the crude extracts was dissolved in 25 mM imidazole–HCl buffer (pH 7.4). The sample was put on a column packed with PBE 94 (0.7 $\times$ 5.2 cm) preequilibrated

with the same buffer. Chromatofocusing was done by elution with 50 ml of the 9-fold diluted polybuffer 74, according to the instructions from Pharmacia LKB Biotechnology.

7) *Con A-Sepharose column chromatography.* The pellet from PEG was dialyzed against phosphate buffer (50 mM, pH 6.5) containing 2 mM dithiothreitol and 0.1 M NaCl, then put on a Con A-Sepharose column (0.7 $\times$ 5.2 cm) preequilibrated with the same buffer. After the column was washed with 50 ml of the buffer, the buffer containing methyl α -D-glucopyranoside (15 mM) was applied.

Results

1) Evidence for the presence of acid and neutral α -glucosidases in banana pulp

Figure 1 shows pH–activity and pH–stability curves of α -glucosidase using crude extracts of ripe yellow banana pulp, giving two optimum peaks, pH 4.5 and pH 6.5 (Fig. 1A); the former is defined as acid α -glucosidase and the latter as neutral α -glucosidase. Acid α -glucosidase was completely stable at pH 3–8 for 20 hr at 4°C, while neutral α -glucosidase was labile at acidic pH but stable at neutral pH (Fig. 1B). Figure 2 shows residual enzymic activities measured at pHs of 4.5 and 6.5 upon heating

Fig. 1. pH–Activity (A) and pH–Stability (B) Curves of α -Glucosidases from a Ripe Yellow Banana.

(A): α -Glucosidase activity at various pHs were measured with crude extracts of banana pulp as an enzyme source. The 100% value is 1.9 mU/ml at pH 6.5. (B): The crude extract was left for 20 h at 4°C at the indicated pHs, followed by measurement of enzyme activities at pH 4.5 (●) and pH 6.5 (○). The 100% values of acid and neutral α -glucosidases are 0.69 mU/ml and 2.60 mU/ml, respectively.

the crude extracts at various temperatures (37–80°C) for 5 min. The temperature which reduced 50% of enzyme activities was found to be 52°C for acid α -glucosidase and 72°C for neutral α -glucosidase, suggesting these enzymes are different proteins. Acid and neutral α -glucosidases were in part retained on a CM-Sepharose CL-6B column at pH 6.2 and were eluted with 0.33 M and 0.13 M sodium

Fig. 2. Heat Stability of α -Glucosidases from a Ripe Yellow Banana.

The crude extracts was used as a enzyme source and heated at various temperatures for 5 min, followed by measurement of enzyme activities at pH 4.5 (●) and pH 6.5 (○). The 100% value is obtained from the non-heated sample and is 0.10 mU/ml at pH 4.5 or 0.72 mU/ml at pH 6.5.

acetate buffer, respectively (Fig. 3). These results demonstrate the presence of acid and neutral α -glucosidases in banana pulp.

2) Changes in activities of α -glucosidases during ripening of bananas

Figure 4 shows the changes in peel color index, the rate of CO_2 production, and activities of acid and neutral α -glucosidases of bananas before and after the ethylene treatment. By measuring of the rate of CO_2 production, the climacteric peak of the sample used in this experiment was reached 24 hr after the end of the ethylene treatment. Activities of acid and neutral α -glucosidases of the sample without ethylene treatment were very low, and they increased by 2 or 3-fold at the climacteric phase, showing complex changes thereafter.

3) Changes in apparent pIs of α -glucosidases during ripening of bananas

We sometimes found that the amount of acid or neutral α -glucosidase retained on a CM-Sepharose column (see Fig. 3) was variable among samples purchased, suggesting that apparent pIs of α -glucosidases are changeable during ripening. We therefore examined this by chromatofocusing (Fig. 5). From the ethylene-untreated green banana, both acid

Fig. 3. Chromatogram of Banana α -Glucosidases on a CM-Sepharose CL-6B Column.

The precipitate with ammonium sulfate (60% saturation) from the crude extracts (40 g banana equivalent) was put on a CM-Sepharose CL-6B column preequilibrated with sodium acetate buffer (10 mM, pH 6.2). After the column was washed with the same buffer, a linear gradient (10–400 mM sodium acetate, pH 6.2) was developed at a flow rate of 15 ml/hr and collected 3.8 ml per tube. ●, acid α -glucosidase; ○, neutral α -glucosidase.

Fig. 4. Changes in Peel Color Index, CO₂ Production, and Activities of Acid and Neutral α -Glucosidases of Bananas before and after Ethylene Treatment.

Bar indicates duration of ethylene treatment (approx. 1,000 ppm, 24 hr). AAG, acid α -glucosidase (●); NAG, neutral α -glucosidase (○); AG, all green; LG, light green; GT, green tip; AY, all yellow; N, samples not treated with ethylene.

and neutral α -glucosidases were shown to have $pI > 7.4$ (Fig. 5A). Similar results were obtained from a green banana 24 hr after the end of the ethylene treatment (0 day of storage) (Fig. 5B) and from the 1st day of storage (data not shown). From a ripe yellow banana (the 4th day of storage), however, the neutral α -glucosidase with $pI > 7.4$ disappeared and enzyme with $pI 5$ appeared instead. Acid α -glucosidase showed near $pI 5$ as major and other various pI s from the same sample (Fig. 5C).

4) Changes in affinity of α -glucosidases for the Con A-Sepharose column during ripening of bananas

Figure 6 shows Con A affinity chromatograms of α -glucosidases from bananas with different stages of ripening. From the ethylene-untreated banana (Fig. 6A) and the sample of the 0 day of storage after the ethylene treatment (Fig. 6B), a large amount of acid α -glucosidase

Fig. 5. Chromatofocusing of Banana α -Glucosidases.

The active fraction was obtained by 20% PEG precipitation from the crude extracts, dissolved in 25 mM imidazole-HCl buffer (pH 7.4) and put on a column packed with Pharmacia PBE 94 equilibrated with the same buffer. The pH gradient was developed by applying the 9-fold diluted Polybuffer 74 to the column. The ordinate scale shows the relative activity (% of total) of the enzyme measured at pH 4.5 (acid α -glucosidase, AAG) or at pH 6.5 (neutral α -glucosidase, NAG). (A): A green banana without ethylene treatment. (B): A light green banana 0 day after the end of the ethylene treatment. (C): A ripe yellow banana 4 days after the end of the ethylene treatment.

was shown to be glycoprotein that bound to a Con A column. However, when ripened (Fig. 6C), the amount of the glycoproteinous enzyme decreased. On the other hand, neutral α -glucosidase from preclimacteric and ripe bananas had no affinity for the Con A column. A small active fraction of neutral α -glucosidase that bound to the column (Fig. 6B) was not true activity of the enzyme, since it disappeared on heating at 60°C for 5 min, conditions in which acid α -glucosidase was stable, as observed in Fig. 2.

Fig. 6. Con A-Sepharose Column Chromatograms of Banana α -Glucosidases.

The active fraction was obtained by 20% PEG precipitation from the crude extracts, dissolved in 50 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl, then put on a Con A-Sepharose column. After the column was washed with the same buffer, the same buffer containing 15 mM methyl α -D-glucopyranoside was put on, as indicated by an arrow. The ordinate scale shows the relative activity (% of total) of the enzyme measured at pH 4.5 (acid α -glucosidase, AAG) or pH 6.5 (neutral α -glucosidase, NAG). The samples, (A), (B), and (C), were the same to those described in the legend of Fig. 5.

Discussion

Garcia and Lajolo⁹⁾ observed a large increase in activity of banana neutral α -glucosidase at the climacteric phase when bananas were ripened naturally, but they did not measure acid α -glucosidase activity. Further these authors reported multiple forms of the enzyme by activity staining that gave no differences in electrophoretic patterns through ripening of bananas. This method, or isoelectric focusing on polyacrylamide gel, has been widely used for investigating protein polymorphism for its high resolution, but it is less quantitative than analysis by chromatofocusing.¹⁴⁾ This study using chromatofocusing showed clearly that

the apparent pIs of banana α -glucosidases varied during ripening, in contrast to the result of Garcia and Lajolo.⁹⁾ At present, however, it is unknown whether heterogeneity of α -glucosidases observed in this study is derived from newly-synthesized isozymes from different genes or post-translational modifications of the preexisted enzymes. It has been shown that acid phosphatase, which is the enzyme that increases most in activity in the course of ripening of bananas,^{6,7,15-17)} was heterogeneous due to a sequential *de novo* synthesis of isozymes.¹⁶⁾

Little attention has been paid to α -glucosidase as a glycoprotein in the research on multiple enzyme forms in plant tissues including banana pulp.¹⁹⁾ As shown in Fig. 6, a large amount of acid α -glucosidase was found to be a glycoprotein that showed affinity for the Con A column. The decrease in the amount of the glycoprotein in a ripe yellow banana suggests that deglycosylation of protein might occur during ripening.

This study provides preliminary information not only on investigating the mechanism of appearance of multiple forms of α -glucosidase or isozymes but also on the strategy for purification of α -glucosidases from bananas at various stages of ripening.

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