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COMBINED EFFECTS OF PECTIC ENZYMES ON THE DEGRADATION OF PECTIN POLYSACCHARIDES OF BANANA FRUIT

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Abstract

Pectin polysaccharide is one of the major components of the primary cellular wall in the middle lamella of plant tissues. The degradation of pectin polysaccharide contributes to fruit softening. In this study, water-soluble pectin (WSP) and acid-soluble pectin (ASP) were isolated from pulp tissues of banana fruit at various ripening stages, and combinations of the enzymes such as polygalcturonase (PG), pectin methylesterase (PME) and β-galactosidase (β-Gal) were used to investigate the effect on the degradation of WSP and ASP. PG promoted the degradation of pectin polysaccharides, especially in ASP. An enhanced effect of the degradation of WSP and ASP from various ripening banana fruit was observed in the presence of PME. In addition, β-Gal accelerated slightly the degradation of WSP and ASP in the presence of PG. Overall, PG, PME and β-Gal can coordinate to promote the degradation of pectin polysaccharides of banana fruit, resulting in fruit softening.

Introduction

Pectin is one of the major components of the primary cellular walls in the middle lamella of plant tissues. The pectic matrix provides an environment for deposition, slippage and extension of the cellulosic-glycan network, and is the major adhesive materials between cells (Willats *et al.*, 2001). Pectin degradation leads to disassembly of the cellulose and hemicellulose network and plays an important role in fruit ripening (Lohani *et al*., 2004; Sañudo-Barajas *et al*., 2009).

Wall degrading enzymes were the major factors to initiate disassembly of cellular walls of harvested fruits (Miller & Fry, 2001). Involvement of pectic enzymes, such as polygalacturonase (PG), pectin methyl esterase (PME) and β-galactosidase (β-Gal), in enzymatic disassembly of cellular walls has been widely reported (Prasanna *et al*., 2007; Nikolić & Mojovic, 2007; Rugkong *et al*., 2010; Wei *et al*., 2010; Almeida & Huber, 2011). PG hydrolyses α-1,4-linked D-galacturonic acid and PME causes de-esterification of pectin while β-galactosidase (β-Gal) hydrolyses β-D-galactan branches. These enzymes contributed to the disassembly of pectin polysaccharides during fruit softening (Rugkong *et al*., 2010; Yoshioka *et al*., 2011). Nikolić and Mojovic (2007) reported that PG and PME cooperatively regulated the disassembly of pectin polysaccharides. However, relatively little information is available for the selectivity of the disassembly of pectin polysaccharides of postharvest fruit at various ripening stages. Furthermore, the role of the combined PG, PME and β-Gal in the disassembly of pectin polysaccharides is not fully understood, and requires further investigation.

Banana is a typical climacteric fruit, which is characterized by rapid softening once ripening is initiated (Jiang *et al*., 1999). Although characteristic of fruit softening has been established, the modification of pectin polysaccharides by various pectic hydrolases during softening of banana fruit is not clear. The major objective of the present study was to examine the interaction of PG, PME and β-Gal in the degradation of pectin polysaccharide from pulp tissues of banana fruit *in vitro* at various ripening stages.

Materials and Methods

Chemicals and plant materials: Endopolygalacturonase (PG, EC 3.2.1.15, one unit corresponding to the amount of enzyme which liberates 1 μmol galacturonic acid from polygalacturonic acid per minute at pH 4.0 and 50°C) from *Aspergillus niger*, pectin methylesterase (PME, EC 3.1.1.11, one unit releasing 1.0 microequivalent of acid from pectin per minute at pH 7.5 and 30°C) from orange peel, β-galactosidase (β-Gal, EC 3.2.1.23, one unit hydrolyzing 1.0 μmol of *o*-nitrophenyl β-D-galactoside to *o*-nitrophenol and *D*-galactose per minute at pH 4.5 and 30 °C) from *Aspergillus oryzae* and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co., USA. All others chemicals used were analytical grade.

Hands of mature green fruit of banana (*Musa* spp., AAA group, cv. Brazil) were obtained from a local farm in Guangzhou. Fruit were cut into fingers and then dipped in 0.1% Sportak® (prochloraz, Bayer) fungicide solution for 3 min to control the postharvest diseases. After air-dried for 1 h, the fruit were kept at 25 ± 1°C and 90% relative humidity (RH). At 0, 10, 15, 17 and 20 days when fruit ripening reached stage I (mature green), II (green), III (green > yellow), IV (yellow > green) and V (yellow), respectively, ten fingers were sampled and then peeled. The pulp tissues were cut into small pieces, frozen immediately in liquid nitrogen, and stored at -80°C.

Preparation of alcohol-insoluble residue: The alcohol-insoluble residue (AIR) was prepared according to the method of Vierhuis *et al.* (2000) with some modifications. The frozen pulp tissues (100 g) were blended for 2 min with 300 mL of 95% (v/v) ethanol using a homogenizer and then maintained in boiling water for 15 min to inactivate endogenous enzymes. After cooling rapidly in an ice bath, the homogenate was centrifuged at 4, 000 *g* for 15 min. The residue was collected, and washed sequentially with 200 mL of mixture solution of chloroform: methanol (1:1, v/v) and 200 mL of acetone. Pulp starch was removed by re-extracting overnight in 90% aqueous Me_2SO. In this study, no starch was detected using the KI-I_2 method (Nelson, 1968). The obtained residue fraction was washed twice with 70% ethanol at 25°C, filtered, and finally dried at 40°C. The dried powder was considered as the AIR and stored in desiccators.

Fractionation of pectin polysaccharides: AIR from banana fruit at five ripening stages mentioned above was subjected to sequential fractionations by the method of Majumder and Mazumdar (2002), with some modifications. AIR (5 g) was stirred for 1 h with 30 mL of distilled water at 25 °C. The suspension was centrifuged at 4, 000 *g* for 15 min, and then the residue was collected and re-extracted twice. The combined supernatants were filtered through Whatman filter paper (No. 1) and lyophilized to obtain water-soluble pectin (WSP). Afterwards, the water-insoluble fraction was incubated for 1 h with 100 mL of 0.5 mol/L HCl at 60°C and then centrifuged for 15 min at 4, 000 *g*. The supernatant was collected, dialyzed with distilled water, and finally lyophilized

to obtain acid-soluble pectin (ASP).

Effect of PG on the degradation of pectin polysaccharides: Effects of PG on the degradation of pectin polysaccharides from pulp tissues of banana fruit at various ripening stages were conducted by the method of Nikolić and Mojovic (2007). Each reaction mixture contained 0.1 mL of 0.5% WSP or ASP solution, 0.8 mL of 0.04 mol/L sodium acetate buffer (pH 4.8) and 0.1 mL of PG at a concentration of 0.25, 0.5 or 1 U/mL. These mixtures were shaken for 1 h at 120 rpm and 40 °C and then used for measurement of reducing base content. Reducing base content was determined by the method of Al-Zuhair (2008), with some modification. Sample (1 mL) was incubated with 2 mL of 3,5-dinitrosalicylic acid (DNS, Sigma Chemical Co., USA) reagent for 5 min in a boiling water bath, and cooled to 25 °C immediately using tap water. The DNS reagent was prepared by mixing 6.5 g of DNS, 325 mL of 2 mol/L NaOH and 45 g of glycerol and adding them into distilled water to a final volume of 1000 mL. The absorbance at 540 nm was recorded. A blank solution consisting of 1 mL distilled water instead of the sample was used. Reducing base content was determined using glucose as a standard.

Effect of PME on the degradation of pectin polysaccharides in the presence of PG: The effect of PME on pectin polysaccharides degradation in the presence of PG was examined by the method of Nikolić and Mojovic (2007). The reaction mixture contained 0.1 mL of 0.5% WSP or ASP solution, 0.7 mL of 0.04 mol/L sodium acetate buffer (pH 4.8), 0.1 mL of PG at 2.5 or 5 U/mL and 0.1 mL of PME at 0.2 or 0.5 U/mL. After the mixture was shaken for 1 h at 120 rpm and 40°C, reducing base content was measured according to the above-mentioned method.

Effect of PG on methanol production in the presence of PME: The effect of PG on methanol production in the presence of PME was evaluated by the method of Nikolić and Mojovic (2007). The reaction mixture contained 0.1 mL of 0.5% WSP or ASP solution, 0.7 mL of 0.04 mol/L sodium acetate buffer (pH 4.8), 0.1 mL of PG at 2.5 or 5 U /mL and 0.1 mL of PME at 0.2 or 0.5 U/mL. Theses mixtures were shaken for 1 h at 120 rpm and 40°C and then collected for measurement of methanol production. Methanol content was analyzed according to the method of Tao *et al*., (2006) with slight modifications. The mixture sample (1 mL) was blended gently with 0.1 mL of 15% (v/v) potassium permanganate/phosphoric acid solution and incubated for 15 min at 25°C. Then, 0.3 mL of 20% (w/v) Na_2SO_3 was added to the mixture solution. Finally, 2 mL of acetylacetone was added, mixed, and finally kept for 3 min in a boiling water bath. After cooling to 25°C, the absorbance at 412 nm was measured. The formaldehyde concentration in the samples was calculated using a standard calibration curve.

Effect of β-Gal on the degradation of pectin polysaccharides in the presence of PG: The degradation of pectin polysaccharides by β-Gal in the presence of PG were analyzed by the method of (Vicente *et al*., 2005). The reaction mixture contained 0.1 mL of 0.5% WSP or ASP solution, 0.7 mL of 0.04 mol/L sodium acetate buffer (pH 4.8), 0.1 mL of PG (2.5 U/mL) and 0.1 mL of β-Gal at 0.5, 1 or 1.5 U/mL. After the mixture was shaken for 1 h at 120 rpm and 40°C, reducing base content was measured according to the above-mentioned method.

Data handling: The experiments were arranged in a completely randomized design with each comprised of three replicates. Data were analyzed by SPSS (Version 13) and presented as means ± standard errors (SE).

Results and Discussion

Effect of PG on the degradation of pectin polysaccharides: Effects of PG on the degradation of pectin polysaccharides from pulp tissues of banana fruit at five ripening stages were shown in Fig. 1. With increasing PG activity in the reaction mixture, the amount of reducing base from ASP increased significantly, but the amount of reducing base from WSP increased only slightly. When PG at an activity of 1 U/mL was used in the reaction system, the amount of reducing base from WSP increased from 35.39 $mg \cdot Glc \cdot h^{-1} \cdot g^{-1}$pectin at stage I to 141.61 $mg \cdot Glc \cdot h^{-1} \cdot g^{-1}$pectin at stage III and then decreased significantly to 16.61 $mg \cdot Glc \cdot h^{-1} \cdot g^{-1}$pectin at stage V (Fig. 1A), indicating that the degradation of pectin polysaccharides by PG might occur before the fruit climacteric phase. A similar change trend of ASP was observed. In fact, the effect of PG on the degradation of ASP was more obvious than that of WSP with about tenfold more reducing base (Fig. 1), which further suggesting that PG may have a major role in the ASP degradation during banana fruit softening.

Pectin polysaccharide is one of the major components of the primary cellular walls in the middle lamella in plants. The modification of pectin polysaccharide results in the dissolution of the middle lamella, cellular wall swelling and an increased porosity, which contributes to fruit softening. The ripening-related changes in the pectic matrix include loss of neutral sugars, demethylation, increased solubility, and reduced molecular mass (Duan *et al*., 2008). Wei *et al*. (2010) reported that some wall degrading enzymes were the major factors initiating cellular wall disassembly in fruit. As the major one of the wall degrading enzymes, PG is the predominant pectin depolymerase in ripening fruit. Although PG is neither necessary nor sufficient to result in fruit softening, PG-mediated pectin dissolution and depolymerization are the important events in banana fruit during ripening (Rugkong *et al*., 2010). The present study indicated that PG may be effective in hydrolyzing ASP before banana fruit reached a climacteric phase (Fig. 1). Furthermore, as PG activity increased *in vitro*, no obvious degradation of pectin polysaccharides was observed in banana fruit at various ripening stages, suggesting that a very low level of PG activity may be sufficient to assure disassembly of the pectin polysaccharides.

Synergistic effect of PME and PG on the degradation of pectin polysaccharides: Fig. 2 presented the effects of PME on the degradation of pectin polysaccharides in the presence of PG. WSP (Fig. 2A) or ASP (Fig. 2B) incubated with the combined PG and PME exhibited more reducing base than those in the absence of PG or PME. Furthermore, the amount of reducing base by PME increased with fruit ripening or increasing PG activity (Fig. 2). Concomitantly, PME efficiently promoted the degradation of pectin polysaccharides by PG, which was more magnificent when a higher PME activity was used in the reaction system (Fig. 2C and 2D). These results suggested that PG and PME may cooperatively contribute to the disassembly of pectin polysaccharides by

hydrolyzing α-1,4-linked D-galacturonic acid (PG activity) following de-esterification of pectin (by PME) during fruit softening (Nikolić & Mojovic, 2007; Rugkong *et al*., 2010).

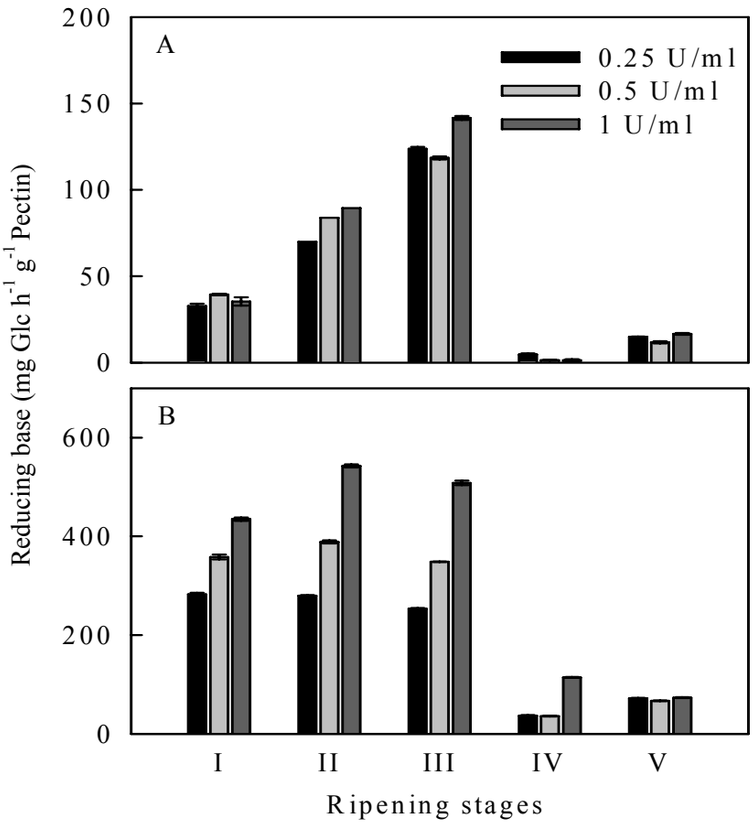

Fig. 1. Effects of PG at 0.25, 0.5 and 1 U/mL on the degradation of WSP (A) and ASP (B) from pulp tissues of banana fruit at five ripening stages. No reducing base from WSP and ASP in the absence of PG was detected.

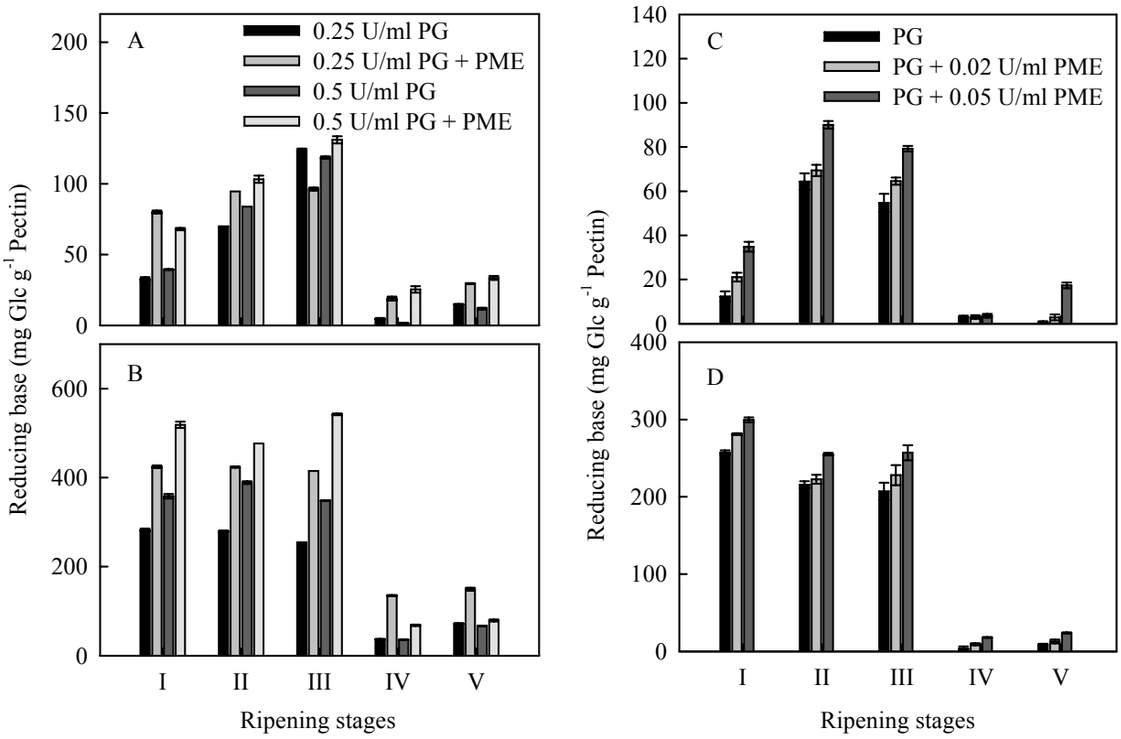

Fig. 2. Synergistic effects of PG and PME on the degradation of pectin polysaccharides from pulp tissues of banana fruit at five ripening stages. WSP (A) or ASP (B) was incubated with various amounts of PG at 0.25 or 0.5 U/mL in the presence of PME at 0.05 U/mL while WSP (C) or ASP (D) was incubated with various amounts of PME at 0.02 and 0.05 U/mL in the presence of PG at 0.25 U/mL. No reducing base from WSP and ASP in the absence of PG and PME was detected.

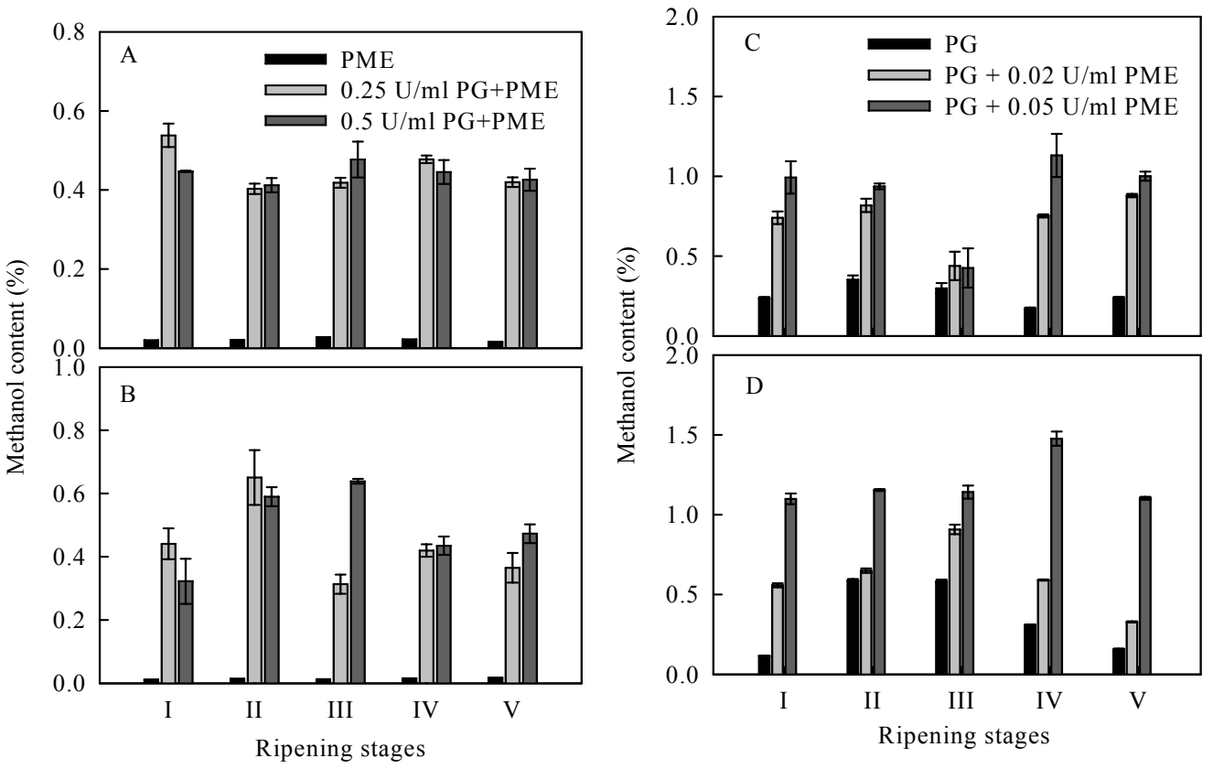

Fig. 3. Synergistic effects of PME and PG on the amount of methanol production from pectin polysaccharides from pulp tissues of banana fruit at five ripening stages. WSP (A) or ASP (B) was incubated with various amounts of PG at 0.25 or 0.5 U/mL in the presence of PME at 0.05 U/mL while WSP (C) or ASP (D) was incubated with various amounts of PME at 0.02 and 0.05 U/mL in the presence of PG at 0.25 U/mL. No methanol production from WSP and ASP in the absence of PG and PME was detected.

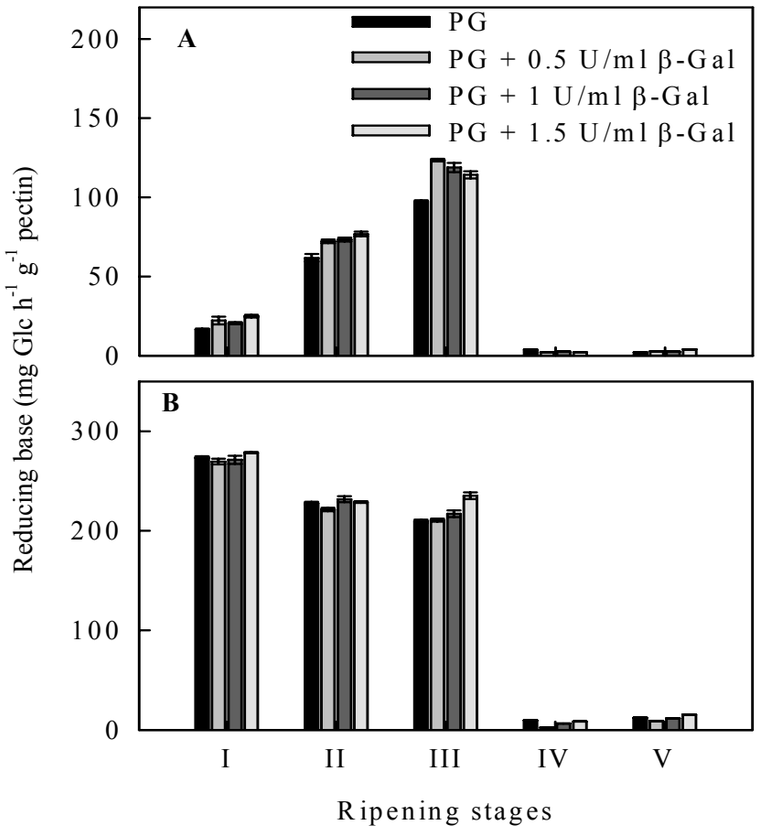

Fig. 4. Synergistic effects of β-Gal and PG on the amount of reducing base from WSP (A) and ASP (B) from pulp tissues of banana fruit at five ripening stages. WSP or ASP was incubated with β-Gal at 0.5, 1 and 1.5 U/mL in the presence of PG at 0.25 U/mL. No reducing base from WSP and ASP in the absence of PG and β-Gal was detected.

Effect of PG on methanol production from pectin polysaccharides in the presence of PME: Both WSP (Fig. 3A) and ASP (Fig. 3B) released only negligible amount of methanol in the absence of PG but the methanol production from pectin polysaccharides after incubation with the combined PG and PME increased significantly (Fig. 3). However, increased PG activity from 0.25 to 0.5 U/mL did not result in a significant increase in methanol production. Furthermore, a low amount of methanol was observed from both WSP and ASP in the presence of PG but in the absence of PME (Fig. 3C and 3D). When PME activity was increased from 0.02 to 0.05 U/mL, methanol production from WSP (Fig. 3C) and ASP (Fig. 3D) increased dramatically. However, no significant increase in methanol production was found when PME activity was increased further. PG also promoted the degradation of the pectin polysaccharide by a pretreatment with PME (Fig. 3), whereas PME accelerated the degradation of the pectin polysaccharides pretreated with PG (Fig. 2), which further indicated that PG and PME cooperatively regulated the disassembly of pectin polysaccharides of banana fruit during softening.

Effect of β-Gal on the degradation of pectin polysaccharides in the presence of PG: β-Gal slightly increased the amount of reducing base from pectin polysaccharides in the presence of PG (Fig. 4). However, no significant increase in the amount of the reducing base released from WSP (Fig. 4A) and ASP (Fig. 4B) was found when β-Gal activity was increased further, suggesting that β-Gal may play a minor role in the degradation of pectin polysaccharides of banana fruit during softening.

Previous studies reported that β-Gal was also involved in banana fruit ripening (Ali *et al.*, 2004; Lohani *et al.*, 2004; Asif & Nath, 2005). In this study, β-Gal slightly promoted the degradation of pectin polysaccharides *in vitro* (Fig. 4). Loss of galactosyl residues from pectin fractions probably caused by β-Gal during ripening of a variety of fruits was reported, such as papaya (Manrique & Lajolo, 2004), apple (Wei *et al.*, 2010) and peach (Manganaris *et al.*, 2006). As β-Gal could act on short chain oligomers of galactose units (Prasanna *et al.*, 2007), the further breakdown of pectin polysaccharides of cellular walls of banana fruit by β-Gal depends largely on their compositions caused by PG and/or PME, which needs to be investigated further.

Conclusions

The degradation of pectin polysaccharides from pulp tissues of banana fruit at various ripening stages required the combined effects of PG and PME. However, β-Gal might play only a minor role in the degradation during fruit softening. Further investigation into the pectin polysaccharide compositions in association with the kinetics of the combined enzymes during fruit softening is needed.

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