Purification and Properties of Cellulase-Free Xylanase from Namwa Banana Fruit *Musa sapientum*

Wanida Phanayingphaisal[1], Wichai Suttimool[1*] and Pairoh Pinphanichakarn[2]

[1]*Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.*
[2]*Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.*

ABSTRACT: Xylanase activity in Namwa banana pulp was measured at different stages of ripening determined as the texture firmness. The maximum specific activity of crude enzyme was found to be 0.66 unit/mg protein at the texture firmness of 210 cN and xylanase from this stage of ripening was purified. Purification of xylanase was achieved through precipitation with 80% saturated ammonium sulfate followed by consecutive chromatography of CM-cellulose and Sephadex G-50 columns. The purification was 14.7 fold and the purified enzyme showed no cellulase activity. The enzyme separated by SDS-PAGE showed a single band with the estimated molecular mass of 19 kDa which was close to the 21 kDa as estimated by Sephadex G-50. The enzyme had a broad range of pH and temperature optima of 5.0 - 5.5 and 45°C - 50°C, respectively.

Key words: xylanase, purification, Namwa banana fruit, *Musa sapientum*

INTRODUCTION

Xylanases hydrolyze β-1,4 glycosidic bonds in xylan which is a major constituent of the hemicellulose complex in plant cell walls.(1) Xylanases received intensive research interest due to their potential industrial applications for pulp biobleaching.(2,3) However, such applications require xylanase with particular properties and which are lacking all cellulase activities which would deteriorate the pulp quality.(4) Most xylanases have been isolated and purified from a wide range of microorganisms such as fungi and bacteria.(5-7) Only a few enzymes have been isolated from plants and fruits.(8) Some isolated enzymes from filamentous fungi and bacteria contain cellulase activity which limit their usage.(9-11) Therefore, plants and fruits might serve as alternative sources for cellulase-free xylanase preparations. There has been one report on a preliminary study of crude xylanase isolated from ripening banana fruit.(12) In this paper, we describe the purification of cellulase-free xylanase from Namwa banana pulp including some properties of the enzyme.

MATERIALS AND METHODS

Freshly harvested mature raw banana fruits (*Musa sapientum*) were bought from fruit dealers. Each batch of fruits was stored at room temperature for normal ripening. Sample of banana fruits, cut from the stem each day for seven consecutive days after the purchase and representing seven different stages of ripening, were peeled and the pulps were used for texture firmness measurement and enzyme preparation.

Measurement of texture firmness

Texture firmness of banana pulp was measured by using a texture analyzer (model TA-XT2i, UK) equipped with a 2 mm-plunger tip. For compression test on the pulp, uniform cylindrical samples of 2 cm length were cut longitudinally and placed for compression by the plunger tip. The texture firmness was recorded as the maximum force, in centiNewton (cN), required for compression.

Enzyme purification procedures

Banana pulps from seven different stages of ripening were cut into small pieces which were then frozen by liquid nitrogen. The frozen pieces were blended into powder and 20 mM sodium acetate

**Correspondence to: e-mail: pwichai@sc.chula.ac.th*

buffer, pH 5.5 was added at the ratio of 1:1 (v/v). The final volume was 450 ml. The suspension of each sample was centrifuged at 12,096×g for 30 min and the supernatant was used for determination of enzyme activity and protein content. The supernatant showing the highest specific activity of xylanase was used as a crude enzyme solution. The enzyme was then fractionated by precipitating with ammonium sulfate at 80% saturation. After centrifugation, the pellet was suspended in a small volume of 20 mM acetate buffer, pH 5.5, and dialyzed against the same buffer. The dialyzed solution was loaded onto a column (1.5 cm × 15 cm) of CM-cellulose (Sigma, U.S.A.) previously equilibrated with the same buffer. The unbound proteins were eluted with the same buffer and the bound proteins were then eluted with a linear gradient of NaCl (0 to 500 mM) in the same buffer. Both unbound and bound fractions were determined for xylanase and cellulase activities including protein content. Fractions containing xylanase were pooled and concentrated by Aquasorb and dialyzed against the same buffer. The dialyzed solution was loaded onto a column (1.5 cm × 90 cm) of Sephadex G-50 (Pharmacia, U.S.A.), and eluted with the same buffer, the fractions containing xylanase activity were pooled and concentrated by Aquasorb. The resulting solution was used as a source of purified xylanase.

Xylanase assay

Xylanase activity was determined by measuring the release of reducing sugars from oat spelt xylan. The reaction mixture contained 0.1 ml of 10 mg/ml xylan in 20 mM acetate buffer pH 5.5, 0.85 ml of 20 mM acetate buffer pH 5.5 and 0.05 ml of appropriately diluted enzyme solution. After the reaction mixture was incubated at 45°C for 10 min, the reaction was stopped by placing the mixture in a boiling water bath for 15 min. The amount of reducing sugar released in the reaction mixture was determined by Somogyi-Nelson method.[13] The unit of xylanase activity (U) was defined as the amount of enzyme that liberated 1 micromole of xylose equivalent per min under the assay conditions.

Cellulase activity

Cellulase activity was determined by measuring the release of sugars from carboxymethyl cellulose (CMC). The reaction mixture contained 0.1 ml of 10 mg/ml CMC in 20 mM acetate buffer pH 5.5, 0.85 ml of 20 mM acetate buffer pH 5.5 and 0.05 ml of appropriately diluted enzyme solution. After the reaction mixture was incubated at 45°C for 10 min, the reaction was terminated by placing the mixture in a boiling water bath for 15 min. The amount of reducing sugar released in the reaction mixture was determined by the dinitrosalicylic acid method.[14] One unit of the enzyme was defined as the amount of enzyme capable of releasing 1 micromole of glucose equivalent per min under the assay conditions.

Effects of temperature and pH on xylanase activity

For the study of the effect of temperature, xylanase activity was assayed under standard conditions except that the reaction mixture was incubated at different temperatures from 35°C to 80°C. For the study of the effect of pH, xylanase activity was assayed under standard conditions except that the reaction mixture contained various buffers at different pH from 3.5 to 9.0 at the optimal temperature.

Protein content

Protein content was determined by the method of Lowry *et.al*.[15] using bovine serum albumin as standard.

SDS-PAGE and molecular mass determinations

SDS-PAGE was performed at room temperature in 15% polyacrylamide gel by the method of Laemmli.[16] Protein was revealed by staining the gel with Coomassie Brilliant Blue R-250 (BioRad). The molecular mass of the xylanase under denaturing conditions was determined by SDS-PAGE using a mixture of standard proteins of known molecular weight. The determination of native molecular mass of xylanase was also performed by gel filtration chromatography on Sephadex G-50 using protein markers of known molecular weight as reference standards.

RESULTS

Texture firmness

The texture firmness during banana ripening as measured by the compression test decreased gradually from the raw stage (Day 1) to the ripe stage (Day 7) as shown in Figure 1.

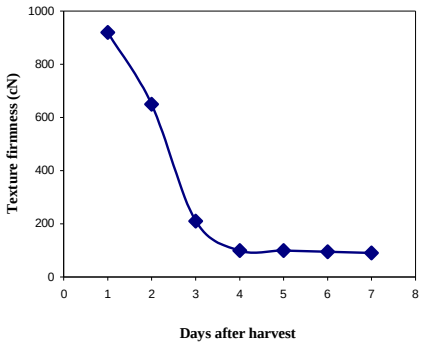

Figure 1. Changes in texture firmness of Namwa banana pulp at different stages of ripening. Results are the average of three replicates.

Purification of cellulase-free xylanase

Crude xylanase from banana pulp of various texture firmnesses was extracted and measured for specific activity (Figure 2). The results indicated that at various stages of banana ripening the xylanase specific activities were also different. The highest specific activity of xylanase was shown at 210 cN. Therefore, in order to obtain the maximum specific activity of the enzyme, banana pulp of texture firmness 210 cN was then used as a source for enzyme extraction and purification. A summary of the purification procedures is presented in Table 1. Cellulase activity was also determined from the crude enzyme preparation and from the fractions obtained from each step of purification. The activity of xylanase was eluted as a single peak from CM-cellulose column with NaCl at the concentrations between 0.06 to 0.14 M (Figure 3) and also a single peak from Sephadex G-50 (Figure 4). No cellulase activity was detected after the step of CM-cellulose chromatography (Table 1). SDS-PAGE of the xylanase eluted from Sephadex G-50 column revealed a single band when stained with Coomassie Brilliant Blue (Figure 5), this result indicated that the enzyme was purified to homogeneity.

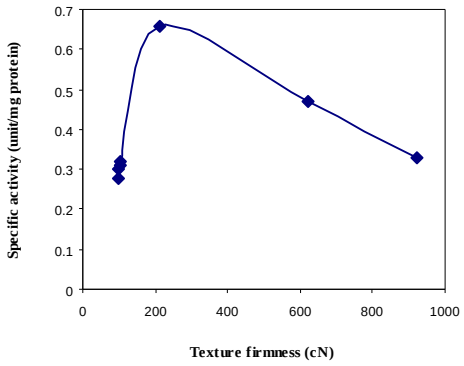

Figure 2. Changes in specific activity of xylanase from Namwa banana pulp at different texture firmnesses. Results are the average of three replicates.

Table 1. Purification of xylanase from Namwa banana pulp and cellulase activity detected in each step of purification.

		Xylanase				Cellulase		
Fraction and purification step	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification (Fold)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
1. Crude enzyme	796	522	0.66	100	1.0	44	0.06	100
2. $(NH_4)_2SO_4$ (0-80% saturation)	274	235.20	0.86	45.1	1.3	7.8	0.03	17.7
3. CM-cellulose	5.5	38.40	6.98	7.4	10.6	0.0	0.00	0.0
4. Sephadex G-50	2.2	21.24	9.64	4.1	14.7	0.0	0.00	0.0

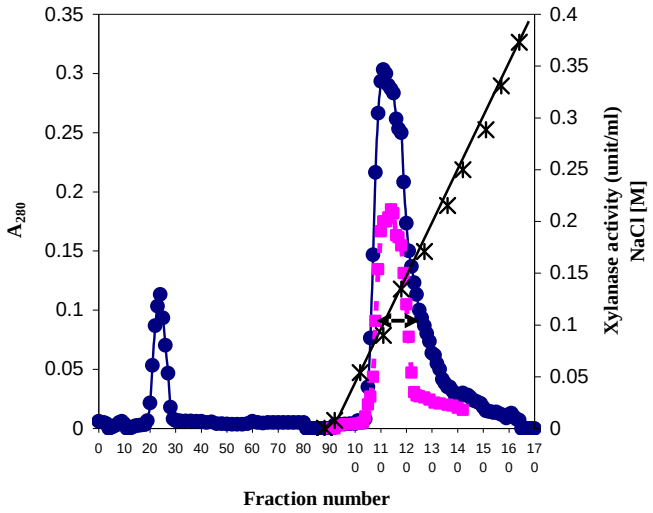

Figure 3. **Purification of xylanase on a cationic exchange column (CM-cellulose). Column was eluted at a flow rate of 0.50 ml/min and the fraction size of 3 ml was collected. Xylanase was eluted with NaCl gradient (–×–×–). Absorbance at 280 nm (–—–) was for protein content. Xylanase activity (¡—¡) was detected. Fractions between arrows were pooled.**

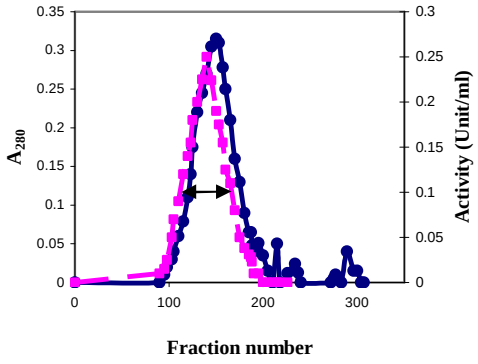

Figure 4. **Purification of xylanase by gel filtration column (Sephadex G-50). Column was eluted at a flow rate of 0.17 ml/min and the fraction size of 1 ml was collected. Absorbance at 280 nm (–—–) was for protein content. Xylanase activity (¡—¡) was detected. Fractions between arrows were pooled.**

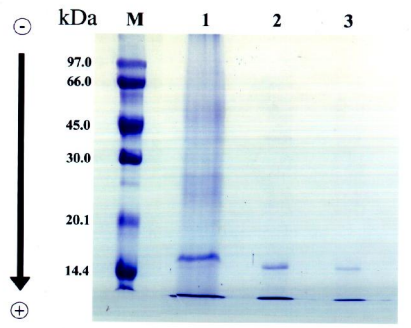

Figure 5. **SDS-PAGE analysis of xylanase. Lane M, molecular mass markers : phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Lane 1 : crude enzyme (17.7 μg) from 80% saturated $(NH_4)_2SO_4$ fraction ; lane 2 : enzyme (6 μg) from CM-cellulose column ; lane 3 : enzyme (2.2 μg) from Sephadex G-50 column.**

Some properties of the purified xylanase

The molecular mass of the purified xylanase under denaturing condition estimated by SDS-PAGE was found to be 19 kDa (Figure 6) which is close to the molecular mass of 21 kDa estimated by Sephadex G-50 (Figure 7). The effect of pH and temperature on the activity of the enzyme are shown in Figure 8 and Figure 9, respectively. The optimum pH and temperature were found to be in the range of 5.0 - 5.5 and 45°C - 50°C, respectively.

DISCUSSION

The present study showed that the texture firmness of banana pulp decreased gradually to the extent of nine fold from the raw stage to the ripe stage. The ripening of banana fruit might be associated with the physical and biochemical changes of banana tissues. It has been reported that many carbohydrate hydrolases including xylanase may contribute to fruit softening.[12] The specific activity of xylanase was highest at a texture firmness of 210 cN. Samples at this level were then used as a source for enzyme preparation. The final step of enzyme purification from Sephadex G-50 gel filtration resulted in a yield of 4.1% of the total activity, 0.28% retention of the total protein and a 14.7-fold increase in specific activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a single band of protein, therefore, the enzyme was purified to homogeneity.

Most xylanases have been reported to have molecular weights ranging from about 20 kDa to 60 kDa.[17-23] Xylanase from the present work had an apparent molecular mass, estimated by SDS-PAGE and Sephadex G-50, of 19 kDa and 21 kDa, respectively. The enzyme appears to be a monomeric protein having low molecular mass and less complex in size. In general, xylanases from various microorganisms and plants are classified into two major families, F/10 and G/11. Xylanase from Namwa banana fruit might be classified in the family G/11 since xylanases in the family F/10 are larger and more complex. Furthermore, molecular mass of this enzyme is within the range detected for xylanases belonging to the family G/11.[24] However, more investigation and information are needed to ensure the family classification of this enzyme.

The enzyme showed relatively high activity under acidic condition and it could work actively in a broad pH range of between 5.0 - 6.0. The enzyme had optimum activity at temperatures of 45°C - 50°C. At 60°C it still had about 80% activity relative to that at the optimum temperature.

In conclusion the enzyme had low molecular weight, the purification steps were simple and after column chromatography it is free of cellulase activity. This enzyme might potentially be useful for pulp biobleaching since its small size can easily penetrate the fibre wall structure and alter, more efficiently, the pulp properties.[25] Furthermore, due to the lack of cellulase activity, this xylanase will not deteriorate the cellulose fibre strength of paper products.

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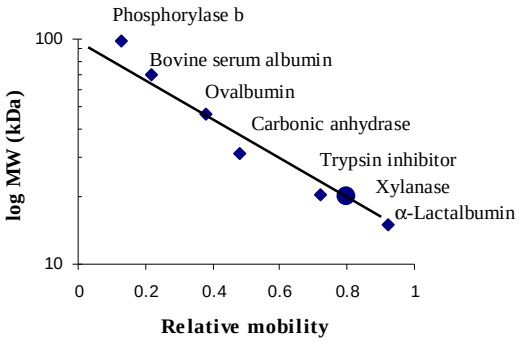

Figure 6. Estimation of molecular mass of xylanase from a plot of log molecular mass (kDa) against relative mobility obtained from SDS-PAGE analysis. Molecular mass of standard protein markers are indicated in Figure 5.

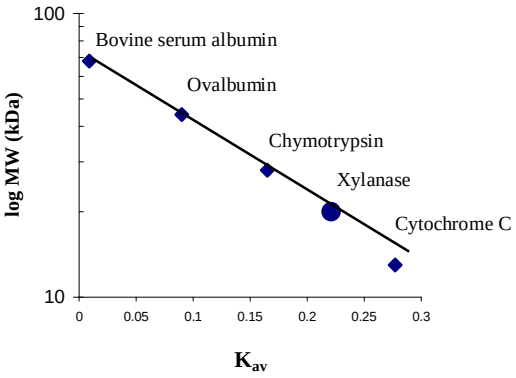

Figure 7. Estimation of molecular mass of xylanase from a plot of log molecular mass (kDa) against partition coefficient (K_{av}) obtained from Sephadex G-50 chromatography. Standard protein markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), chymotrypsin (27 kDa) and cytochrome C (12.5 kDa).

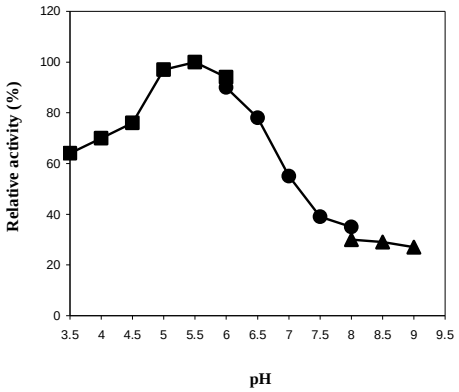

Figure 8. Effect of pH (¢ 20 mM acetate buffer, ˜ 20 mM phosphate buffer, p 20 mM Tris-HCl buffer) on activity of xylanase. Results are the average of three replicates.

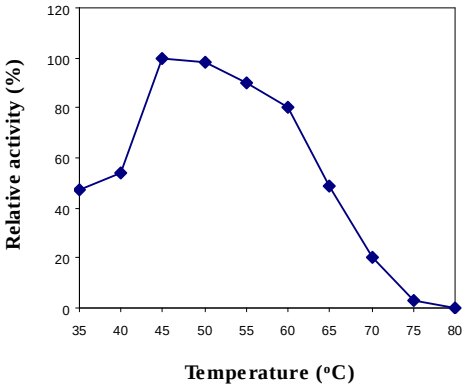

Figure 9. Effect of temperature on activity of xylanase. Results are the average of three replicates.

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