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# COCONUT α-D-GALACTOSIDASE ISOENZYMES: ISOLATION, PURIFICATION AND CHARACTERIZATION*

CESAR V. MUJER†, DOLORES A. RAMIREZ and EVELYN MAE T. MENDOZA

Biochemistry and Genetics Laboratories, Institute of Plant Breeding, University of the Philippines at Los Baños, College, Laguna 3720, Philippines

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**Key Word Index**—*Cocos nucifera*; Palmae, coconut; α-D-galactosidase; enzyme purification; regulation; mutant endosperm.

**Abstract**—α-D-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) from normal coconut endosperm were isolated and partially purified by a combination of ammonium sulfate fractionation, SP-Sephadex C50-120 ion-exchange chromatography and Sephadex G-200 and G-100 gel filtration. Two molecular forms of the enzyme, designated as A and B, were eluted after SP-Sephadex C50-120 ion-exchange chromatography. α-D-Galactosidase A, which is the major isoenzyme, was partially purified 43-fold on Sephadex G-200 and has a MW of about 23 000 whereas α-D-galactosidase B was partially purified 23-fold on Sephadex G-100 and has a similar MW of about 26 600. Both isoenzymes exhibited optimum activity at pH 7.5. The apparent $K_m$ and $V_{max}$ of α-D-galactosidase A were obtained at $3.46 \times 10^{-4}$ M and $1.38 \times 10^{-3}$ M *p*-nitrophenyl α-D-galactoside, respectively. A distinct substrate inhibition was noted. The enzyme was inhibited strongly by D-galactose and to a lesser extent by myo-inositol, D-glucose-6-phosphate, L-arabinose, melibiose and iodoacetic acid. Similarly, makapuno α-D-galactosidase was localized in the 40–70% $(NH_4)_2SO_4$ cut but its optimum activity at pH 7.5 was considerably lower as compared to the normal. Its $K_m$ was obtained at $6.75 \times 10^{-4}$ M *p*-nitrophenyl α-D-galactoside while the $V_{max}$ was noted at $5.28 \times 10^{-3}$ M *p*-nitrophenyl α-D-galactoside. Based on the above kinetic data, the possible cause(s) of the deficiency of α-D-galactosidase activity in makapuno is discussed.

## INTRODUCTION

α-D-Galactosidase catalyses the hydrolysis of an α-D-galactopyranosyl linkage from an alkyl, aryl or a glycosyl (mono or oligo) residue or group [1]. In plants, one of its functions is to cleave α-D-galactosyl groups from α-D-galactose-containing oligo- and polysaccharides. The degradation products in turn serve as a ready source of energy and cell metabolites [2]. In particular, the enzyme activity has been shown to increase during seed germination concomitant with the depletion of galactomannans and other reserve carbohydrates. α-D-Galactosidases have been implicated also with the metabolism of galactolipids [3] and with chloroplast-membrane function [4,5]. The isolation, properties and characterization of these enzymes from animals, plants and micro-organisms have been discussed in two reviews [1,2].

In another paper [6], we reported on the deficiency of α-D-galactosidase activity in the mutant (makapuno) endosperm of coconut and suggested that the deficiency may have caused the aberrant cellular behaviour and properties of makapuno. In order to understand better the regulation of α-D-galactosidase activity, the effects of various effectors on and the physico-chemical and kinetic properties of the partially purified enzyme were investigated. The results may provide vital information on why α-D-galactosidase activity is deficient in makapuno. In this paper, we report the isolation, partial purification, and characterization of α-D-galactosidases from mature coconut endosperms.

## RESULTS AND DISCUSSION

### *Purification of α-D-galactosidases*

*$(NH_4)_2SO_4$ fractionation.* A summary of the purification scheme is presented in Table 1. α-D-Galactosidase was obtained from the 40–70% $(NH_4)_2SO_4$ cut. This step resulted in a 1.8-fold purification with 85% recovery. The fraction accounted for about 47% of the original proteins.

*SP-Sephadex C50-120 ion-exchange chromatography.* The elution profile after ion-exchange chromatography is shown in Fig. 1. Two molecular forms of α-D-galactosidase, designated as A and B, were eluted separately at 0.14 M and 0.24 M NaCl, respectively. α-D-Galactosidase A and B were purified respectively 21- and 5-fold with 63% and 8% yield.

*Sephadex G-200 gel filtration.* The pooled active fractions of α-D-galactosidase A, which was the major isoenzyme, were concentrated to 7 ml by ultrafiltration and applied on a Sephadex G-200 column. A 43-fold purification with 50% recovery was achieved after gel filtration. However, PAGE resolved two broad protein bands with $R_f$ values of 0.63 and 0.72 indicating that the enzyme was not yet pure. SDS-polyacrylamide gel electrophoresis of the same enzyme resolved only one protein band.

*Part 7 in the series on the "Genetics and Biochemistry of Makapuno Coconut Endosperm". For part 6 see ref. [6].

†Present address: Department of Entomology, The International Rice Research Institute, Los Baños, Laguna.

Table 1 Purification of α-D-galactosidase A and B from 11 to 12 month old normal coconut endosperm

| Fraction | Vol (ml) | Total activity (units) | Total protein (mg) | Specific activity (units/mg protein) | Purification factor* | Recovery* (%) |
|---|---|---|---|---|---|---|
| Total crude extract | 752 | 1083 | 195 5 | 5.5 | 1 | 100 |
| 40–70% $(NH_4)_2SO_4$ cut | 41 | 922 | 91.8 | 10.0 | 1.8 | 85.2 |
| SP-Sephadex C50-120 | | | | | | |
| α-D-Gal A | 110 | 678 | 5.9 | 115 5 | 21 | 63 |
| α-D-Gal B | 190 | 89 | 3.2 | 28 | 5 | 8.2 |
| Sephadex G-200 | | | | | | |
| α-D-Gal A | 110 | 542 | 2.3 | 235.8 | 43 | 50 |
| Sephadex G-100 | | | | | | |
| α-D-Gal B | 75 | 3 8 | 0 03 | 127 | 23 | 0.35 |

*The purification factor and % recovery were calculated with respect to the crude extract.

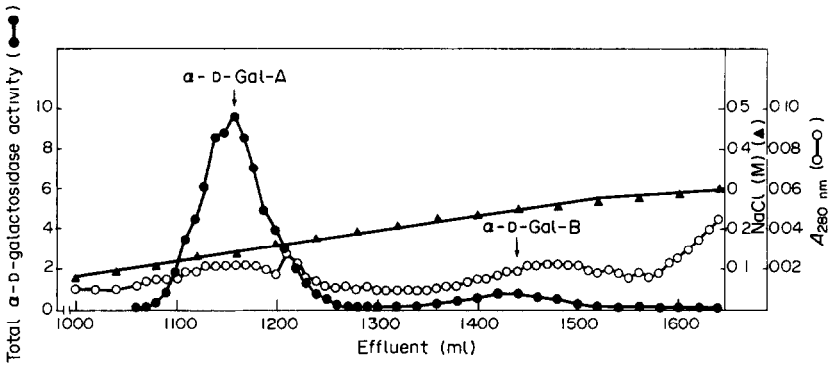


Fig. 1 Elution profile of α-D-galactosidase A and B after SP-Sephadex C50-120 ion-exchange chromatography

*Sephadex G-100 gel filtration.* α-D-Galactosidase B was purified 23-fold with a very low recovery of 0.35% after gel filtration. At least two protein peaks were observed to be under the activity curve indicating the presence of non-enzyme protein impurities.

### *Characterization of α-D-galactosidases*

*pH–activity profile.* Both α-D-galactosidase A and B exhibited optimum activity at pH 7.5. The pH–activity curve of α-D-galactosidase A was more steep as compared to those of B and the crude enzymes from the normal and makapuno. The pH optimum obtained for coconut α-D-galactosidases was close to that reported for *E. coli* which has a pH optimum of 7.6 [7]. However, this is in contrast to a previous report [8] that α-D-galactosidases from coconut kernel exhibited a more acidic pH optimum of 5.6.

*pH stability and optimum temperature.* The α-D-galactosidase obtained from the 40–70% $(NH_4)_2SO_4$ cut was stable over the pH range 4–6.5, when incubated in the appropriate buffer for 30 min at 24°. On further purification with Sephadex G-200, α-D-galactosidase A exhibited a broader range of stability over pH 4–8, when incubated under similar conditions. The enzyme retained 71% and 50% of its activity after incubation at pHs 2.6 and 9 respectively.

The optimum assay temperature of crude α-D-galactosidase was noted at 50°. At higher temperatures of up to 60°, the enzyme activity declined gradually due to thermal denaturation. The enzyme was inactivated completely at 70°. A similar temperature optimum was reported for *M. vinacea* [9].

*Kinetic constant.* A Lineweaver–Burk plot for crude α-D-galactosidase gave apparent $K_m$ and $V_{max}$ values of $3.13 \times 10^{-4}$ M and $2.19 \times 10^{-3}$ M *p*-nitrophenyl α-D-galactoside respectively, but a distinct substrate inhibition was noted. A similar substrate inhibition pattern was observed when α-D-galactosidase A from Sephadex G-200 gel filtration was used (Fig. 2). The apparent $K_m$ of the partially purified enzyme was obtained at $3.46 \times 10^{-4}$ M *p*-nitrophenyl α-D-galactoside while the apparent $V_{max}$ was noted at $1.38 \times 10^{-3}$ M *p*-nitrophenyl α-D-galactoside. The anomalous Lineweaver–Burk plot obtained for α-D-galactosidase has been reported previously by other workers [10,11]. The kinetic properties of makapuno α-D-galactosidase were obtained after partial purification of the enzyme at 40–70% $(NH_4)_2SO_4$ saturation. Makapuno α-D-galactosidase exhibited a slightly higher $K_m$ at $6.75 \times 10^{-4}$ M *p*-nitrophenyl α-D-galactoside. The $V_{max}$ was obtained at $5.28 \times 10^{-3}$ M *p*-nitrophenyl α-D-galactoside. The above kinetic data indicate that the enzymes from the normal and makapuno endosperms share almost similar catalytic properties. Hence, the deficiency of activity in makapuno is not due to a possible mutation of the structural gene for α-D-

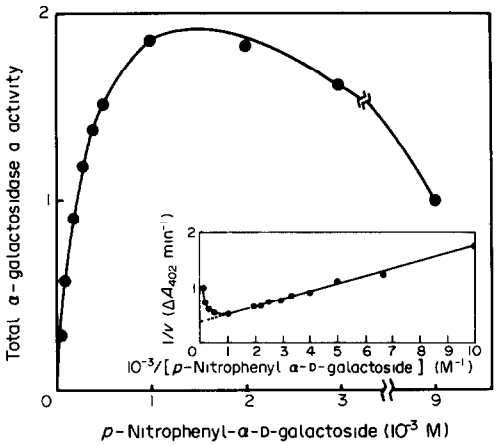


Fig. 2. Saturation curve and double reciprocal plot (inset) of α-D-galactosidase A activity after Sephadex G-200 gel filtration versus *p*-nitrophenyl α-D-galactoside concentration.

galactosidase, but may be due to either a continuous repression of enzyme synthesis or the presence or absence of specific effectors.

*Molecular weights.* Both α-D-galactosidase A and B were monomers with MWs of 23 000 and 26 600 respectively. The MWs of α-D-galactosidase A and B were calculated after Sephadex G-200 and G-100 gel filtration respectively. The elution volumes of the enzymes nearly coincided with that of trypsinogen which has a known MW of 24 000. The MWs of the two isoenzymes were similar to those reported previously from other sources [12, 13]. However, they differed from those of a previous report [8] on the galactosidases of coconut kernel which indicated MWs of 123 000 and 21 400 for α-D-galactosidases A and B respectively.

*Inhibition by sugars and related compounds.* The inhibitory effects of various sugars and related compounds on α-D-galactosidase A activity are summarized in Table 2. The inhibition by galactosides follows the order: galactose > myo-inositol > glucose-6-phosphate > arabinose > melibiose. D-Galactose was found to be a powerful inhibitor. It is possible that the formation of this hexose by α-D-galactosidase-catalysed hydrolysis of galactomannans during the germination of coconut would eventually inhibit the further utilization of the polysaccharides. Dey and Pridham [1] reported also that galactose is a powerful competitive inhibitor of α-D-galactosidases from a number of sources.

The purified galactomannans from coconut endosperms did not inhibit significantly α-D-galactosidase A activity. Likewise, the crude extracts from mature makapuno and young normal endosperms had no inhibitory effects on the enzyme. However, it is possible that the extracts were diluted considerably. Hence, if galactose or other sugar inhibitors were present, their amount may be insignificant as compared to that in the intact cell.

The ability of galactose to inhibit strongly α-D-galactosidase may yet find its application in the artificial induction of makapuno. Exogenous incorporation of this inhibitor or its analogues to the growth medium of normal endosperms cultured *in vitro* or its direct application to naturally growing coconuts may possibly lead to the formation of the makapuno endosperms. Preliminary investigations in this area are currently being undertaken in this laboratory.

Table 2. Inhibition of α-D-galactosidase A-catalysed hydrolysis of *p*-nitrophenyl α-D-galactoside by sugars and related compounds at 30°

| Inhibitor | [Inhibitor] (mM) | Inhibition (%) |
|---|---|---|
| D-Arabinose | 75 | 3 |
| L-Arabinose | 75 | 35 |
| | 50 | 25 |
| | 25 | 20 |
| | 5 | 8 |
| L-Fucose | 75 | 9 |
| D-Galactose | 5 | 88 |
| | 1 | 68 |
| | 0.5 | 46 |
| | 0.1 | 24 |
| D-Glucose | 75 | 5 |
| D-Glucose-6-phosphate | 75 | 60 |
| | 50 | 42 |
| | 25 | 22 |
| | 5 | 4 |
| D-Mannose | 75 | 3 |
| | 25 | 0 |
| Myo-inositol | 75 | 86 |
| | 50 | 80 |
| | 25 | 67 |
| | 5 | 26 |
| Melibiose | 75 | 25 |
| Sucrose | 75 | 0 |
| Coconut endosperm galactomannans | 0.005% | 5 |
| | 0.010% | 7 |
| | 0.025% | 24 |

*Inhibition by sulfhydryl-specific reagents.* The inhibition of α-D-galactosidase A by sulfhydryl specific reagents is presented in Table 3. Iodoacetic acid, but not iodoacetamide and dithiothreitol, inhibited the enzyme. This suggests that a sulfhydryl group participates in the enzyme catalysis. Other α-D-galactosidases that require –SH groups for activity were reported in several microorganisms [14–16]. At 0.5 M, potassium and sodium did

Table 3. Inhibition of α-D-galactosidase A by cations and sulfhydryl-specific reagents at 30°

| Inhibitor | [Inhibitor] (mM) | Inhibition (%) |
|---|---|---|
| Iodoacetic acid | 0.075 | 96 |
| | 0.050 | 66 |
| | 0.025 | 27 |
| | 0.005 | 6 |
| Iodoacetamide | 0.075 | 0 |
| Dithiothreitol | 0.075 | 0 |
| $Mn^{2+}$ | 0.002 | 0 |
| $K^+$ | 0.5 | 0 |
| $Na^+$ | 0.5 | 0 |

not inhibit or activate α-D-galactosidase A. Similarly, manganese (0.002 M) had no effect on enzyme activity.

## EXPERIMENTAL

*Endosperms.* Makapuno (mmm) coconut endosperms (11–12 mo) were collected from embryo-cultured makapuno trees (*Cocos nucifera* L, var Laguna). Likewise, normal (MMM) coconut endosperms were obtained from true-breeding normal trees. The endosperms were cut into small cubes, placed inside a perforated plastic bag and frozen immediately They were lyophilized to constant weight and stored dessicated at −10° until use

*Enzyme extraction.* The lyophilized normal endosperm (240 g) was homogenized with 1.44 l 0.05 M acetate buffer, pH 4, for 5 min in a pre-chilled Waring blendor. The slurry was centrifuged at 12 000 rpm for 15 min at 2–8° and the supernatant was filtered by suction through Whatman filter paper no. 3. Further purification steps were conducted at 4° unless specified otherwise.

$(NH_4)_2SO_4$ *fractionation.* 752 ml of the crude extract was brought to 40% satn by adding 174 g of solid $(NH_4)_2SO_4$. The soln was stirred continuously for 30 min at 0° After standing 18 hr, the soln was centrifuged at 12 000 rpm for 15 min at 0–8° and the ppt was discarded. The supernatant (821 ml) was brought from 40% to 70% satn by adding 156 5 g solid $(NH_4)_2SO_4$ and centrifuged as described after continuous stirring for 30 min and standing for 2 hr. The ppt was dissolved with 40 ml of the same buffer and dialysed twice against 4 l of the same buffer for 24 hr. A small amount of inactive ppt was removed by centrifugation as described.

*SP-Sephadex C50-120 ion-exchange chromatography* 43 ml of the 40–70% $(NH_4)_2SO_4$ dialysed fraction containing 92 mg protein was applied to a SP-Sephadex C50-120 column equilibrated with 0.05 M acetate buffer, pH 4. The proteins were eluted with a linear gradient of from 0 to 0.5 M NaCl in the same buffer in a total vol. of 1700 ml at a flow rate of 171.4 ml/hr. 10 ml fractions were collected and alternate fractions were assayed for α-D-galactosidase activity and protein concentration.

*Sephadex G-200 and G-100 gel filtration.* The active fractions from SP-Sephadex C50-120 ion-exchange chromatography were pooled and concd by ultrafiltration using Amicon stirred cells with UM 10 Diaflo ultrafilters The system was operated at 60 psi (4 atm) $N_2$ pressure at 24° The major peak (α-D-galactosidase A) containing 5.9 mg proteins in 7 ml 0.05 M acetate buffer, pH 5, was subjected to Sephadex G-200 gel filtration. Likewise, the minor peak (α-D-galactosidase B) containing 3 24 mg proteins in 3 ml of the same buffer was subjected to Sephadex G-100 gel filtration in a separate column equilibrated under similar conditions The proteins were eluted with the same buffer at a flow rate of 18 ml/hr 10 and 5 ml fractions were collected for α-D-galactosidase A and B respectively, and alternate fractions were assayed for enzyme activity and protein content. The active fractions were pooled and stored at 4° MWs of α-D-galactosidase A and B were determined by using the following protein standards cytochrome *c* (12 384), trypsinogen (24 000), egg albumin (43 000) and catalase (240 000). All gel permeation chromatography runs were done at 23°

*Electrophoresis.* PAGE was performed as described by Davis [17] on 7.5% polyacrylamide gel (10 × 0.5 cm i.d.) at 4° and at a current of 4 mA/gel cylinder. Protein band patterns were visualized by incubating the gels with 1% amido black in 7% HOAc for 30 min and destaining in 7% HOAc for 18 hr. SDS-PAGE was performed as described by Weber and Osborn [18].

*α-D-Galactosidase assay* α-D-Galactosidase activity was determined spectrophotometrically by measuring the increase in absorbance at 280 nm or by the method of Lowry *et al.* [19] but described previously [6].

*Protein determination* Total protein was determined either by absorbance at 280 nm or by the method of Lowry *et al.*[19] but with some modifications.

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