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GALACTOMANNAN DEGRADING ENZYMES IN MATURING NORMAL AND MAKAPUNO AND GERMINATING NORMAL COCONUT ENDOSPERM

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Key Word Index—*Cocos nucifera*; coconut; α-D-galactosidase; β-mannanase; β-mannosidase; galactomannan; makapuno.

Abstract—β-Mannosidase activity in the maturing normal endosperm of coconut decreased from 1.40 milliunit/mg protein at 7–8 months to 0.29 milliunit/mg protein at 11–12 months. It was 6–10 times lower at 7–8 and 8–9 months and was not detected at the more mature stages of makapuno. In contrast, the liquid endosperm from normal nuts increased in α-D-galactosidase, β-mannanase and β-mannosidase activity as development progressed. In makapuno liquid endosperm, the values for all the enzymes were consistently low during endosperm maturation. During germination, α-D-galactosidase activity in the solid endosperm increased from 0.51–0.84 unit/mg protein. β-Mannanase and β-mannosidase activity increased from 33.12 to 67.76 milliunit/mg protein and 5.13 to 43.05 milliunit/mg protein, respectively. In the haustorium, α-D-galactosidase activity remained at 0.25–0.27 unit/mg protein while β-mannanase increased from 55.34 to 138.12 milliunit/mg protein. β-Mannosidase decreased from 371.79 to 165.17 milliunit/mg protein. The implications of enzyme levels of α-D-galactosidase, β-D-mannanase and β-D-mannosidase on the mode of galactomannan degradation in the developing and germinating nut and phenotype determination of both normal and makapuno nuts are discussed.

INTRODUCTION

Makapuno is a mutant coconut found among normal nuts. These nuts are characterized by a thick, soft and fluffy solid endosperm and a very viscous fluid which completely fills the centre of the nut. Earlier studies in our laboratory revealed that makapuno endosperm contains significantly higher amounts of viscous component than the normal [1]. This viscous component was found to consist of water-soluble galactomannan with a mannose:galactose ratio of 3:1 [2, 3]. These results indicate that the makapuno phenotype is a consequence of the accumulation of galactomannan in the makapuno endosperm.

Makapuno nut is considered a lethal mutant because of its inability to germinate. However, embryo culture technique has been successfully employed to grow makapuno trees which could yield 100% makapuno nuts [4].

Galactomannans are common major carbohydrate reserves in seed endosperm of plants that belong to the families *Anonaceae*, *Convulaceae*, *Leguminosae*, *Palmae* and *Rubiaceae*. Degradation of galactomannans involves three enzymes: α-D-galactosidase [α-D-galactoside galactohydrolase, EC 3.2.1.22], β-mannosidase [β-D-mannoside mannohydrolase 3.2.1.25] and β-mannanase [(1–4) β-D-mannan mannohydrolase EC 3.2.1.78]. Several studies on mobilization of galactomannans during germination have been reported for fenugreek (*Trigonella foecum-graecum*) [5] and some legume seeds such as lucerne, guar, carob and soybean [6].

Some of the abnormal cellular properties and the non-germination of the makapuno could result from an altered galactomannan metabolism. Thus, as part of our efforts to elucidate the abnormal cell growth of makapuno, we have worked on the enzymes involved in galactomannan degradation. Earlier reports showed a much greater level of the α-D-galactosidase activity in the normal than in the makapuno [7]. Kinetic data also showed the similar properties for the enzymes from the two types of coconut [8]: This paper reports on the two other enzymes, β-mannosidase and β-mannanase, involved in galactomannan degradation during endosperm development and on all three enzymes during germination.

RESULTS AND DISCUSSION

During maturation

α-D-*Galactosidase*. A previous study [7] showed that α-D-galactosidase activity continually increased significantly with age in the normal solid endosperm from 0.07 to 1.71 unit/mg protein (Table 1). In contrast, this enzyme in the makapuno solid endosperm was detectable only at 11–12 months and was 8300-fold lower than normal.

In the liquid endosperm of the normal coconut, α-D-galactosidase also increased significantly and was 200- tc

*The major portion of this study was obtained from the MS thesis of the senior author.

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Table 1. α-D-Galactosidase activity in developing normal and mutant (makapuno) coconut solid and liquid endosperms

Age* (months)	Specific activity (unit/mg protein)			
	Solid endosperm†		Liquid endosperm ($\times 10^3$)	
	Normal	Makapuno	Normal	Makapuno
7–8	0.07 d‡	0	0.74 d	0
8–9	0.23 d	0	0.64 d	0
9–10	0.59 c	0	1.46 c	0
10–11	0.99 b	0	2.09 b	0
11–12	1.71 a	slight§	3.06 a	0

*Age in months after pollination.

†Data from Mujer *et al.* [7]

‡Means followed by the same letter within each category are not significantly different from each other at 0.05 level of significance [Duncan's Multiple Range Test (DMRT)].

§8300-fold lower.

1000-fold lower than in the solid endosperm (Table 1). This enzyme was not detected in the makapuno liquid endosperm.

The increase in α-D-galactosidase activity in both the solid and the liquid endosperms of the maturing coconut was accompanied by a two-fold decrease in water-soluble galactomannan and a four-fold increase in its mannan fraction [2, 3]. In maturing makapuno, opposite trends were observed: a 50–100% increase in water-soluble galactomannan and a two-fold decrease in mannan. An apparent increase in α-D-galactosidase activity has been observed to accompany the synthesis of galactosylsucrose derivatives in maturing seeds such as *Vicia faba* [9] and *Phaseolus vulgaris* [10].

β-Mannanase. β-Mannanase activity decreased significantly in both the normal and makapuno solid endosperm (4- and 30-fold from 7–8 months to 11–12 months old endosperm, respectively; Table 2). It was also consistently higher in makapuno than in normal except in the 11–12 month sample.

β-Mannanase in the liquid endosperm was eight- and 300-fold lower than in the solid endosperm. The trend, however, was different; there was a significant increase from 0.34 milliunit/mg protein at 7–8 months to 1.86 milliunit/mg protein at 11–12 months in the normal nut. However, in makapuno, the increase was from 0.22 milliunit/mg of protein at 7–8 months to 0.78 milliunit/mg protein at 10–11 months followed by a 50% drop in activity at 11–12 months. The differences in the β-mannanase activity with regard to phenotype and age were both highly significant.

These results indicate an active degradation of the polymer in both types of nuts during maturation. The higher mannanase activity in makapuno solid endosperm could also account for its shorter and less viscous galactomannan [2].

β-Mannosidase. β-Mannosidase activity decreased significantly from 1.40 milliunit/mg protein at 7–8 months to 0.29 milliunit/mg protein at 11–12 months in the endosperm of the normal (Table 3). In the makapuno endosperm β-mannosidase also decreased significantly from 0.19 milliunit/mg protein at 7–8 months to zero at 11–12 months.

In the liquid endosperm of the normal coconut, β-mannosidase increased significantly from 0.19 milliunit/mg protein at 7–8 months to 1.52 milliunit/mg protein at 11–12 months. However, this enzyme only slightly increased in the liquid endosperm of the makapuno. The difference between normal and makapuno for both solid endosperm and liquid endosperm was highly significant.

The lower levels of β-mannosidase and β-mannanase in the mature samples suggest that less amounts of sources of energy and metabolites were being produced by the cell and indicate a slowing down of the mature cell's metabolism.

Table 2. β-D-Mannanase activity in developing normal and makapuno coconut solid and liquid endosperms

Age* (months)	Specific activity (milliunit/mg protein)			
	Solid endosperm		Liquid endosperm	
	Normal	Makapuno	Normal	Makapuno
6–7	69.38 a†	—	—	—
7–8	28.31 b	60.02 a	0.34 c	0.22 c
8–9	29.14 b	48.01 a	0.38 c	0.26 c
9–10	17.21 b	36.08 b	0.50 c	0.55 b
10–11	16.93 b	41.63 a	0.95 b	0.78 a
11–12	16.10 b	2.22 c	1.86 a	0.31 c
Normal vs makapuno	**		**	
Age	**		**	
Interaction			**	

*Age in months after pollination.

†Means followed by the same letter are not significantly different at 5% level of probability (DMRT).

**Highly significant.

Table 3. β-Mannosidase activity in developing normal and makapuno coconut solid and liquid endosperms

Age* (months)	Specific activity (milliunit/mg protein)			
	Solid endosperm		Liquid endosperm	
	Normal	Makapuno	Normal	Makapuno
7–8	1.40 a†	0.19 a	0.19 c	0.11 b
8–9	0.57 b	0.11 b	0.14 c	0.12 b
9–10	0.43 c	0.00 c	0.20 c	0.16 ab
10–11	0.26 d	0.00 c	0.69 b	0.22 a
11–12	0.29 d	0.00 c	1.52 a	0.21 a
Normal vs makapuno	**		**	
Age	**		**	
Interaction	**		**	

*Age in months after pollination.

†Means followed by the same letter within each category are not significantly different at 5% level of probability (DMRT).

**Highly significant.

During germination

During germination the solid endosperm decreases four-fold from zero to six months. At one month the liquid endosperm has decreased by 50% and the haustorium has grown to almost one-third of the space inside the nut. At two months, the liquid endosperm has disappeared and the haustorium fills the space inside the nut [11]. Therefore, for germination, enzyme activities were determined in the solid endosperm and haustorium only.

α-D-Galactosidase. α-D-Galactosidase activity increased from 0.51 to 0.837 unit/mg protein at 0.5 months and 7–8 months, respectively, in the endosperm (Table 4). Its activity in the haustorium ranged from 0.25 to 0.45 unit/mg protein. The enzyme levels in the endosperm and haustorium during germination were comparable with the activities observed in the developing endosperm. These results indicate that degalactosylation occurs extensively throughout the normal nut's development and germination, a step needed for more efficient action by the two other enzymes on the resulting mannan polymer. In cotton seeds [12] and carob seeds [13], an increase in α-D-galactosidase levels was observed during seed germination concomitant with the depletion of reserve polysaccharide. McCleary and Matheson [6] observed an increase and then decrease in α-D-galactosidase activity in seeds of lucerne, guar, carob and soybean accompanied by a depletion of galactomannan.

β-Mannanase. β-Mannanase activity in the solid endosperm of the germinating nut increased significantly by two-fold from 33.12 milliunit/mg protein at 0.5 month to 67.76 milliunit/mg protein at six months (Table 5). In the

Table 4. α-D-galactosidase activity in germinating normal coconut solid endosperm and haustorium

Age† (months)	Specific activity (unit/mg protein)	
	Solid endosperm	Haustorium
0.5	0.511 d‡	— a
1	0.412 e	0.249 a
2	0.391 e	0.251 a
5	0.782 c	0.450 a
6	0.917 a	0.305 a
7	0.837 b	0.270 a
Endosperm vs Haustorium	*	
Age	*	

†Age in months after sowing nuts in a seed bed.

‡Means in a column followed by the same letter are not significantly different at 5% level of probability (DMRT).

*Significant.

Table 5. β-Mannanase activity in germinating normal coconut solid endosperm and haustorium

Age* (months)	Specific activity (milliunit/mg protein)	
	Solid endosperm	Haustorium
0.5	33.12 b†	55.34 d
1	33.83 b	72.39 cd
2	53.36 ab	89.36 c
3	58.68 ab	21.32 e
4	39.66 b	220.50 a
5	43.73 ab	131.86 b
6	67.76 a	138.12 a
Endosperm vs Haustorium	**	
Age	**	
Interaction	**	

*Age in months after sowing nuts in a seed bed.

†Means followed by a common letter within each category are not significantly different at 5% level of probability (DMRT).

**Highly significant.

Table 6. β-Mannosidase activity in germinating normal coconut solid endosperm and haustorium

Age* months	Specific activity (milliunit/mg protein) Solid endosperm	Haustorium
0.5	5.13 b†	371.79 a
1.0	30.94 ab	299.51 b
2.0	32.33 ab	169.02 d
3.0	27.58 ab	206.75 c
4.0	14.04 ab	112.44 e
5.0	33.98 ab	163.95 d
6.0	43.05 a	165.17 d
Endosperm	**	
Age	**	
Interaction	**	

*Age in months after sowing nuts in a seed bed.

†Means followed by a common letters within each category are not significantly different at 5% level of probability (DMRT).

**Highly significant.

haustorium the activity also increased significantly from 55.34 to 138.12 milliunit/mg protein at 0.5 and 6 months, respectively. β-Mannanase activities in lucerne, soybean and carob seeds increased and decreased during germination, paralleling galactomannan degradation [6].

β-Mannosidase. This enzyme was also found to be very active in the solid endosperm of the germinating nut. Its activity increased eight-fold from 5.13 milliunit/mg protein at 0.5 month to 43.05 milliunit/mg protein at 6.0 month. In the haustorium the activity decreased more than two-fold from 371.79 milliunit/mg protein at 0.5 month to 165.17 milliunit/mg protein at 6.0 month. β-Mannosidase levels in lucerne and honey locust varied little during germination although certain isozymes of mannosidase in guar and carob started very high and decreased during germination [6].

The high activity of α-D-galactosidase and even much higher activities of β-mannanase and β-mannosidase account for the large decrease in hydrolysable polysaccharides in the haustorium and solid endosperm of the germinating coconut [11]. This is accompanied by a four-fold decrease in total soluble sugars and reducing sugars in the solid endosperm and 10-fold increase in the haustorium [11]. Similar trends for the soluble sugars were earlier reported by Balasubramaniam *et al.* [15].

CONCLUSIONS

Previous results showed the deficiency of α-D-galactosidase in makapuno which permits the accumulation of the viscous water-soluble galactomannan giving makapuno its soft and fluffy endosperm phenotype [7]. The results of this study further show that the shorter and less viscous galactomannan of makapuno [2] could be due to the higher activity of β-mannanase in makapuno than in normal.

On the other hand, barely detectable levels of α-D-galactosidase and β-mannosidase in the makapuno compared to the much higher levels in the normal could result in the little or non-mobilization of the galactomannan indicating lack of nutrients and energy for the embryo, and thus, could contribute primarily to the non-germination of makapuno.

In the germinating normal nut, all three enzymes were active in both solid endosperm and haustorium. β-Mannanase and β-mannosidase activities were notably higher in the haustorium than in the endosperm indicating that the greater rate of degradation of smaller galactomannan polymers to smaller units occurs in the haustorium for uptake by the growing plant.

EXPERIMENTAL

Materials. The plant materials used in the study were normal and makapuno nuts of the Laguna Tall Green Variety. The developing and germinating nuts were obtained from the Department of Horticulture, University of the Philippines at Los Baños, College of Agriculture. The classification of developmental stage was based on the age of the nut in months after pollination for the developing nut and in months after sowing for the germinating nut.

Preparation of samples for enzyme determination. The endosperms were diced into small pieces. The endosperm (10 g) was homogenized in 20 ml of 0.05 M McIlvaine buffer pH 5 at 4° for 10 min. The resulting slurry was pressed through 3 layers of cheesecloth and the filtrate was centrifuged at 10000 rpm for 15 min at 4°. The clear supernate was then placed in an Erlenmeyer flask and analysed for enzyme activity. The same extraction procedure was done on the haustorium of germinating coconuts. Coconut water of developing normal and makapuno nuts was also analysed for galactomannan degrading enzymes after filtration through Whatman filter paper no. 1 at 4°. The conditions used for the assay of coconut β-mannanase and β-mannosidase were first determined and will be reported in another paper.

α-D-Galactosidase assay. Enzyme soln (25 μl) was incubated with 5 μl of 0.01 M *p*-nitrophenyl α-D-galactopyranoside in 300 μl of 0.05 M K-Pi buffer pH 7.5 for 5 min at 30°. Reaction was stopped with 2.5 ml of 0.1 M Na_2CO_3 and absorbance at 420 nm determined [14]. One unit of α-D-galactosidase activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min at 30°, pH 7.5 under specified conditions as earlier reported [7].

β-Mannanase. Coconut galactomannan solution (1%, 100 μl) was pre-incubated with 0.05 M McIlvaine buffer pH 5.8 (300 μl) for 5 min at 4° after which the enzyme preparation (50 μl) was added and further incubated for 15 to 30 min. The reaction was stopped with dinitrosalicylic acid reagent (1 ml). The resulting mixture was heated at 100° for 15 min, cooled to room temp. and read at 540 nm [16]. One unit of β-mannanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar and estimated as glucose at 40° and pH 5.8 under the specified conditions.

β-Mannosidase assay. Enzyme soln (25 μl) and 0.05 M McIlvaine buffer pH 5.0 (300 μl) were incubated for 5 min at 50°. To this mixture, 0.01 M *p*-nitrophenyl mannopyranoside (25 μl) was added. The mixture was further incubated for 5 min, the reaction stopped by adding 0.1 M Na_2CO_3 soln (2.5 ml) [5] and absorbance read at 402 nm.

One unit of β-mannosidase activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min at 50° and pH 5.0 under the specified conditions.

Protein determination. Total protein was determined by the method of ref. [17] with some modifications.

Statistical design and analysis. Each enzyme was analysed from a minimum of 3 nuts coming from 3 different trees.

Two subsamples were done per nut. Split-plot in complete randomized design was used in the statistical analysis of the data.

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