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Starch Properties of the Sago Palm (*Metroxylon sagu* Rottb.) in Different Soils

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Abstract. We investigated the relationships between starch concentrations and activities of starch synthetic enzymes in sago palms (*Metroxylon sagu* Rottb.) under acid sulfate and mineral soil conditions. Plants grown naturally that had reached their maturated stage were sampled. We found that the growth in acid sulfate soil is lower than that in mineral soil and that starch granules were larger and there was more amylase activity in acid sulfate soil than in mineral soil. Lower amylase activity in mineral soil could eliminate the degradation of starch, making the smaller granules suitable for storing large amounts of starch in a limited space inside cells.

Key words: Acid sulfate soil, *Metroxylon sagu,* Oxisol, Sago palm, Starch accumulation, Starch enzymes

Introduction

The sago plant (*Metroxylon sagu* Rottb.) may be a good alternative starch-producing plant [1]. Furthermore, the advantage of this plant is its ability to survive in adverse environments, including peat soil, submerged soil, acid soil, and saline soil. The importance of starch production by sago palm has been focused especially in Asia-Pacific region [2] and South East Asia [3]. And the amount of starch accumulated in the trunk of the sago palm can reach about 250 kg (dry weight per plant) [4], the starch accumulation is not high in those adverse soils [5]. When sago starch was compared among samples obtained from different location in Papua New Guinea, the morphological characteristics were identical [6]. The sago palm grows more slowly in peat soil than in mineral soil [7]. Starch quality of sago is studied from the viewpoint of size [8], amylose and amylopectin concentration [9, 10], and gelatinization and viscosity [11–15]. Recently, the importance of sago starch increased not only for the production of glucose, but for the production of generic fermentation medium [16]. In this report, we focus on the effect of growth condition (peat soil vs. mineral soil) on the formation of starch in the sago palm and the biochemical properties of starch-synthesizing enzymes. We determined the activity of amylase and starch branching enzyme to make clear the difference in the accumulation of starch in sago palms under different environmental conditions. Granule-bound starch synthase was purified to compare the amino acid sequence among plant species.

Materials and Methods

All the reagents except for acrylamide were purchased from Wako Pure Chemicals (Japan), and acrylamide was purchased from Sigma Aldrich (USA).

Study Site

Sago palms were collected from two different soils which are located very near each other in To Daeng, Narathiwat Prefecture, Thailand. It is said that one site's soil is typically acid sulfate soil (Lu-Po-Sa-Ma) and the other site's soil is mineral soil (oxisol) (Ban-Mai); we also sampled soil from each site to confirm the soil characteristics.

Plant Material

Sago palms (*Metroxylon sagu*) used in experiments were grown naturally in acid sulfate and mineral soils for around 10 years. One plant was selected from each soil which has average height and diameter in each site. Each plant was cut at the bottom, then separated into 50-cm length beginning at the bottom. Sampling was done within 1 week in July, hence the seasonal variation can be omitted.

The fresh weight of each segment was measured, and then a part was cut out of each segment to determine the dry weight. Fresh samples were scraped from the cut end of each segment and then stored at 4 °C in the field and kept under −20 °C in the laboratory until their starch concentration, enzyme activity, and protein extraction were analyzed.

Soil Analysis

Twenty-five milliliters of distilled water or 1 M KCl was added to 10 g of soil prior to shaking at room temperature for 1 hour, and then pH (H_2O) and pH (KCl) were determined. Available phosphate was determined by the Bray II method. To determine exchangeable potassium, magnesium, calcium, and sodium, 10 g of soil was extracted by 1 *M* ammonium acetate, and then potassium and sodium concentration were determined by the flame photoemission method, and calcium and magnesium were determined by the atomic absorption method. Total nitrogen was extracted

with 2 *M* KCl solution, and then the amount of nitrogen was determined by the Kjeldhal method [17]. Organic carbon concentration was determined by the Tyurin method [18].

Determination of Starch Concentration

Plant tissue (3 g) was incubated in 30 ml of 0.2% NaOH for 6 hours to remove protein, and then homogenized by Polytron (Kinematica, Switzerland) and filtered with doubled cheese cloth. Next, the filtrate was washed with acetone followed by washing with distilled water three times to remove fat. After washing, the filtrate was dried at room temperature, and we determined its starch concentration by the Phenol-H_2SO_4 method [19]. Although this procedure also counted for other glucose present in other components, as the starch content of the sample was high, we considered that the obtained value can represent the starch content in the sample.

Observation of Starch Granules by Optical Microscope

To observe the shape of the starch granules, starch was extracted by mashing the plant tissue on slide glass. Staining of starch granules was done by iodine staining [20]. We observed them with an optical microscope (Olympus, BH-2). As the diameters of observed starch granules were lower than 15 μm and we have divided them into two groups: large granules of 8- to 15-μm diameter, and small granules of <8 μm. The classification was done on the randomly collected sample under microscopy.

Preparation of Enzyme Extract

Plant tissue (5 g) was homogenized with the extraction buffer [20 ml of 20 mM Tris-HCl containing 1 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid (pH 7.5)] by Polytron. The homogenate was further extracted for 30 min at 4 °C with stirring, and then the supernatants were obtained by centrifugation (10,000 *g*, for 20 min) for the subsequent enzyme assay. The purified starch was prepared from the precipitates in the extraction buffer. The filtrate was washed and dried by the same method as was used for starch concentration determination as shown above. It seems that as the precipitation was not containing other visible contaminant, we have decided that the precipitation has enough purity for the subsequent analysis.

Assay of Starch Branching Enzyme

Starch branching enzyme (SBE) activity was determined by two different methods. Assay I was the iodine-staining method of Boyer and Preiss [21]. One unit of activity was defined as the amount of enzyme decreasing 0.1 of absorbance at 660 nm per min at 30 °C. The second method was a phosphorylase *a* stimulation assay (assay P) also described by Boyer and Preiss [21]. One unit of activity was defined as the amount of enzyme incorporating 1 μmol of glucose into methanol precipitate per minute at 30 °C The absorbance was determined by colorimeter (Shimadzu UV-1600, Japan). These methods are distinct because assay P could evaluate the SBE activity only, while assay I evaluates not only SBE activity but also amylase degradable activity. Thus, by comparing both assays, we could estimate the contribution of amylase activity.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done using 10% polyacrylamide gel [22]. Subjected samples of enzyme extract were prepared by boiling them for 10 min in an equal volume of SDS sample buffer. The proteins bound to starch granules were prepared by boiling the samples for 10 min followed by adding SDS sample buffer to the purified starches at a ratio of 15:1 (v/w). After centrifugation, each aliquot (10 μl for enzyme extract and 30 μl for starch granule-bound protein) was subjected to SDS-PAGE with a molecular marker. After electrophoresis, proteins were detected by silver staining (Nakalai Tesque, Sil-Best stain, Japan). Electrophoresis was done by using Bio-Rad Protean II (USA).

Purification and Sequencing of Granule-Bound Protein

Four percent SDS containing 6% 2-mercaptoethanol was added to 20 g of the purified starch at a ratio of 20:1 (v/w). After being boiled for 10 min, the paste was cooled at -80 °C for 30 min. Subsequently the frozen paste was melted at room temperature and then centrifuged to obtain the supernatant. Three volumes of cold-acetone were added to the supernatant and centrifuged again. The obtained precipitate was subjected to SDS-PAGE, and the proteins in the gel were then transferred onto the PVDF membrane electrophoretically (Bio-Rad Trans Blot SD, USA). The 58-kDa protein on the PVDF membrane was cut and sequenced in the protein sequencer (Applied Biosystems 477A, USA).

Results

Soil Characteristics

Lu-Po-Sa-Ma soil was acid sulfate soil with very low pH (4.0 in water and 3.3 in KCl), the pH of mineral soil in Ban-Mai was also low (4.6 in water) (Table 1). The low organic carbon concentration seems to reflect the poor vegetation in acid sulfate soil (about one-third of that in mineral soil). Cation exchange was very low in both sites, and the relative concentration of K was very low in acid soil. These

Table 1. Soil characteristics in Lu-Bo-Sa-Ma (acid sulfate soil) and Ban-Mai (mineral soil) sites

Soil name	pH (H_2O)	pH (KCl)	Exchangeable cations (meq/100 g)				Total *N* (%)	Org. C (%)
			Ca	Mg	K	Na		
Lu-Bo-Sa-Ma	4.00	3.30	0.99	0.57	0.14	0.13	0.34	4.4
Ban Mai	4.57	4.25	0.32	0.27	0.29	0.14	0.84	12.3

characteristics observed in acid soil indicates its poor productivity of above vegetation.

Vertical Distribution of Fresh Weight of Sago Trunk

The vertical distribution of fresh weight is shown in Figure 1. Although the palms at both sites were assumed to have reached their maturation stage, their amounts of growth are quite different. The plant height at Lu-Po-Sa-Ma was a little less than that at Ban-Mai. At the Ban-Mai site, the fresh weight was equally distributed up to 800 cm from the bottom, while the distribution was relatively low at lower parts of plant at Lu-Po-Sa-Ma. Dry matter concentration is higher in mineral soil (about 0.40) than in acid sulfate soil (about 0.30) (data not shown).

Starch Concentration

The starch concentration at each position within the palms is shown in Table 2. The starch storage positions differed in the palms grown in acidic and mineral soils. In plants grown in mineral soil, most starch was accumulated below the 600-cm height, with the maximum at the position of 300–350 cm, and almost no starch contained in the top (850–920 cm). Conversely, starch concentrations of plants grown in acidic soil were higher at the top, with the maximum at the height of 700–780 cm as shown in Table 3.

Microscopic Observation of Starch Granules

Figures 2 and 3 showed micrographs of starch granules obtained from different heights of sago palms grown in mineral and acid sulfate soils. There were no morphological differences in the starch granules among the soil conditions and the positions. Their shapes were ellipsoidal and contained granules lacking some of their parts, concentric circular rings inside as observed in most starch granules from other plants were not clearly observed. On the other hand, the granules were clearly divided into two groups according to size. The large granules were 8–15 μm, and the small ones were less than 8 μm in lengthwise diameters. Few middle-sized granules were found. Table 3 shows the relative ratios between large and small granules at the different positions. In plants grown in both types of soil

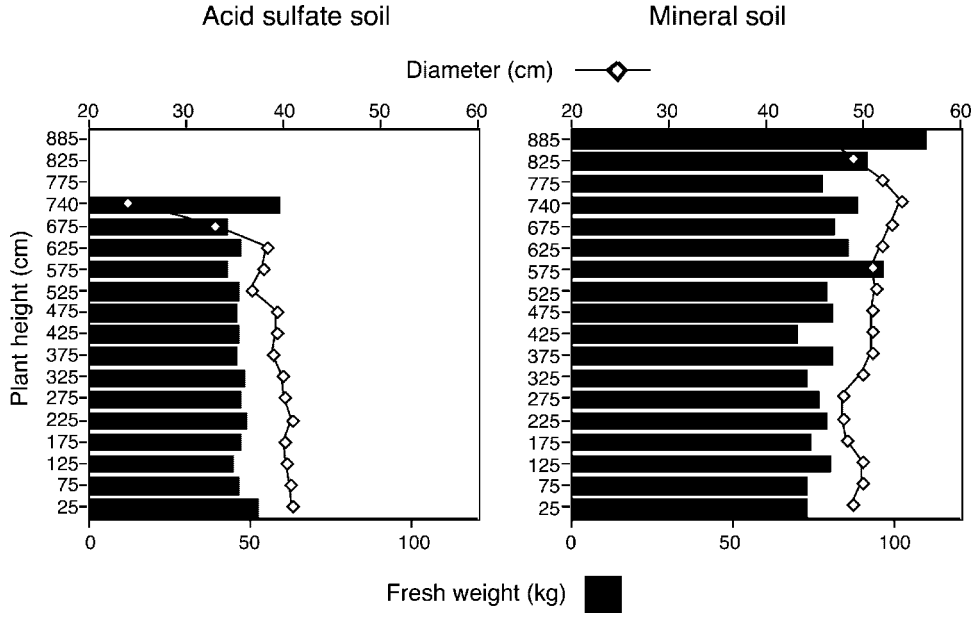

Figure 1. Horizontal distribution of fresh weight and diameter. Sago palms were cut down and cut into segments. Height is expressed from the bottom. Diameter is measured from the bottom of each side section.

Table 2. Starch concentration in each position at height

Soil condition and plant height	Starch contents mg of dried starch/g of fresh tissue
Mineral soil (cm)	
0–50	101.3
300–350	147.1
600–650	32.4
850–920	1.1
Acid sulfate soil (cm)	
0–50	22.5
300–350	11.7
600–650	71.3
650–700	108.7
700–780	81.2

Table 3. Consist of large and small starch granules

Soil condition and plant height	No. of large granules (a)	No. of small granules (b)	$b/(a+b)$
Mineral soil (cm)			
0–50	81	60	0.43
300–350	116	62	0.35
600–650	119	61	0.34
850–920	110	27	0.20
Acid sulfate soil (cm)			
0–50	115	10	0.08
300–350	117	19	0.14
600–650	81	16	0.16
650–700	117	16	0.12
700–780	133	40	0.23

Note. The granule more than 8 μm in long diameter belongs to the large and the shorter belongs to small granule.

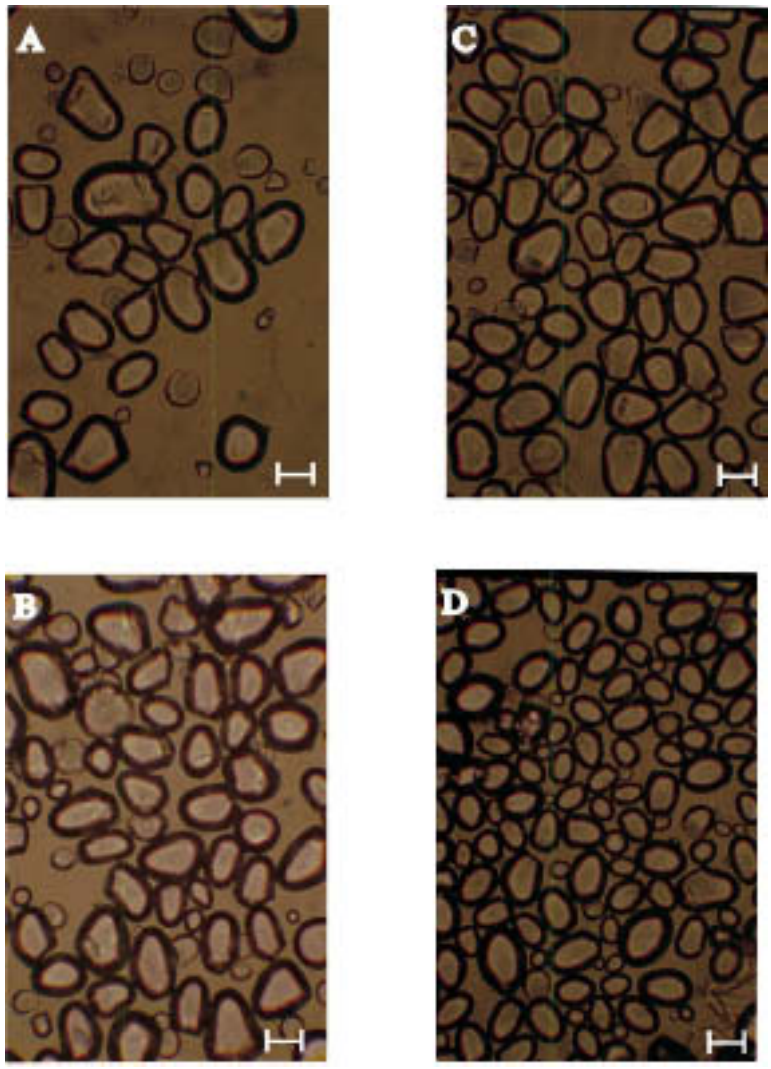

Figure 2. Micrographs of starch granules grown in mineral soil. A, B, C, and D indicate the iodine-stained starch granules from the positions of 0–50, 300–350, 600–650, and 850–900 cm heights, respectively. Bars indicate 6.9 μm.

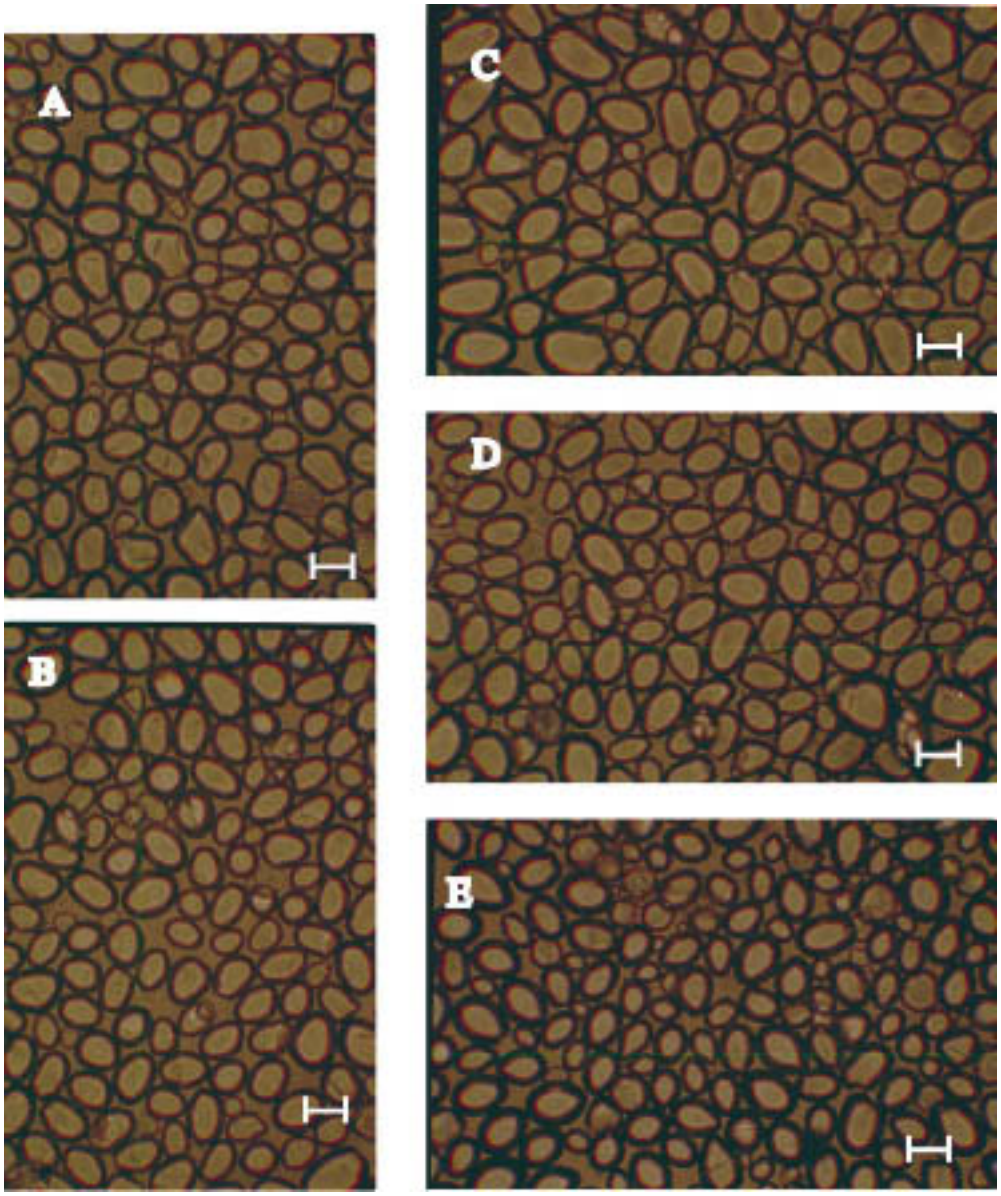

Figure 3. Micrographs of starch granules grown in acid sulfate soil. A, B, C, D, and E indicate the iodine-stained starch granules from the positions of 0–50, 300–350, 600–650, 650–700, and 700–780 cm heights, respectively. Bars indicate 6.9 μm.

studied, the ratio of the number of large granules is higher than that of small ones (Figures 2 and 3). However, the ratio of small granules in plants grown in mineral soil was clearly higher than that of small granules in plants grown in acidic soil at most positions. In particular, in plants grown in mineral soil, 43% of granules were small at the position of 0–50 cm.

SBE Activity

Figure 4 shows SBE activities in the enzyme extracts from different positions. In plants grown in mineral soil, SBE activities by assay I markedly increased at the top. On the other hand, the activity at the middle position was fairly low compared to activity at the bottom and top in plants grown in acidic soil.

The specific assay (assay P) for SBE was performed because assay I determines amylose degrading activity together with SBE activity. The SBE activity revealed by assay P showed different behavior from that shown by assay I, that is, the SBE activity from plants in mineral soil was higher than that one detected in acidic soil in all positions. The differences between assay I and assay P may reflect amylase activity.

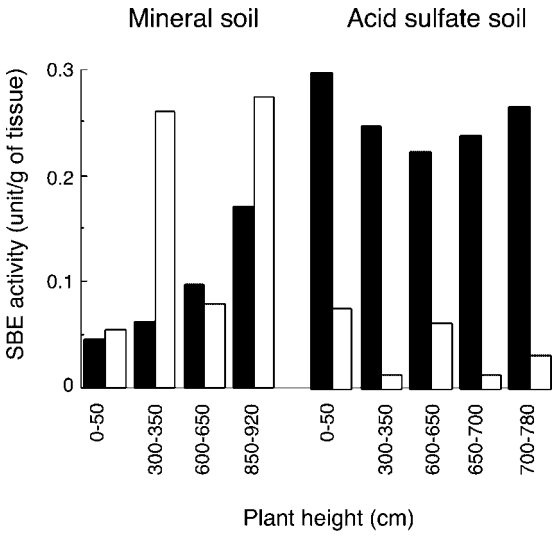

Figure 4. SBE activities at each height. SBE activities were assayed by assay I (■) and assay P (□).

SDS-PAGE

Figure 5 shows SDS-PAGE of granule-bound proteins from the different positions in plants grown in mineral and acid sulfate soils. A single band appeared in all samples. The molecular mass of GBSS from sago palm was estimated to be 58 kDa, which is similar to the values from GBSS reported previously in other plants.

Protein Sequencing of 58-kDa Protein

Figure 6 shows the N-terminal amino acid sequence of a 58-kDa protein aligned with the reported Granule-bound starch synthases (GBSSs) from several plants. The protein from sago palms showed high similarities to the sequences of all GBSSs. The residues conserved among the other GBSSs corresponded especially well with the ones from

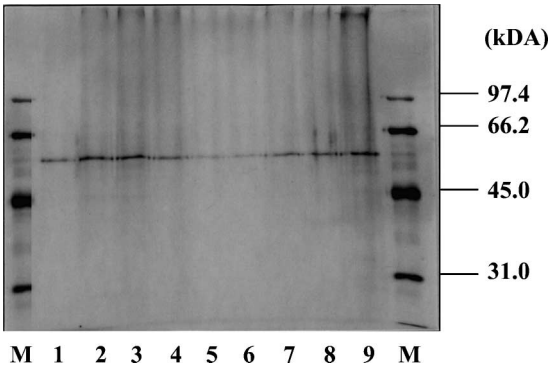

Figure 5. SDS-PAGE of starch-bound proteins. The starch-bound proteins were subjected to SDS-PAGE. Lanes 1–9 correspond with the samples in Fig. 4 in order.

Figure 6. Alignment of amino acid sequences of GBSS from several plants. Sequences of eight GBSSs are aligned to produce a consensus sequence. Black boxes indicate identical residues. Cassava [23]; Potato [24]; Pea [25]; Maize [26]; Wheat [27]; Barley [28]; Rice [29].

sago palms. On the basis of these results, we identified the protein as GBSS.

Discussion

Sago palms were collected from mineral soil and acid sulfate soil, and the starch properties were evaluated. The starch concentration was lower in sago palms grown in acid sulfate soil than in palms grown in mineral soil, and this difference was more obvious at lower positions on the trunk. The reason for this difference is unclear, but we speculate that in acid sulfate soil, plants are forced to use storage compounds to overcome the adverse circumstances. Smaller sized granules were abundant in sago palms grown in mineral soil. The existence of two groups (or three groups) of sizes of starch granules has been studied on wheat and barley [30–32]. The physiological role of differentiation of group is still unclear, while Takeda et al. [33] suggested that there exists genetical control for the synthesis of different size of granule. It is reported that large granule size ratio to small one increased along growth [33]. And the differentiation in size is not only observed in barley and wheat, the similar observation was reported on tulip [34] and kidney bean [35]. Current paper is the first report about the existence of two groups of size in starch granule in sago palm, while the effect of environmental factor on the synthesis (or degradation) of starch granule needs to be studied more precisely. Although the degradation rate of small granules was considered to be faster than with larger ones, but the physiological roles have not been revealed yet. It may be effective for a plant to align small granules among large ones to accumulate as much starch in a limited space as possible. Ahamad et al. [36] compared eight different sampled from Malaysia, Indonesia, and Thailand, and they have reported that the shape of starch granule of sago palm was oval and the diameter was between 20 and 40 μm in every sample. Although our samples show smaller granules, it is suspected that one of the reasons seems that in their case the sample was obtained from manufacture. It is plausible

that the properties of sago starch had been adjusted for the market. Thus we considered that the observed difference in sized group in our case is reflecting their environmental condition, that is relatively lower distribution ratio to small sized might be representing the higher requirement for the usage of starch stock as the energy source under acidic soil, which decreased the ratio of small- to large-sized group.

The starch from sago palms contained GBSS with a molecular mass of 58 kDa. N-terminal amino acid sequence (25 amino acids) of GBSS had high similarity to GBSSs of other plants. SBE activities revealed by assay P were remarkably low as compared with the activities in other plants (rice 466 U/g of tissue [37], maize 29.2 U/g of tissue [21]). We have observed that higher activities of SBE in acidic soil compared with those in mineral soil might be a response to the stressful soil conditions. Although the starch concentration was not related to SBE activity, the higher activity of amylase seems to explain the lower starch concentration in plants grown in acid sulfate soil to some extent.

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