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Molecular Characterisation of Sago Starch Using Gel Permeation Chromatography Multi-Angle Laser Light Scattering

(Pencirian Molekul Kanji Sago Menggunakan Kromatografi Penelapan Gel Serakan Cahaya Laser Pelbagai Sudut)

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ABSTRACT

The molecular characteristics of sago starch (native and debranched) were determined using a gel permeation chromatograpy multi-angle laser light scattering (GPC-MALLS) method. The method involves the optimisation of sample solubilisation and GPC operating conditions. The weight-average molecular weight M_w of native and debranched sago starch determined was 29.1 ± 2.1 × 10^6 and 1.87 ± 0.4 × 10^5 gmol^{-1} respectively while radius of gyration R_g was 123.6 and 59.3 nm respectively. The reduction in M_w and R_g in debranched sago was attributed to the hydrolysis of α-1,6 glycosidic linkages by pullulanase to smaller oligosaccharides.

Keywords: GPC-MALLS; radius of gyration; sago starch; weight-average molecular weight

ABSTRAK

Pencirian molekul kanji sagu (asli dan dinyahcabang) telah dijalankan menggunakan kaedah kromatografi penelapan gel serakan cahaya laser pelbagai sudut (GPC-MALLS). Kaedah pencirian ini melibatkan pengoptimuman pelarutan sampel dan keadaan operasi kromatografi. Berat purata berat molekul M_w kanji sago asli adalah 29.1 ± 2.1 × 10^6 berbanding 1.87 ± 0.4 × 10^5 gmol^{-1} untuk kanji sago dinyahcabang dan legaran jejari R_g adalah 123.6 nm untuk kanji asli berbanding 59.3 nm untuk kanji dinyahcabang. Penurunan nilai M_w dan R_g dalam kanji sago dinyahcabang adalah hasil hidrolisis ikatan glikosidik α-1,6 oleh enzim pullulanase ke oligosakarida yang lebih kecil.

Kata kunci: Berat purata berat molekul; GPC-MALLS ; kanji sagu; legaran jejari

INTRODUCTION

Starch is one of the most important biopolymers and is widely used in numerous industrial applications such as in the paper, textile, food, pharmaceutical and cosmetics industries. Starch is a polymeric mixture of essentially linear (amylose) and branched (amylopectin) alpha-glucans. Starch functionality depends to a great extent on the molecular structure, size and weight of these components as demonstrated in gels, extrusion products and starch pastes.

Starch molecular weight is often influenced by botanical source, starch isolation procedures, amylose and amylopectin separation methods, and especially the technique used to determine polymer molecular weight (Mua & Jackson 1997). Gel permeation chromatography coupled on line to a multi-angle laser light scattering detector (GPC-MALLS) is currently the best available technique for the absolute determination of polysaccharide molecular weights and their distribution (Al-Assaf et al. 2005). It provides information about weight-average molecular weight M_w, molecular weight distribution and other structural information such as radius of gyration R_g and the second virial coefficient A_2 that relates to the interaction with solvent. Multi-angle laser light scattering (MALLS) utilises the principle that the intensity of light scattered elastically by a molecule (Raleigh scattering) is directly proportional to the product of the weight average molecular weight and concentration of the polymer. The theory for the absolute characterization of macromolecules by GPC light scattering was described by Wyatt (1993).

Macromolecular features of starches from various botanical sources such as corn, wheat and potato have been analysed using GPC-MALLS (Bello Perez et al. 1998a). The present study described a procedure to determine the molecular characteristics of native sago starch and the changes after enzyme debranching treatment.

MATERIALS AND METHODS

Dry sago starch (*Metroxylon sagu*) was obtained from Songiing Holding Sdn. Bhd. in Sarawak and used as received. Debranched sago was prepared by hydrolysis using debranching enzyme pullulanase “Promozyme”. Sago starch (3.0%) was gelatinised in 0.1 M sodium acetate buffer (pH 4.6) at 90°C for 30 min in a shaking water bath (220 rpm). The starch suspension was cooled to 54°C before pullulanase (0.55% v/w starch) was added and the mixture was incubated for 5 h at 54°C. Upon enzyme

deactivation, the starch slurry was filtered to remove insoluble particles and the supernatant subjected to spray drying to obtain the debranched sago.

The schematic diagram of the GPC-MALLS system is shown in Figure 1. The system utilised a Waters (Division of Millipore, USA) Solvent Delivery System Model 6000A. The column used was dual linear (connected in series) 10 μm Hemabio column (300 × 8 mm^2) packed with hydrophilic modified Hema gel (hydroxyethyl methacrylate copolymer) obtained from Polymer Standard Services, Germany. The columns were maintained at 60°C in a temperature regulated oven. Injection into the GPC column was made with a manual Rheodyne Model 7725i syringe loading sample injector equipped with a 1 mL sample loop. The output of the column was connected to the DAWN EOS laser light scattering photometer in conjunction with the concentration dependent detector Wyatt Optilab rEx operated at 632.8 nm equipped with a 10-mm P100 cell (Wyatt Technology Corporation, USA). The DAWN EOS is equipped with a 690 nm He–Ne laser (Wyatt Technology Corporation, USA) with 15 detectors. The mobile phase used was 0.1 M potassium thiocyanate with 0.005% sodium azide that had been degassed and filtered through 0·2 μm cellulose nitrate membrane filter (Millipore) at flow rate 0·5 ml/min.

Starch dissolution for GPC was achieved by microwave heating in a high pressure vessel (microwave bomb) after pretreatment of sample with dimethyl sulphoxide (DMSO) as described by Bello Perez et al. (1998a). The starch sample in 0.1 M potassium thiocyanate was hydrated for 48 hours before transferring into the Teflon cup of a model 4782 polycarbonate microwave bomb (Parr Instrument Co., Moline, IL, USA). The capped Teflon cup was then fitted into the bomb and this assembly was heated in a Powerwave 800 W microwave oven (Proline, Korea). After cooling, the sample was centrifuged at 2500 rpm for 10 min and the sample (1 mL) was filtered using 0.8 μm cellulose acetate filter before injection into the HPSEC system. Data analyses were processed using the Astra 4.50 software package (Wyatt Technology). The specific RI increment value (dn/dc) of 0.146 cm^3/g was used. The weight-average molecular weight M_w, molecular weight distribution and radius of gyration R_g (defined as the average distance from the centre of gravity of a polymer coil to the chain end) of sago starch were determined.

Optimization of the sample preparation and GPC-MALLS conditions for molecular weight characterisation of sago starch was studied by varying the (1) the microwave heating time (30 s, 45 s and 60 s), (2) storage time after sample preparation (1 to 60 h), (3) storage temperature of solubilised sample (25 and 60°C), and (4) column temperature (25 and 60°C)

Results and Discussion

OPTIMIZATION OF SAMPLE SOLUBILISATION AND ANALYSIS BY GPC

A prerequisite step for determining the molecular weight distribution of amylose and amylopectin in starch, by any technique, is the complete dissolution of the starch sample in an appropriate solvent without any degradation of the constitutive macromolecules. In this study, the microwave bomb treatment is used to solubilize the starch and the effect of microwave bomb exposure time was determined to maximise both molecular weight and percentage mass. Percentage mass indicate the mass recovery of the material (proportion of injected material) after passing through column using a given refractive index increment (dn/dc) for a polymer solvent system.

Table 1 shows the effect of microwave heating time on the M_w of sago starch. An increase was recorded from (17.7 ± 2.3) × 10^6 to (26.8 ± 2.9) × 10^6 g mol^{-1} for exposure times of 30 s and 45 s respectively. A longer exposure time of 60 s resulted in a molecular weight reduction. It is suggested that for exposure time t ≤45 s, disaggregation of starch polymer occur without degradation. However, for t≥60 s, disaggregation is accompanied by degradation

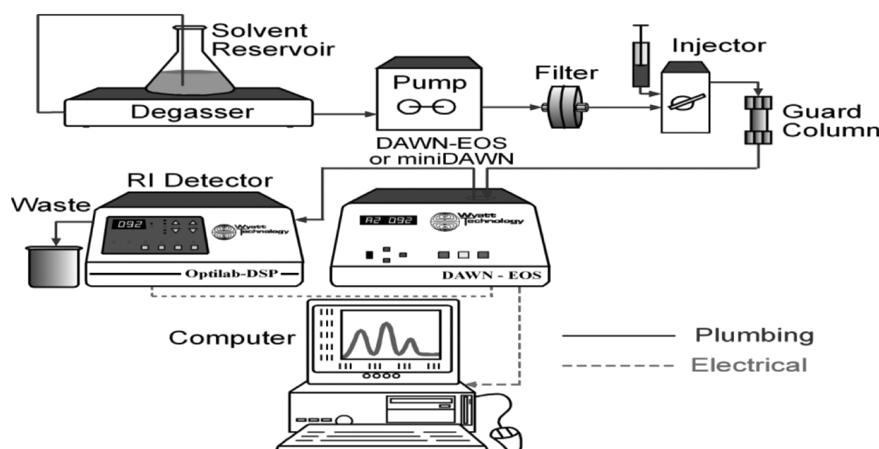

FIGURE 1. The schematic diagram of the GPC-MALLS system (adapted from Wyatt Technology)

TABLE 1. Effect of microwave heating on M_w of sago starch

Microwave regime: exposure time (s)	M_w ($gmol^{-1}$)	Mass recovery (%)
30 s	$17.7 \pm 2.3 \times 10^6$	81.3
45 s	$26.8 \pm 2.9 \times 10^6$	79.9
60 s	$2.4 \pm 0.2 \times 10^6$	86.2

of the polymer as indicated by the reduction in M_w to 2.4 ± 0.2 × 10^6 $gmol^{-1}$. Molecular weight reduction during microwaving occurred through breaking of covalent bonds by heating (Fishman & Hoagland 1994). At exposure time of 30 s, the lower M_w obtained also suggest that the sago starch was not fully solubilised. The microwave heating time to solubilise starch varies slightly with botanical origin. Using similar solubilisation technique, an optimum time of 40 s was determined for mango and okenia, and 50 s for banana (Millan-Testa et al. 2005) while Bello Perez et al. (1998a) used 35 s for maize starch. The different requirements presumably reflect the inherent structural heterogeneity within the granules.

Assuming molecular dispersion of the starch components was achieved by the above treatment, the amylose and amylopectin should be present as individual molecules prior to injection. However, in aqueous solution, amylose molecules can rapidly associate to build up molecular aggregates from assemblies of double helices that soon exceed colloidal dimensions and precipitate or form a gel (Gidley & Bulphin 1989). The retrogradation tendency depends on amylose molecular size and concentration, temperature, pH, and the presence of other chemicals in the solution. To determine the storage time and temperature effect on sample stability, the prepared sago starch solution was injected immediately after being prepared or allowed to stand at room temperature or incubated at 60°C using water bath for up to 60 h before injection into the column.

Table 2 shows the effect of storage temperature and time prior to injection on the M_w of sago starch analysed at column temperature of 25°C. The samples stored at 6°C appeared stable for longer period compared to 25°C. Reduction of molecular weight with lower mass recovery (41.9%) was observed for sample stored for 60 h at 25°C as compared to that stored at 60°C suggesting retrogradation of amylose taking place after long storage time at 25°C. The observation agreed with Lu et al. (1997) who found that the retrogradation rate of a dilute amylose solution decreased as incubation temperature increased and that different molecular sized amylose subfractions have different retrogradation tendencies. Cornell et al. (2002) reported similar finding whereby the area of the refractometer signal (that is directly related to the quantity of polymer eluted) is always smaller for the solution after 24 h than when injected immediately after being prepared. This indicates that, after a long time, a process of aggregation and/or retrogradation occurs, resulting in the formation of a structure retained during filtration. Bello Perez et al. (1998b) also observed the diminution in M_w value of corn starch when storage time increased. Thus, when studying the molecular characteristics of starch, it is necessary to work with freshly solubilised samples to make a quantitative analysis.

The effect of column temperature on the M_w was studied to determine for aggregation of sample during the GPC analysis. The M_w of sago was increased to 10.1 ± 0.3 × 10^6 when the analysis was carried out at column temperature of 60°C compared to M_w 5.7 ± 0.1 × 10^6 at 25°C. The result suggests aggregation of amylose in the sample at 25°C which precipitated out at beginning of run and was excluded, thus only the lower molecular weight components were determined. Mass recovery of starch at 60°C was also higher (77.5%) suggesting more sample passed through the column. Lu et al. (1997) reported that a substance shown at the void volume was attributed to entangled amylose. Following incubation, amylose molecule can be in 3 states: free in the supernatant, entangled and eluted at void volume, and retrograded and precipitated. Amylose of large molecular weight does not retrograde when its temperature is maintained above this critical retrogradation temperature. Thus, at 60°C, amylose does not retrograde. Based on the data obtained, the optimum GPC conditions were identified and used for the subsequent M_w and R_g determination of native and debranched sago starch.

MOLECULAR CHARACTERISATION OF SAGO STARCH

Sokhey and Chinnaswamy (1993) reported that the molecular weight distribution profile of amylose containing

TABLE 2. Effect of sample storage temperature and time on M_w (column temperature 25°C)

Storage temperature	Storage time (h)	M_w ($gmol^{-1}$)	Mass Recovery (%)
25°C	1	2.92×10^6	87.6
	3	3.21×10^6	82.5
	60	1.47×10^6	49.1
60°C	1	3.22×10^6	83.9
	7	3.10×10^6	82.0
	60	3.68×10^6	54.1

starch is composed of two fractions. Fraction I consists of the branched and higher molecular weight amylopectin, eluting early owing to its very large size, while fraction II, partially overlapping with Fraction 1 represents the mainly linear and lower molecular weight fraction of amylose.

Figure 2 presents the GPC chromatogram of native sago starch which showed a bimodal distribution as a function of elution volume, although no clear separation between the amylopectin and amylose component was observed. Results of all chromatographic studies of starch showed that amylose and amylopectin cannot be strictly separated on the basis of their respective sizes in solution. All fractionation procedures lead to intermediate fractions between amylose and amylopectin, which suggests a continuous change between these two well-identified chemical species (Roger et al. 1999). In this study, the M_w and R_g determined for the native sago starch were 29.1 ± 2.1 × 10^6 gmol^{-1} and 123.6 nm, respectively with mass recovery of 75.5%. The Debye peak maxima obtained at elution volume 10.24 mL was 49.1 ± 0.8 × 10^6 gmol^{-1}. No values have been reported for M_w and R_g of sago starch using GPC-MALLS. However, the M_w of sago amylose determined by light scattering batch mode ranged between 1.41 × 10^6 and 2.23 × 10^6 gmol^{-1} while for sago amylopectin, the value was from 6.70 × 10^6 to 9.23 × 10^6 gmol^{-1} (Ahmad et al. 1999).

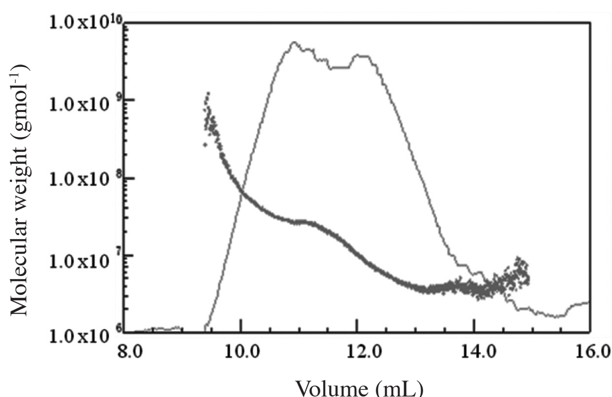

FIGURE 2. GPC chromatogram of molecular weight distribution of sago starch

Figure 3 shows the molecular weight distribution of debranched sago, yielding two distinct separate peaks. The M_w of debranched sago was 1.87 ± 0.4 × 10^5 gmol^{-1} and the R_g was 59.3 nm with mass recovery of 74.9%. The reduction in M_w and R_g was attributed to the hydrolysis of α-1,6-glycosidic linkages of sago starch by pullulanase to smaller oligosaccharides. Praznik et al. (1994) reported a similar observation in both wrinkled pea and high amylose corn starch whereby the M_w of pea starch (1 × 10^6) and corn starch (0.52 × 10^6) decreased to 0.38 × 10^6 and 0.19 × 10^6 gmol^{-1} respectively after debranching with pullulanase. Similarly, Vorwerg et al. (2002) found that after debranching the amylopectin of wrinkled pea starch using pullulanase Promozyme, the M_w was reduced from 12.2 × 10^6 to 2.67 ×10^5 gmol^{-1}. As a consequence of the debranching reaction, the molecular weight distribution of the debranched starch was very broad (elution volume 11-19 mL) compared to native sago (elution volume 9.5-15 mL). This is due to the presence of products containing amylopectin short side chains and some lower oligosaccharides due to the glycosidic activity of the Promozyme as observed by Vorverg et al. (2002).

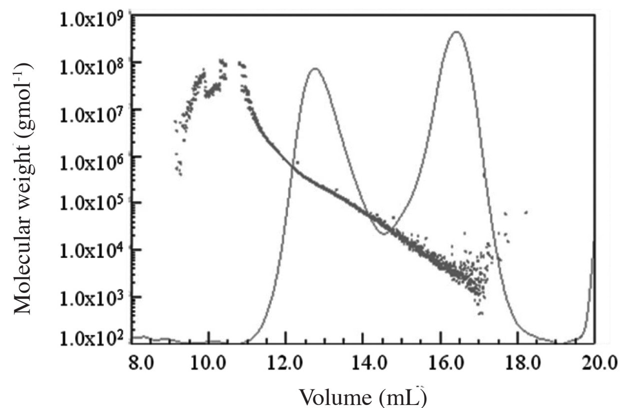

FIGURE 3. GPC chromatogram of molecular weight distribution of debranched sago starch

CONCLUSION

The weight-average molecular weight and radius of gyration of native sago starch determined using GPC-MALLS was found to be 29.1 ± 2.1 × 10^6 gmol^{-1} and 123.6 nm, respectively. Enzyme debranching treatment effectively decreased the molecular weight and size of sago starch. Optimisation of starch solubilisation and GPC-MALLS operating conditions improved the accuracy of the molecular characterisation of the sago starch.

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