

Fluorescence-labelling for analysis of protein in starch using asymmetrical flow field-flow fractionation (AF4)

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(Received January 5, 2017; Accepted February 8, 2017)

Abstract: Starch is a mixture of amylose (AMY) and amylopectin (AMP) which are different in physical properties such as molar mass (M), rms radius (R_g) and hydrodynamic diameter (d_{H1}). The rheological and functional properties of starch are influenced by various factors including the molecular size, molar mass distribution (MD) and the concentration ratio of AMY and AMP. It is also important to analyze proteinaceous material in starch as they affect the flavor and texture of food to which starch is added. In this study, asymmetrical flow field-flow fractionation (AF4) was employed for separation and quantitation of AMY and AMP in starches (Amaranth, potato, taros and quinoa). AF4 was coupled with a multi-angle light scattering (MALS) and a refractive index (RI) detector for determination of the absolute M , MD and molecular structure. It was found that AMP has the M and R_g ranging $3.7 \times 10^7 \sim 6.5 \times 10^8$ g/mol and 84 ~ 250 nm, respectively. Also the existence of branch was confirmed in higher M . In addition, proteinaceous material in starch was analyzed by AF4 coupled with a fluorescence detector (FS) after fluorescence-labeling. AF4-FS with fluorescence-labelling showed a potential for investigation on existence of proteinaceous material and the interaction between proteinaceous material and polysaccharide in starch.

Key words: Asymmetrical flow field-flow fractionation (AF4), Starch, Amylose, Amylopectin, Fluorescence labeling, Proteinaceous materials

1. Introduction

Starch consists of large polydisperse glucose homopolymers of amylose (AMY) and amylopectin (AMP). AMY is a linear polymeric molecule with α (1→4) linked glucose units and is primarily of long chains. AMP has a branched structure with a mixture of α - (1→4) and α -(1→6) linked glucose linkages.¹

Starch is one of the most abundant biopolymers on earth and one of the principal sources of energy for human and animal. Plant product by starches is most important, and their derivatives are wide-spread in various industries, e.g., food, paper, adhesive, textile and cosmetic industries.² Often it is important to optimize the modification and the processing of starch materials. Further knowledge of the relationship

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between the processing parameters and the properties of the final starch product is required. Thus, the chemical and physical properties of the starch materials need to be investigated, especially the molar mass distribution (MD) and size.¹⁻⁴

It is also important to analyze minor components such as lipids and proteins that are present in small quantities in the starch granule. Heating increases the complexity of the reactions between protein and starch, and thermal changes in protein lead to denaturation of starch.⁵ Denaturation is accelerated in the presence of moisture. Denaturation of proteins leads to disulfide-sulfhydryl interchange reaction resulting in the extensive cross-linking of proteins, protein-protein interactions.⁶ Starch undergoes a loss of crystallinity, swelling of granules, and leaching of amylose out of the granules leaving mostly amylopectin behind in starch. The granules collapse and the matrix of amylose forms a part of the gel network.⁷ When protein and starch are in contact, a stable protein-starch matrix involving hydrogen bonding, covalent bonding, and ionic linkages may form. These moieties can be distributed freely in the granule or interact with the present carbohydrates.⁸

Protein and starch constitute the major components in various food systems such as bakery food, pasta, snack food and breakfast cereal. The characteristic flavor and texture of these foods are due partially to the protein-starch interaction. This means, when a starch sample is dissolved by the so-called 'DMSO method' (will be explained in more detail later), proteinaceous materials exist in the starch suspension. These proteinaceous materials are insoluble flocculent proteins. It is thus difficult to isolate starch with protein.⁹

An elemental analyzer can be used to determine the protein content in food materials. However, it can only provide the content, and not the information on the distribution or the interaction of proteinaceous materials with starch. For more complete study, separation is required.^{6, 10}

Some classical methods (e.g. osmometry, viscometry and light scattering) have been employed for starch analysis.¹¹⁻¹³ However, they can only provide the

averages of some physical properties such as the size and molar mass (M). Again, a separation is required for more reliable analysis of starches

Size-exclusion chromatography (SEC) has been also used for analysis of proteins in starch.^{14,15} In SEC, however, materials with high M such as AMP are often eluted unresolved at the "excluded" volume, and may suffer from shear degradation in the SEC packed column. Also the interactions between the sample components and the column stationary phase tend to interrupt analysis of starches.

The asymmetrical flow field-flow fractionation (AF4) is well suited to rapid size-based separation of ultrahigh M biopolymers and bio-colloidal particles ranging in size from nano- to micrometer.¹⁶⁻²⁰ Unlike SEC, AF4 uses an open channel with no stationary phase (or packing material). The shear force is thus minimized in AF4 during separation. The size-based separation is achieved by AF4 as the retention time (t_r) is inversely proportional to the diffusion coefficient of the sample component, which is dependent on the molecular weight or particle size and shape. Conversely, the diffusion coefficient (D) of a sample component can be determined from the observed experimental t_r , and, in turn, transformed into the hydrodynamic diameter (d_{H1}), which is one of the most important characteristics of macromolecules.

AF4 coupled with multi-angle light scattering (MALS) and refractive index detector (RI) has shown applicability to the determination of the MD of various ultrahigh M polymers.²¹⁻²³ There have been some reports on application of FFF for analysis of starches.^{24,25} Successful separations of AMY and AMP in four maize starches having various AMY content (0, 30, 50, and 70 %) have been reported using flow FFF (FIFFF) coupled with MALS-RI with a cross flow-programming.²⁶ FIFFF with a cross flow-programming also yielded successful separation of AMY from AMP in barley starch.²⁴ A dual flow-programming (channel and cross flow) has been employed for FIFFF separation of AMY and AMP in maize.²⁷ AF4 has also been used for fractionation of AMP.²⁸

In this study, the molecular conformation as well

as the molecular mass and size were analyzed using an AF4-MALS-RI for various types of starches. Also the fluorescence labeling was tested for analysis of proteinaceous materials in starches using AF4 and fluorescence (FS) detector (AF4-FS).

2. Theory

2.1. Asymmetrical flow field-flow fractionation (AF4)

In AF4 with constant cross flow, the D of an eluted component is given as a function of the t_r by³

$$D = \frac{t^0 F_c w^2}{6V^0 t_r} \quad (1)$$

where w is the channel thickness, V^0 is the channel volume, t^0 is void time, t_r is retention time and F_c is the cross flow rate. The void time t^0 can be calculated by³

$$t^0 = \frac{V^0}{F_c} \ln \left(1 + \frac{F_c}{F_{out}} \left[1 - \frac{w(b_0 z' - \frac{b_0 - b_L}{2L} z' 2 - y)}{V^0} \right] \right) \quad (2)$$

where F_{out} is the flow rate through the outlet of the channel. The terms b_0 , b_L , L , z' , and y are the breadth of the trapezoid at the inlet end, the breadth of the trapezoid at the outlet end, the length of the channel, and the distance from the channel inlet end to the point where the sample zone is focused immediately before the elution starts.

By combining Eq. (1) with the Stokes–Einstein equation, a direct relationship between the d_H , of a component and its t_r is obtained by³

$$d_H = \frac{2kTV^0}{\pi\eta t^0 F_c w^2} t_r \quad (3)$$

Thus the d_H of an eluted molecule can be experimentally determined directly from the observed t_r . In AF4, sample components are eluted in the order of increasing d_H .

2.2. Multi-angle light scattering (MALS)

The M and cross flow R_g can be obtained from MALS combined with RI by applying the Rayleigh-

Gans-Debye theory given by²⁹

$$\sqrt{\frac{K_c}{R_\theta}} = \sqrt{\frac{1}{M_w} + \frac{16\pi^2}{3\lambda^2} \times R_g^2 \times \sin^2\left(\frac{\theta}{2}\right)} \quad (4)$$

where K is the optical constant, c is the sample concentration, R_θ is the Rayleigh ratio, M_w is the weight-average molecular weight, λ is the wavelength. MALS data processing was performed using the Astra software. The Berry method was used for data-fitting in this study.

2.3. Total nitrogen content determination

The total nitrogen content was measured at duplicates, from which the %protein in starch was determined.

3. Experiment

3.1. Starch dissolution

In this study, four starch varieties from botanical origin: potato, taros, amaranth and quinoa were used. Starch samples were received from Department of Food Technology, Engineering and Nutrition in Lund University (Lund, Sweden). Starch solutions were prepared by the so-called ‘DMSO method’. The starch sample of about 100 mg was weighed in a 8 mL glass vial, and dispersed in 0.3 mL 80 vol% ethanol with magnetic stirring for 3 ~ 5 min, after which 3.0 mL of DMSO was added while being stirred for 6 ~ 10 min. The sample vial was capped, and then heated for 1 h in a boiling oil bath with magnetic stirring at 160 rpm, to a temperature of 100 °C, after which the sample solution was cooled down to room temperature. The sample solution was quantitatively diluted with water to 100 mL, and injected into the AF4 channel.²⁵ The sample injection volume was 50 μ L.

3.2. Elemental analyzer

Total protein content of starches was determined by measuring nitrogen content using the elemental analyzer Flash EA 1112N (Thermo Fisher Scientific, Delft, Netherlands). The small amount of sample was weighted and packed into a small package with aluminum foil. The package was heated up to

1000 °C. The sample (30 ~ 50 mg) was combusted in a sealed furnace and the nitrogen content was determined with thermal conductivity detection.

3.3. Fluorescence-labeling for proteinaceous material

For analysis of proteinaceous material in starch using AF4, fluorescence labeling was tested using amine-reactive fluorescent dye method. The labeling solution was prepared by mixing 10 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and 10 mM 7-methoxycoumarin-3-carboxylic acid in the volume ratio of 1:1 for 3 h. The labeling solution was added to the starch solution in the volume ratio of 1:1 and mixed for 1 h, and then the solution was injected into the AF4 channel.³⁰ For detection of labeled components, the wavelength of the fluorescence detector was set at 336 nm for excitation and 402 nm for emission, respectively.

3.4. UV/Vis spectrometer

A UV-3101PC UV-Vis-NIR scanning spectrophotometer (Shimadzu, Japan) was employed to obtain UV/Vis spectra of normal and fluorescence-labelled samples in the wavelength range of 190 ~ 600 nm at ambient temperature.

3.5. AF4 system

The AF4 system used in this study was an Eclipse 2 Separations System (Wyatt Technology Europe, Dernbach, Germany). It was connected with a RI detector (Shimadzu, Kyoto, Japan), a DAWN EOS MALS detector (Wyatt technology, Santa Barbara, CA, USA) operating at the wavelength of 690 nm, and a RL-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). A refractive index increment, dn/dc , for starch of 0.146 g/mL was taken from the literature,³¹ and was used for all starch samples. Agilent 1100 HPLC pump (Agilent Technologies, Waldbronn, Germany) equipped with an in-line vacuum degasser delivered the carrier liquid into the AF4 channel. A 0.1 µm membrane filter (Millipore Corp. MA, USA) was placed after the pump to ensure the carrier liquid entering AF4 channel is particle-free.

The channel was assembled with a 350 µm thickness Mylar spacer and a regenerated cellulose membrane with the molecular weight cut-off (MWCO) of 10 kDa. The channel thickness was determined to be 273 µm from the t_r of bovine serum albumin (BSA). The channel geometry was trapezoidal with the tip-to-tip length of 26.5 cm and breadths at the inlet and the outlet of 2.2 and 0.6 cm, respectively. The carrier liquid was deionized water containing 50 mM sodium nitrate (Sigma-Aldrich, St. Louis, USA) and 0.02% sodium azide (Sigma-Aldrich, St. Louis, USA).

In order to avoid excessive retention of sample, a cross flow rate was programmed, where it decays exponentially with time according to Eq. (5)³²:

$$V_c(t) = V_c(0)e^{-\frac{\ln 2}{t_{1/2}}t} \quad (5)$$

where $V_c(t)$ is the cross flow rate as a function of time t . $V_c(0)$ is the initial cross flow rate, $t_{1/2}$ is the half-life time. An exponential cross flow-programming usually yields a good resolution and a uniform selectivity across the entire size distribution of polydisperse macromolecules. The initial cross flow $V_c(0)$ was 2.0 mL/min and was decreased exponentially with $t_{1/2}$ of 3 min for all AF4 analysis of starches.

4. Result and Discussion

4.1. Analysis of molar mass (M) and rms radius (R_g) of starch

Starch solution was directly injected into the AF4 channel and analyzed with MALS-RI. Results are shown in *Fig. 1*. The RI signal shows two polysaccharide populations. The earlier eluting population corresponds to low molecular weight species such as amylose. It was eluted after about 2 min. The second population was eluted after about 18 min and corresponds to high molecular weight species such as amylopectin. *Fig. 1* clearly shows AF4 separates the amylose and amylopectin for all four starch samples. The RI elution profiles of four starch samples are somewhat different, probably because they are different in the molecular shape or

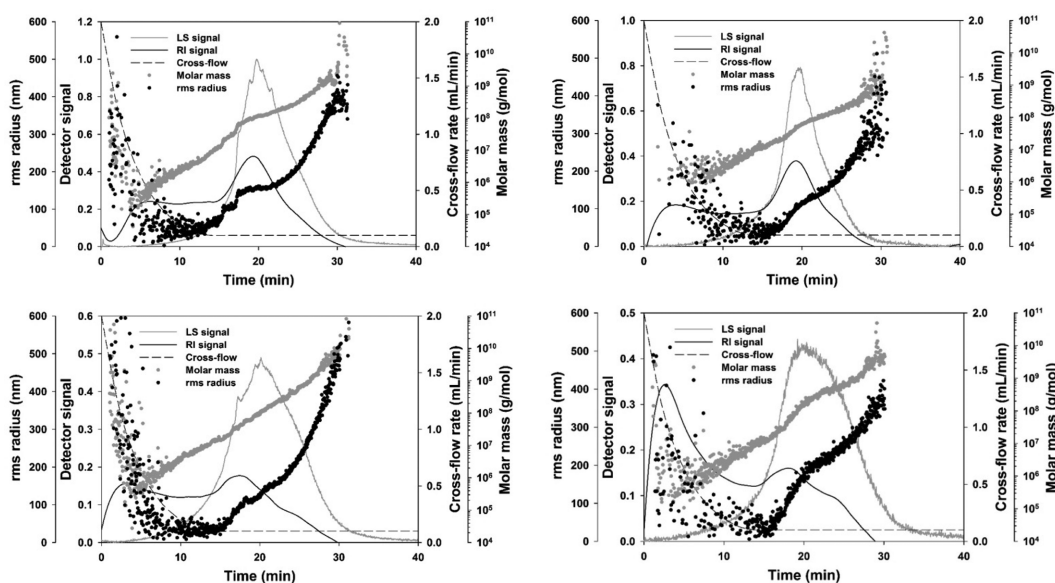


Fig. 1. AF4-MALS-RI fractograms, rms radius (R_g) and molar mass (M) distribution for four starches: (a) Taros (b) Potato (c) Amaranth (d) Quinoa.

in the branching degree, which should affect the relationship between the t_r and the M .

LS signal is proportional to both the sample concentration and the M , and shows the second population only. LS intensity is stronger for higher M components. It seems the LS signal from larger mass components (amylopectin) masks the signal from lower mass components (amylose). For the species of the same d_H and M of amylopectin is much higher than that of amylose. For this reason, in MALS measurement, acceptable precisions for the M and size are realized only down to about 5×10^6 g/mol and 50 nm, respectively. The M and R_g were determined only for the second population as the scattering intensity was too weak for the first population.

To determine the M and size from MALS signal, a 2nd order Berry fitting was employed, which has been reported to be the most appropriate fitting method for amylopectin analysis. The M and the R_g measured for amylopectin were in the range of $3.7 \times 10^7 \sim 6.5 \times 10^8$ g/mol and 84 ~ 250 nm, respectively. These results agree reasonably well with those cited in previous reports.³³⁻³⁶

4.2. Reproducibility

Fig. 2 shows the reproducibility of distributions obtained by AF4 for four starch samples. Generally, AF4 fractograms of four starch sample show good reproducibility. The RI responses show the taros and potato are typical normal starch (Fig. 2(a), (c)). The quinoa has higher amylose content than other starches (Fig. 2(d)), and the second population is eluted earlier than that of other starch samples, indicating the quinoa is mostly composed of lower molecular weight species than other samples. The amaranth has broader (or polydisperse) size distribution than others (Fig. 2(c)), and the reproducibility was lower.

4.3. Conformation of starch

The plot of R_g vs. M , more accurately the slope of the plot, gives information on molecular density (and thus the molecular conformation (Fig. 3)). The slopes are in the range of 0.25 ~ 0.42 for the starch samples. The slope is higher for the quinoa than that for others. For the quinoa, the amylopectin content was lower, while the molecular density of the amylopectin fraction was higher than other starches. Results

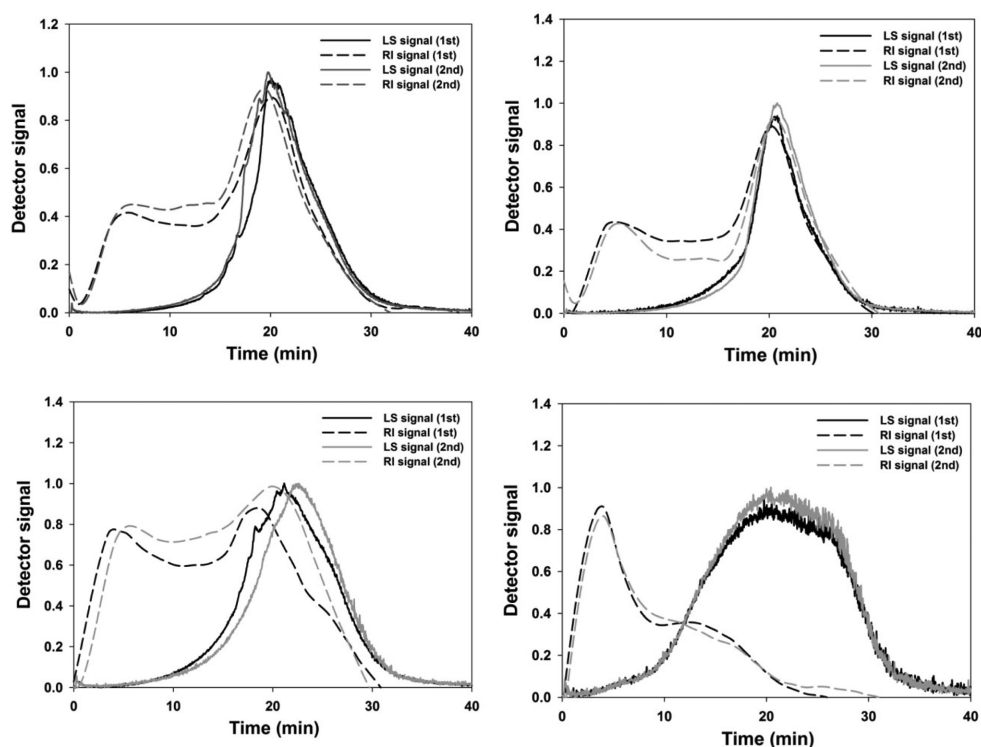


Fig. 2. AF4-MALS-RI results of reproducibility test for four starches. (a) Taros, (b) Potato, (c) Amaranth, (d) Quinoa

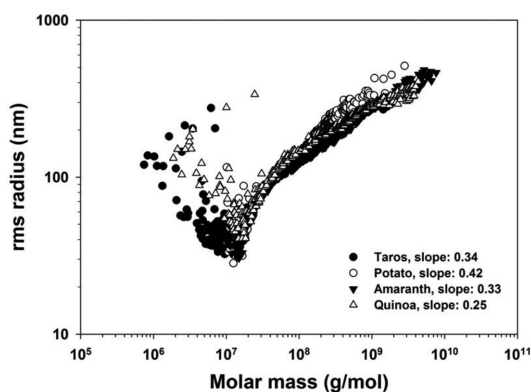


Fig. 3. Conformation plot ($\log R_g$ vs. $\log M$) for four starches.

suggest, although there are somewhat of difference in the slope among the four samples, all four starches were composed of randomly branched polymers, and those in higher M population are of high degree of branching. The plot reaches a plateau as the M increases. This means further increase in the M is absorbed inside the polymer coil without an increase in the molecular size, suggesting the amylopectin is

of high degree of branched. It is noted that the data in Fig. 3 are scattered below M of about 10^7 , due to low LS signal.

4.4. Analysis of proteinaceous material in starch using AF-FS

Fig. 4 shows the protein contents of four starch samples obtained by an elemental analyzer (see Experimental section). The amaranth starch has the highest protein content among the four starches studied in this study. The protein contents were similar for the quinoa and taros starches. Potato has almost no protein.

When the UV/Vis spectra of the unlabeled and fluorescence-labeled starches were compared, increased band intensity was observed from the labelled starch. The first absorption band at 190 ~ 230 nm indicates that a peptide bond was formed by reaction between the amine group in the protein and the carboxylic group in the 7-methoxycoumarin-3-carboxylic acid. The second band at 336 nm indicates the dye

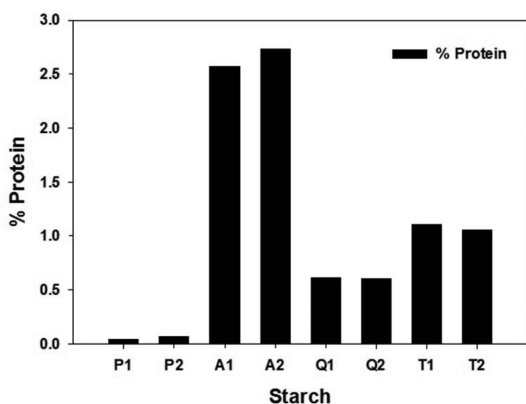


Fig. 4. Histogram of protein content in four starches obtained by elemental analysis: P – Potato, A – Amaranth, Q – Quinoa, T – Taros

component shows improved fluorescence signal for the proteins in starch.

The fluorescence-labelling method for protein analysis was tested using bovine serum albumin (BSA), a protein standard, as shown in Fig. 5. In AF4-FS analysis of the labelled BSA, the channel and the cross flow rates were 1.0 and 4.0 mL/min, respectively. All other AF4 conditions were the same as those used earlier for the starch analysis. Fig. 5(b) shows that the FS signal was intensified after the fluorescence-labelling. It seems that aromatic amino acids such as tryptophan and tyrosine in protein give fluorescence response.

The same labelling method was applied to starches,

followed by AF4-FS analysis. First the samples were analyzed by a fluorescence spectrometer to see if the proteinaceous materials in starches were labeled. As shown in Fig. 6, the non-labeled starches still show fluorescence signals, although the intensities are low, probably due to presence of some fluorescent molecules such as vitamins or aromatic amino acids in the starch. They are also shown as a small shoulder at 280 nm in Fig. 5.

The fluorescence of starch comes from the transition from the non-bonding N-electrons in the hetero-atom (O) of the functional group (C-O-C), called ‘ether linkage’ to the anti-bonding σ orbital.³⁷ The intensities of the FS signals for the potato and quinoa are different in the amylopectin region. There are more of heteroatoms of the ether in the amylopectin than in the amylose, and thus the FS signal from amylopectin is expected to be stronger than that from the amylose. The FS signals are also observed in the AF4 void time (t^0) for the amaranth and quinoa. This indicates fluorescent proteins or vitamins having low M are eluted without being retained in AF4.

There will also be somewhat of light scattering in the fluorescence detector, yielding stronger signal for the amylopectin region than for the amylose region. This is shown in Fig. 6(d) as compared to the results in Fig. 6(a) ~ (c).

By above-mentioned reasons, the fluorescence (FS)

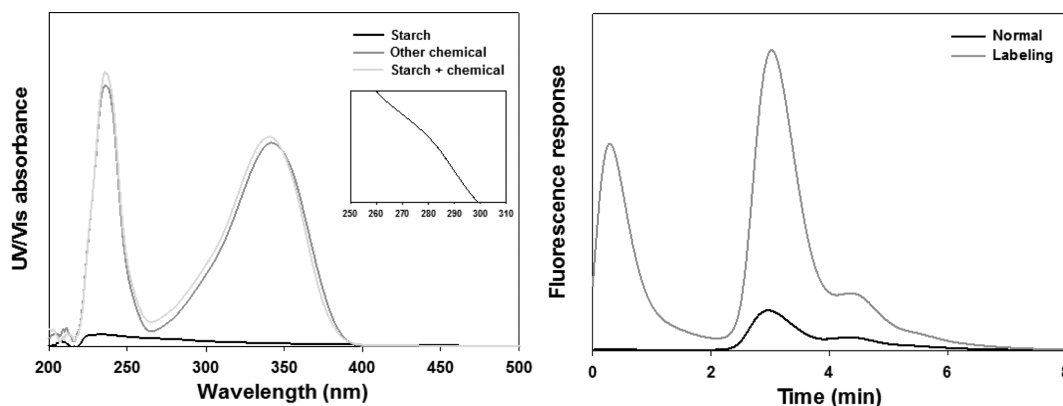


Fig. 5. Result about fluorescence labeling test to starch (a) Fluorescence labelling test using UV/Vis spectrometer: (i) starch (ii) other chemical (DMSO + ethanol + EDAC + 7-methoxycoumarin-3-carboxylic acid) (iii) Starch + other chemical. (b) AF4-FS of BSA results for comparison of normal and labeling

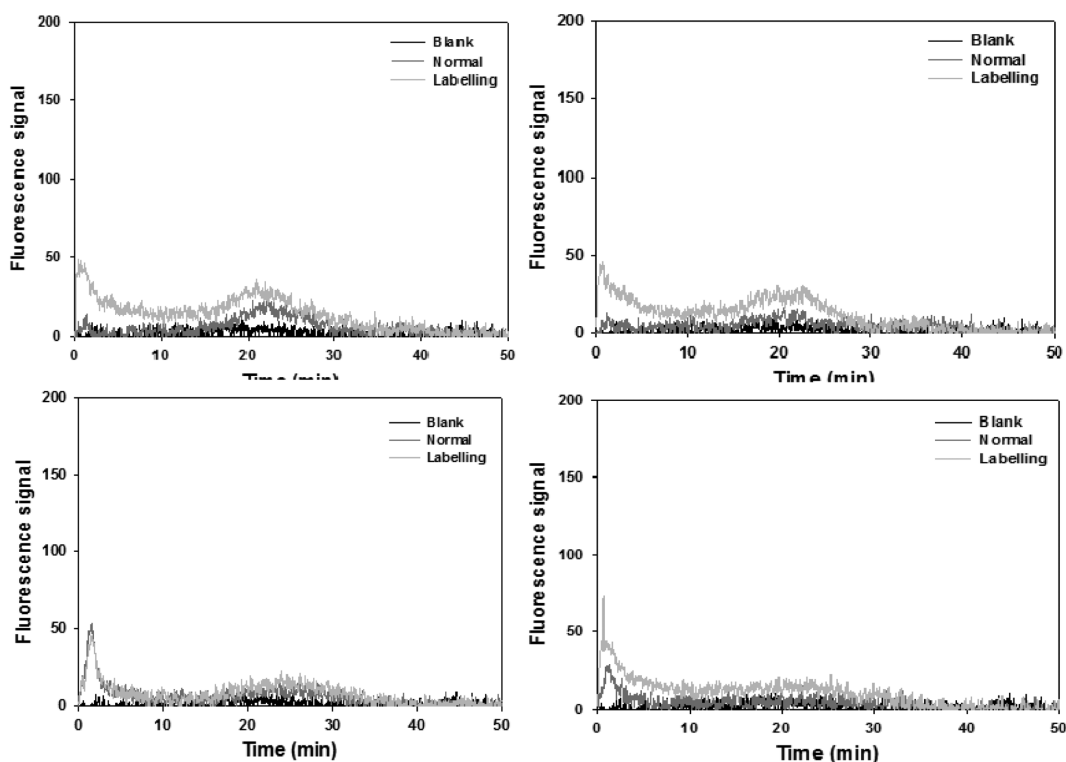


Fig. 6. AF4-FS results of normal and labeled starch: (a) Taros (b) Potato (c) Amaranth (d) Quinoa

signals of starches are lower than those of BSA. It seems there exist proteinaceous materials in starches. After the fluorescence-labeling, FS signal was increased at the elution time of 1~5 min in all starches except the amaranth. The FS signal observed before the elution time of 5 min is due to proteins in the starch, which are not attached to the starch molecules.

Although the FS signals were too low for an in-depth study of the interaction between proteinaceous materials and polysaccharide, the potential of labelling method for improvement of FS signal is clearly shown.

5. Conclusions

Separation of starches using AF4-MALS-RI-FS allowed characterization of M and size distribution of various types of botanical starches. The information on molecular conformation (or the molecular density) was also obtained from the plot of molecular size vs. M . AF4 combined with a fluorescence detector (AF4-FS)

after fluorescence-labeling allowed analysis of proteinaceous materials in starches. Results show that AF4 can be a useful tool for separation of component in starches and for analysis of their M and size. Results shown this study may provide a basis for application of FFF and its related techniques in the starch-related food industries.

Acknowledgement

The authors acknowledge the support provided by 2015 Hannam University Research Fund (HNU).

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