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Determination of molecular mass distribution of amylopectin using asymmetrical flow field-flow fractionation

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ABSTRACT

The molar mass (M_w), radius of gyration (R_g), diffusion co-efficient (D), and hydrodynamic radius (R_h) of amylopectin (waxy maize starch) has been determined using asymmetrical flow field-flow fractionation coupled with multi-angle light scattering and refractive index detectors (AF4/MALS/RI). A high pressure microwave vessel (HPMV) was used to dissolve amylopectin in 1 M KSCN. The effect of varying the flow rates on the M_w and R_g distributions of amylopectin were investigated to determine the optimum flow rates. The average M_w and R_g values of amylopectin determined using the Berry and Debye equations. D and R_h values were determined directly from the AF4 retention times and compared to values obtained by dynamic light scattering.

1. Introduction

Starch is an abundant material and is obtained commercially mainly from maize, potato, wheat, tapioca and rice (Murphy, 2000). It occurs in the form of granules, which have varying degrees of structural order and consists of two polysaccharides, namely amylose and amylopectin. Amylose consists of linear α-(1,4)-linked glucopyranose chains with very little branching (10 branch points per molecule), while amylopectin also contains sequences of α-(1,4)-linked glucopyranose units, however, it has extensive branching via α-(1,6) linkages. The molecular mass of amylose may range from 10^5 to 10^6 g/mol while that for amylopectin may range from 10^7 to 10^8 g/mol (Banks & Greenwood, 1975; Murphy, 2000).Considerable efforts made over the last thirty years or so to determine the molecular mass distribution and radius of gyration, R_g of starch using various techniques such as Size Exclusion Chromatography (SEC) (Bello-Perez, Roger, Baud, & Colonna, 1998; Fishman & Hoagland, 1994; Fishman, Rodriguez, & Chau, 1996; Han & Lim, 2004a, 2004b; Han, Lim, & Lim, 2005; Tetchi, Rolland-Sabaté, Amani, & Colonna, 2007; Yoo & Jane, 2002), Static Light Scattering (SLS) (Aberle, Burchard, Vorwerg, & Radosta, 1994; Bello-Perez, Paredes-Lopez, Roger, & Colonna, 1996; Bello-Perez, Roger, Colonna, & Paredes-Lopez, 1998; Elfstrand et al., 2004; Millard, Wolf, Dintzis, & Willet, 1999; Roger, Bello-Perez, & Colonna, 1999; Vorwerg & Radosta, 1995; Yang et al., 2006) and Flow Field Flow Fractionation (FFFF) (Bowen et al., 2006; Hanselmann, Burchard, Ehrat, & Widmer, 1996; Roger, Boud, & Colonna, 2001; Rolland-Sabate, Colonna, Mendez-Montealvo, & Planchot, 2007; van Bruijnsvoort, Wahland, Nilsson, & Kok, 2001), as shown in Table 1. However, SEC is incapable of achieving the desired resolution for very large molecules such as amylopectin due to shear scission (Cave, Seabrook, Gidley, & Gilbert, 2009; Gidley et al., 2010). Complete dissolution of starch is required to determine the molar mass and its limited solubility in water is a major obstacle for its characterization. Researchers have adopted various strategies and protocols to disrupt the crystalline structure of starch to facilitate dissolution. In order to produce meaningful data it is necessary to completely disaggregate the amylopectin and amylose molecules within the granules without inducing molecular degradation. The dissolution methods that have been adopted include: solvation in DMSO (Bello-Perez et al., 1996; Han & Lim, 2004a; Han et al., 2005; Millard et al., 1999), treatment with alkali or acid (Han & Lim, 2004b; Yang et al., 2006), high pressure treatment (Bowen et al., 2006), high temperature treatment (Aberle et al., 1994; Bultosa, Hamaker, & BeMiller, 2008; Elfstrand et al., 2004; Hanselmann et al., 1996; van Bruijnsvoort et al., 2001; Vorwerg & Radosta, 1995; Yoo & Jane, 2002), sonication (Jackson, Waniska, & Rooney, 1988), homogenization (Modig, Nilsson, Bergensthal, & Wahlund, 2006), ionic liquid (Stevenson, Biswas, Jane, & Inglett, 2007) and high pressure microwave vessel (HPMV) (Bello-Perez, Roger, Baud, et al., 1998; Bello-Perez, Roger, Colonna, et al., 1998;

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Table 1
Literature M_w and R_g values for waxy maize starch.

Dissolution process	Method	M_w ($\times 10^6$ g/mol)	R_g (nm)	R_h (nm)	Ref.
DMSO, heated for 1 h at 90–100 °C, stored for various time periods	SEC/MALS/RI	171–254	226–244	–	Han and Lim (2004a)
90% DMSO/H_2O, heated for 50 min in hot H_2O bath	SEC/MALS/RI	254	241	–	Han et al. (2005)
90% DMSO–H_2O	SLS & DLS	560	342	348	Millard et al. (1999)
DMSO, heated for 1 h at 90–100 °C, stored for 8 h	SLS	274	255	–	Han et al. (2005)
10% DMSO, 90% H_2O	SLS & DLS	53	242	58	Bello-Perez et al. (1996)
1 M NaOH, rt, vortexed for various time periods	SEC/MALS/RI	88–185	198–215	–	Han and Lim (2004b)
0.5 M NaOH stirred for 18 h, various temperatures	SLS, DLS	129–530	223–276	172–282	Yang et al. (2006)
Boiled in water (30 min)	SEC/MALS/RI	830	372	–	Yoo and Jane (2002)
Autoclaved at 155 °C for 20 min	SLS	77	234	–	Aberle et al. (1994)
Heated at 135 °C for 20 min	SLS	77	–	–	Vorwerg and Radosta (1995)
Heated in 0.1 M $NaNO_3$ at 120 °C, 15 min	SLS	47	62	–	Elfstrand et al. (2004)
Heated in H_2O	AF4/MALS/RI	10–1000	Over 300	–	van Bruijnsvoort et al. (2001)
Autoclaved at 175 °C, 20–60 min	SdFFF/MALS	38–360	121–320	–	Hanselmann et al. (1996)
Heated at 140–50 °C in a pressure cell	AF4/MALS/RI	4092	–	–	Bowen et al. (2006)
Heated for 90 s, (HPMV 700 W)	SEC/MALS/RI	19.5	81		Fishman and Hoagland (1994)
Heated for 80 s (HPMV 700 W)	SEC/MALS/RI	26	76	–	Fishman et al. (1996)
Heated for 35 s, stored for 0–24 h (HPMV 900 W)	SEC/MALS/RI	140–200	220–234	–	Bello-Perez, Roger, Baud, et al. (1998)
Heated for 35 s (HPMV 900 W)	SEC/MALS/RI	318	241	–	Tetchi et al. (2007)
Heated for 35 s, stored for 0–72 h (HPMV 900 W)	SLS, DLS	110–270	200–259	183–203	Bello-Perez, Roger, Colonna, et al. (1998)
Heated for 35–90 s (HPMV 900 W)	SLS, DLS	78–270	170–260	148–221	Roger et al. (1999)
Heated for 35 s (HPMV 900 W)	FFFF/MALS/RI	450	334	–	Roger et al. (2001)
Heated for 40 s (HPMV, 900 W)	AF4/MALS/RI	318	241	–	Rolland-Sabate et al. (2007)

Fishman & Hoagland, 1994; Fishman et al., 1996; Roger et al., 1999; Roger et al., 2001; Rolland-Sabate et al., 2007; Tetchi et al., 2007).

This paper describes experiments undertaken to determine the molecular mass distribution of amylopectin from waxy maize starch sample using AF4/MALS/RI.

1.1. AF4/MALS/RI

The theory of AF4 has been described elsewhere in the literature (Andersson, Wittgren, & Wahlund, 2001; Bowen et al., 2006; Hanselmann et al., 1996; Roger et al., 2001; Rolland-Sabate et al., 2007; van Bruijnsvoort et al., 2001; Viebke & Williams, 2000; Wahlund, 2000; Wittgren & Wahlund, 1997). The fractionation of molecules takes place in a thin channel, which consists of a permeable lower wall, known as the accumulation wall, and an upper impermeable solid wall (Viebke & Williams, 2000). The separation of the molecules is governed by the differences in the diffusion coefficient D. The diffusion coefficient, D, of the molecules can be determined directly from AF4 as shown in Eq. (1) below, where t^0 is the void time, F_{cr} is the cross flow, w is the channel thickness, V^0 is the geometric void volume, and t_r is the retention time (Viebke & Williams, 2000).

$$D = \frac{t^0 F_{cr} w^2}{6V^0} \times \frac{1}{t_r} \tag{1}$$

Void time t^0 is calculated using Eq. (2) (Wahlund, 2000).

$$t^0 = \frac{V^0}{F_{cr}} \ln\left\{1 + \frac{F_{cr}}{F_{ch}}\left[1 - \frac{w(b_0 z - ((b_0 - b_L)/2L)z^2 - y)}{V^0}\right]\right\} \tag{2}$$

The geometric void volume, V^0 is determined from the channel parameters. The channel thickness, w is determined experimentally, b_0 and b_L are the breadths at the channel inlet and outlet respectively, z is the distance between the channel inlet and the focusing point, y is the channel area lost by the tapered inlet, F_{cr} is the cross flow rate, F_{ch} is the channel flow rate and L is the channel length.

The hydrodynamic radius, R_h of the molecule is related to D according to the Stokes–Einstein relationship (Andersson, Wittgren, & Wahlund, 2003),

$$R_h = \frac{kT}{6\pi\eta D} \tag{3}$$

R_h can therefore be determined directly from the retention time as shown in Eq. (4), where, k is Boltzmann's constant, T is the temperature (K), F_{cr} is the cross flow, η the viscosity coefficient of the solvent and t_r is the retention time (Wahlund, 2000).

$$R_h = \frac{kTV^0}{\pi\eta t^0 F_{cr} w^2} \times t_r \tag{4}$$

The retention of the molecules is dependent on the cross flow (F_{cr}) and channel flow (F_{ch}) ratio, F_{cr}/F_{ch} which may be determined from Eq. (5), if the diffusion coefficient of the molecule is known (Wahlund, 2000).

$$\frac{F_{cr}}{F_{ch}} = \frac{\exp(6Dt_r/w^2) - 1}{A} \tag{5}$$

In online multi-angle laser light scattering (MALS) the scattered light is measured at various angles ranging from 26° to 143°, the excess Rayleigh ratio is extrapolated to zero angle using the Berry (Eq. (6)) and Debye (Eq. (7)) fits (Andersson et al., 2003), where c is the concentration of the macromolecules, M_w is the weight-average molar mass, $P(\theta)$ is the particle scattering function, R_θ is the excess Rayleigh ratio, K an optical constant, λ_0 is the wavelength of the laser in vacuum.

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} + \frac{16\pi^2}{3\lambda^2} \times R_g^2 \times \sin^2\left(\frac{\theta}{2}\right) \tag{6}$$

$$\sqrt{\frac{Kc}{R_\theta}} = \sqrt{\frac{1}{M_w} + \frac{16\pi^2}{3\lambda^2} \times R_g^2 \times \sin^2\left(\frac{\theta}{2}\right)} \tag{7}$$

The molecular mass of the macromolecule is obtained from the intercept and the radius of gyration from the gradient.

2. Materials and methods

A sample of waxy maize starch was provided by National Starch. Amylopectin Azure (Sigma–Aldrich; Gillingham, UK), a blue-colored water-soluble potato amylopectin starch was employed to visualize the focusing step. Bovine serum albumin (BSA) was also obtained from Sigma–Aldrich, Gillingham, UK to calibrate the channel thickness.

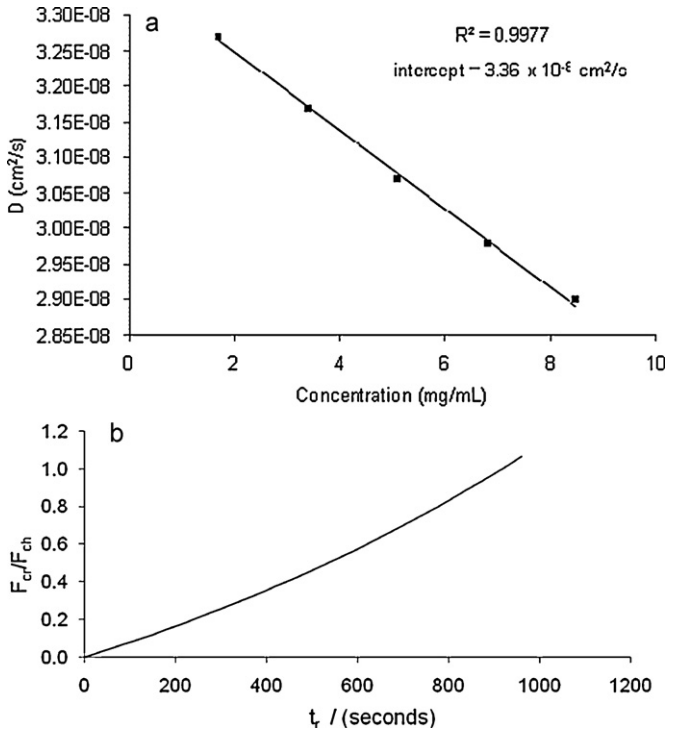

Fig. 1. (a) Plot of D as a function of waxy maize starch concentration determined using DLS. (b) F_{cr}/F_{ch} as a function of t_r.

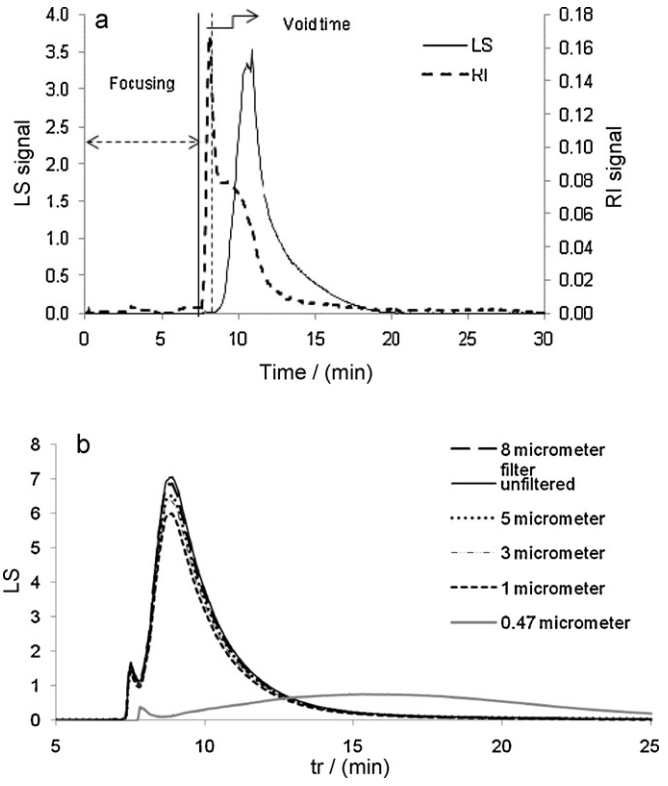

Fig. 2. (a) LS and RI as a function of time for waxy maize starch. The data corresponding to focusing and void time were not included in the determination of M_w and R_g of amylopectin. (b) LS signal as a function of t_r for waxy maize starch solutions filtered through 8 μm, 5 μm, 3 μm, 1 μm and 0.47 μm cellulose nitrate filters.

2.1. Dissolution using a high pressure microwave vessel (HPMV)

HPMV has been employed by a number of researchers for the dissolution of starch (Bello-Perez, Roger, Baud, et al., 1998; Bello-Perez, Roger, Colonna, et al., 1998; Fishman & Hoagland, 1994; Fishman et al., 1996; Roger et al., 1999, 2001; Rolland-Sabate et al., 2007; Tetchi et al., 2007) and polysaccharides (Ratcliffe, Williams, Viebke, & Meadows, 2005). Pregelatinisation of waxy maize starch samples was carried out by dissolving each sample in DMSO (95%), stirring for 12 h and then precipitating with ethanol. The pregelatinised waxy maize starch sample was dried at room temperature. 20–30 mg of the pregelatinised waxy maize starch sample was left to stir in 50 ml of 1 M KSCN for 12 h, following which 20 ml of the solution was then transferred into the Teflon cup of a model 4782 polycarbonate HPMV (total volume 45 ml) (Parr Instrument Co., Moline, IL) and placed in a microwave oven (800 W) and heated at 90% power for 60 s. The HPMV was cooled in an ice bath for 1 h after which the solution was filtered through an 8 μm cellulose nitrate filter.

2.2. AF4/MALS/RI

Starch solutions were analysed using AF4/MALS/RI. The eluent (0.035 M KSCN) was passed through a vacuum degasser (Cambridge Scientific Instruments Ltd., Ely, UK) into the Flow Box P 2.1 (ConSenxus, Ober-Hilbersheim, Germany) and then through a 0.22 μm inline filter (Millipore (U.K.) Ltd., Watford, UK) into the Control-Box V3 valve box (Control-Box V3, ConSenxus, Germany) before entering the aFFFF channel (ConSenxus, Ober-Hilbersheim, Germany). The dimensions of the channel were 320 mm × 100 mm × 145 mm, and the area of the accumulation wall was 36.09 cm^2. A polysulphone membrane, with a M_w-cut-off of 10 kDa (ConSenxus, Ober-Hilbersheim, Germany), and a 190 μm spacer were employed. The sample was injected through a ChemInert® injection valve (VICI Valco Instruments Ltd., Houston, Texas) with a sample loop volume of 100 μl. The channel outlet was connected both to the Valve

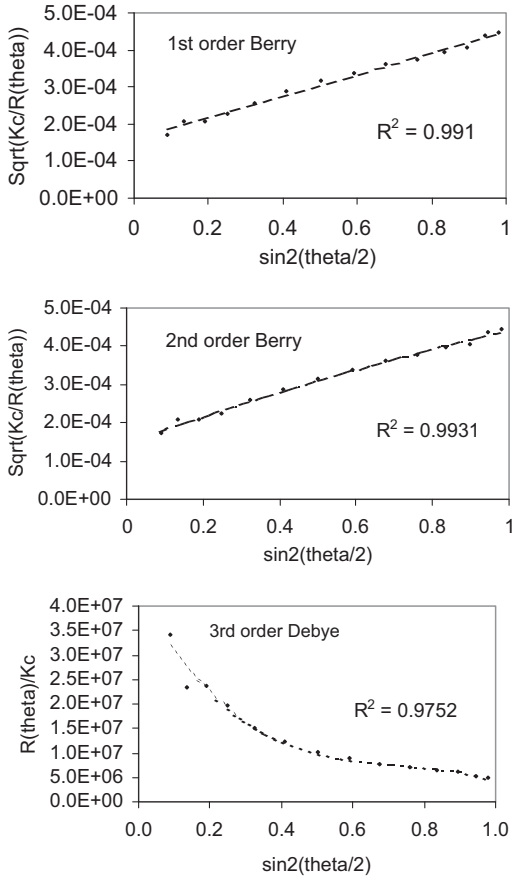

Fig. 3. Comparison of Debye and Berry fits for waxy maize starch.

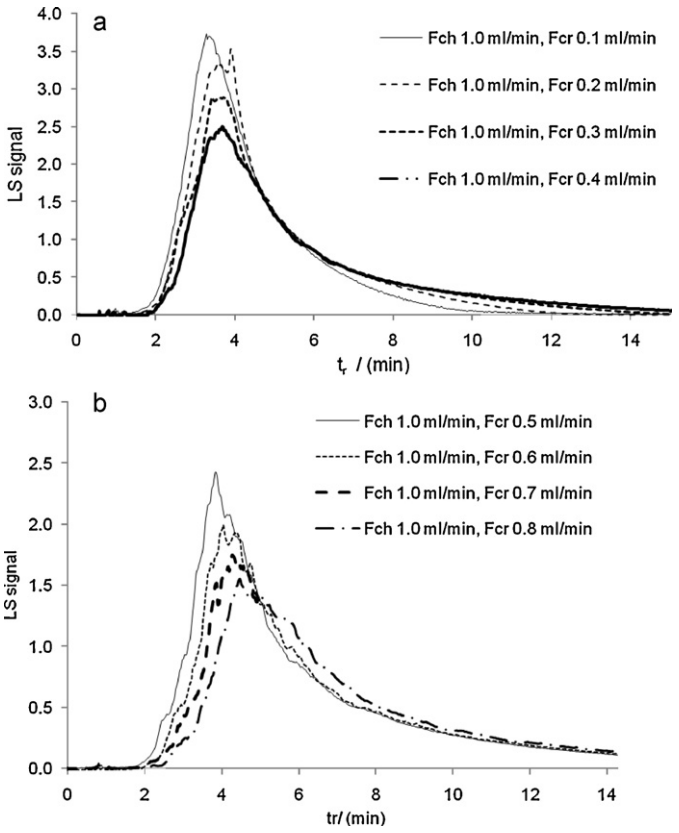

Fig. 4. (a) Effect of varying F_{cr} from 0.1 to 0.4 ml/min at a fixed F_{ch} rate of 1.0 ml/min upon LS peaks. (b) Effect of varying F_{cr} from 0.5 to 0.8 ml/min at a fixed F_{ch} rate of 1.0 ml/min upon LS peaks.

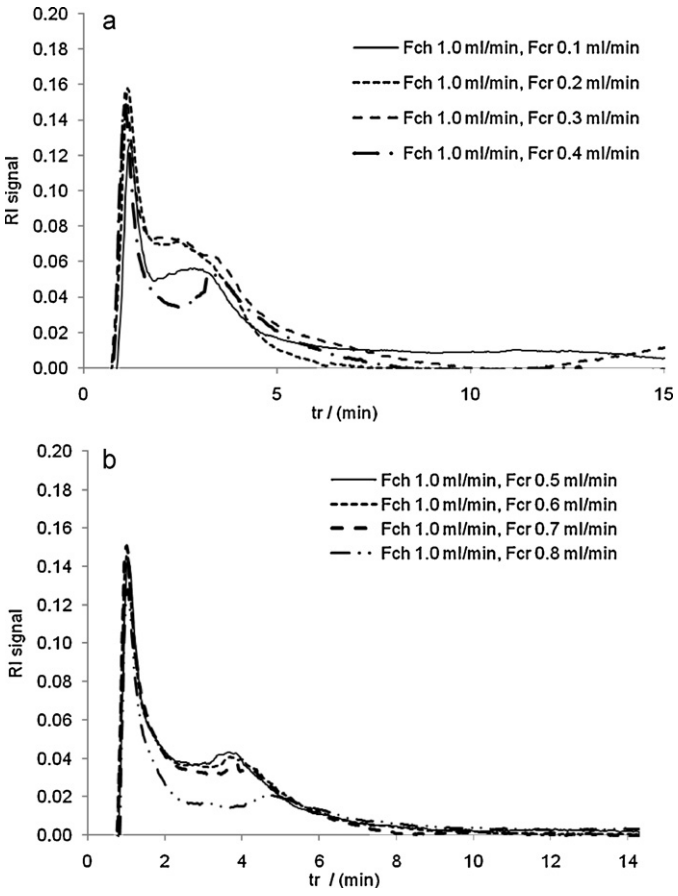

Fig. 5. (a) Effect of varying F_{cr} from 0.1 to 0.4 ml/min at a fixed F_{ch} rate of 1.0 ml/min upon RI peaks. (b) Effect of varying F_{cr} from 0.5 to 0.8 ml/min at a fixed F_{ch} rate of 1.0 ml/min upon RI peaks.

Box and the detectors. The flow rates were controlled employing WinFFF_Control V.30 software (ConSenxus, Ober-Hilbersheim, Germany). The on-line detectors employed were multi-angle laser light scattering Wyatt Dawn® EOS ($\lambda = 690$ nm) (Wyatt Technology Corporation, Santa Barbara, CA) and the RI detector was an interferometric refractrometer Optilab DSP (Wyatt Technology Corporation, Santa Barbara, CA). The results were collected and processed employing ASTRA for Windows 4.90 (Wyatt Technology Corporation, Santa Barbara, CA).

The channel thickness was calculated using Eq. (4) from experiments using BSA, which has a diffusion coefficient of 5.96×10^{-7} cm^2/s at 294 K, and was found to be 165 μm.

The RI detector was calibrated with sodium chloride. The calibration constant of 2.369×10^{-5} was employed to process the data. The MALS detector was calibrated with toluene, and the calibration constant determined was 9.04×10^{-6}. A dn/dc value of 0.156 was determined for the waxy maize starch in 0.035 M KSCN experimentally.

2.3. Analysis of waxy maize using AF4/MALS/RI

Aqueous solutions of waxy maize starch were prepared by heating for 60 s in the HPMV. The focusing time was determined by direct observation with amylopectin-azure solution and found to be 7 min. The eluent employed was 0.035 M KSCN with 0.05% NaN_3 filtered through a Millipore 0.2 μm cellulose nitrate filter. A 100 μl sample of waxy maize starch solution in 1 M KSCN was focused for 7 min, a F_{ch} rate of 2.5 ml/min and a F_{cr} rate of 2.4 ml/min was used. The separation of waxy maize starch molecules was studied for a range of channel flow, F_{ch}, and cross flow, F_{cr}, rates.

2.4. Determination of D and R_h of waxy maize starch using DLS

D and R_h of waxy maize starch in 0.035 M KSCN was measured using the Malvern Zeta Nano ZS (Malvern Instruments Ltd., Malvern, UK). A stock solution of waxy maize starch (8.48 mg/ml in 1 M KSCN) was prepared by heating in a HPMV for 60 s followed by an hour of cooling in an ice bath. Further dilutions were prepared to yield five different concentrations ranging from 8.48 mg/ml to 1.69 mg/ml. D was determined at room temperature (21 °C) and plotted as a function of concentration. The diffusion coefficient at zero concentration, D_0 was determined by extrapolation. The hydrodynamic radius R_h was determined using Eq. (3).

3. Results and discussion

The D values for waxy maize starch solutions at varying concentrations are presented in Fig. 1a. D_0 was determined by extrapolation to zero concentration and was found to have a value of 3.35×10^{-8} cm^2 s^{-1} which corresponds to an R_h value of 73 nm. Theoretical F_{cr}/F_{ch} values were calculated using Eq. (5) and the values are plotted in Fig. 1b. The sample will not elute if F_{cr} value is greater than that of F_{ch} value, therefore the value of F_{cr}/F_{ch} should not exceed 1. The resolution of the peak can be improved by fine tuning F_{cr}/F_{ch} values and the estimation of the t_r of the peak can also be undertaken from these values. At F_{cr}/F_{ch} values of 0.05–1.00, waxy maize starch peak is expected to elute within 16 min, as shown in Fig. 1b.

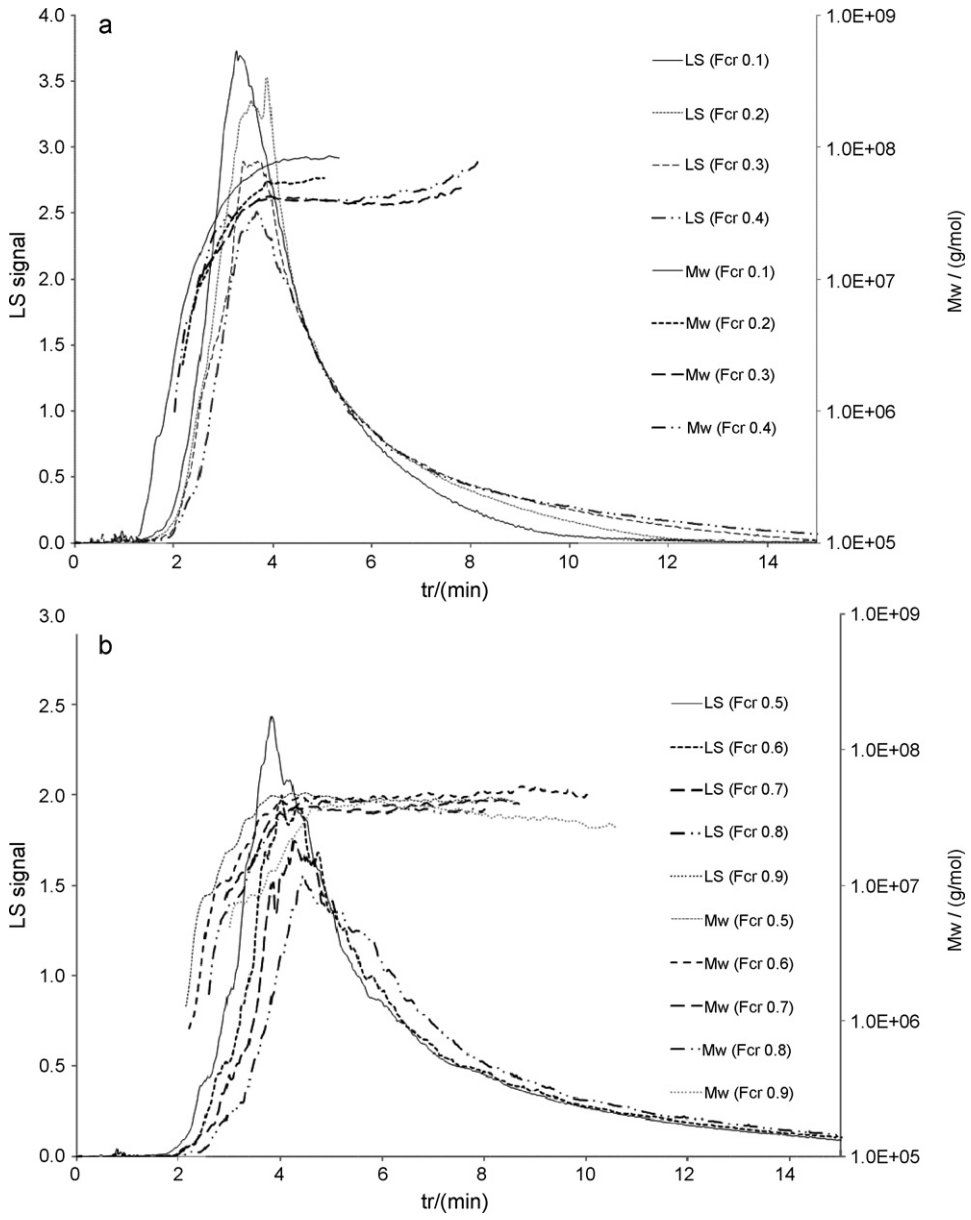

Fig. 6. (a) M_w as a function of t_r with LS peaks superimposed determined using the 1st order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) M_w as a function of t_r with LS peaks superimposed determined using the 1st order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

3.1. Typical elution profiles of waxy maize starch

A solution of waxy maize starch (2.76×10^{-2} mg/ml) filtered through an 8 μm cellulose nitrate filter was analysed with AF4/MALS/RI at F_{ch} of 1 ml/min and F_{cr} of 0.2 ml/min. The resultant LS and RI peaks are shown in Fig. 2a and indicate that the waxy maize starch is highly disperse, since the LS and RI peaks do not superimpose. The LS peak is very large in contrast to the RI peak. The former is due to the fact that the eluting molecules have a very high molecular mass and the latter is due to the fact that only a very low concentration of sample is used otherwise the light scattering signal becomes saturated. The LS signal increases steadily from 8.5 min and reaches a maximum intensity at around 10.5 min, thereafter the LS peak gradually decreases. Focusing of the waxy maize starch occurs for the initial ~7 min. The overall pressure of the system during focusing increases from 10 bar to 18 bar and then returns to 10 bar in the transition from focusing to elution mode. The small spike in the RI peak observed at ~8 min is an experimental artifact caused by the pressure drop and corresponds to the void time. The peak area corresponding the void peak was excluded from the analysis of light scattering and refractive index peak as shown in Fig. 2a.

3.2. Effect of filtration upon waxy maize starch AF4/MALS/RI elution profiles

The effect of filtration was investigated by filtering waxy maize starch solutions through 8 μm, 5 μm, 3 μm, 1 μm and 0.47 μm cellulose nitrate filters. These solutions were analysed by AF4/MALS/RI at a F_{ch} rate of 1.0 ml/min and a F_{cr} rate of 0.1 ml/min. The resultant LS peaks are shown in Fig. 2b. These were compared against LS peaks obtained with an unfiltered solution. The LS signal obtained for waxy maize starch solution filtered through a 0.47 μm cellulose nitrate filter is very low due to the retention of high M_w fractions on the filter. However, the LS signal for corresponding waxy maize starch solutions filtered through 8 μm, 5 μm, 3 μm and 1 μm filters

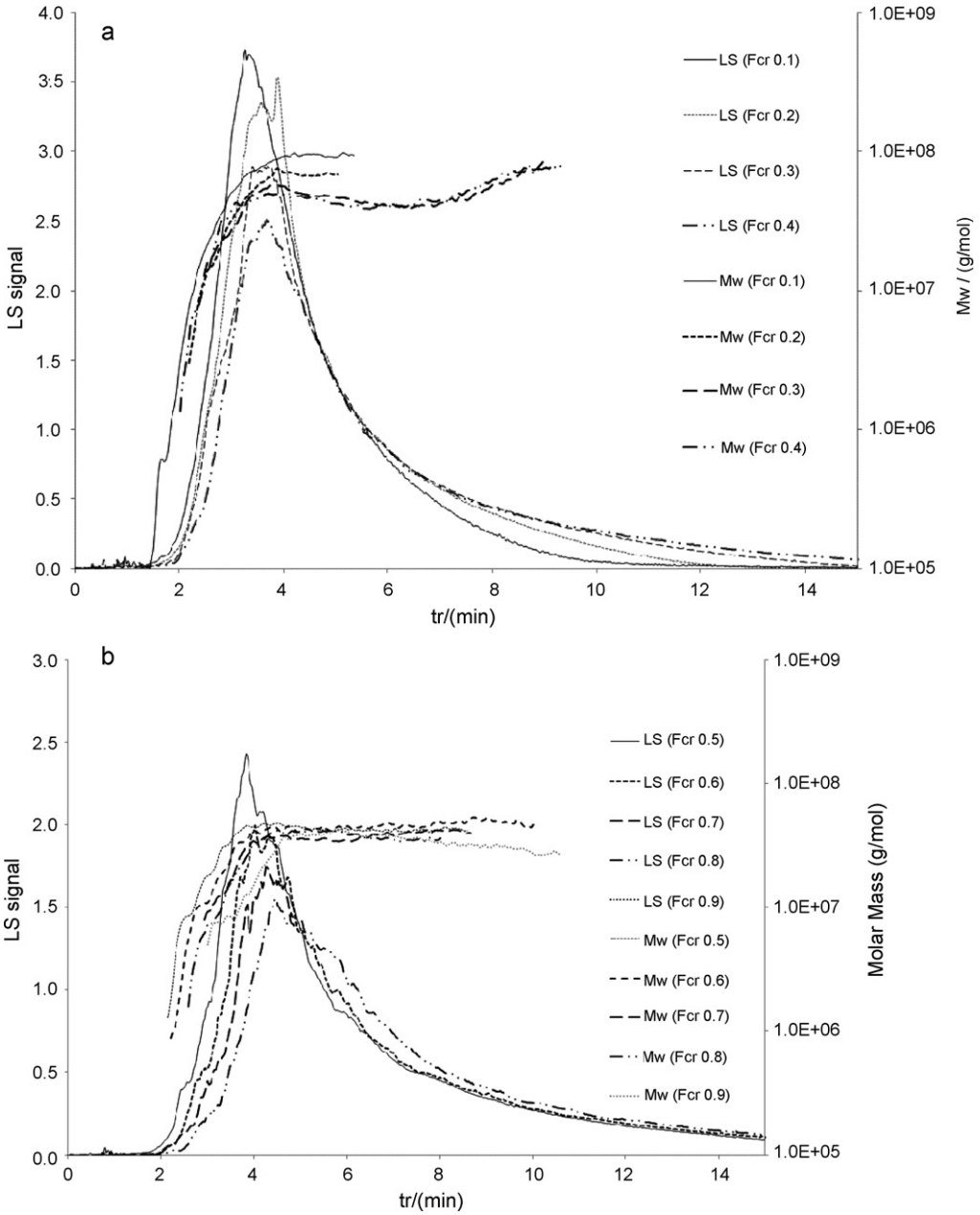

Fig. 7. (a) M_w as a function of t_r with LS peaks superimposed determined using the 2nd order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) M_w as a function of t_r with LS peaks superimposed determined using the 2nd order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

are unaffected. An 8 μm cellulose nitrate filter was chosen to filter waxy maize starch solutions for AF4/MALS/RI analysis.

3.3. Analysis of light scattering data

The MALS data was evaluated using Eqs. (6) and (7) by plotting the excess Rayleigh scattering against $\sin^2(\theta/2)$ at different angles ranging from 14° to 163°. Low angle detectors 14°, 26° and 35° were not included to process the results in some cases due to oversaturation. The Berry and Debye equations were found to provide the best fits to the light scattering data as shown in Fig. 3. The Berry method has been reported to be the most appropriate by others for starch (Bello-Perez, Roger, Baud, et al., 1998; Rolland-Sabate et al., 2007).

3.4. Effect of varying F_{cr} rates upon LS and RI elution profiles

At a fixed F_{ch} of 1.0 ml/min, F_{cr} was varied from 0.1 to 1.0 ml/min in 0.1 ml/min increments. The retention time, t_r, was determined by subtracting the focusing time from the overall collection time. The LS and RI peaks as a function of retention time are shown in Figs. 4a, b and 5a, b, respectively. Both Figures show that the peaks become broader with increasing F_{cr} rates, due to the increase in retention time of the polymer molecules in the channel. The initial spike observed in the RI peak which appears from ~1 min to ~2 min (Fig. 5a and b) is due to the pressure drop in the transition from focusing to elution. Due to the low signal-to-noise ratio the pressure drop is more noticeable with RI peaks. The peak areas corresponding to the void peak and the RI spike were excluded for the determination of the molar mass and size measurements.

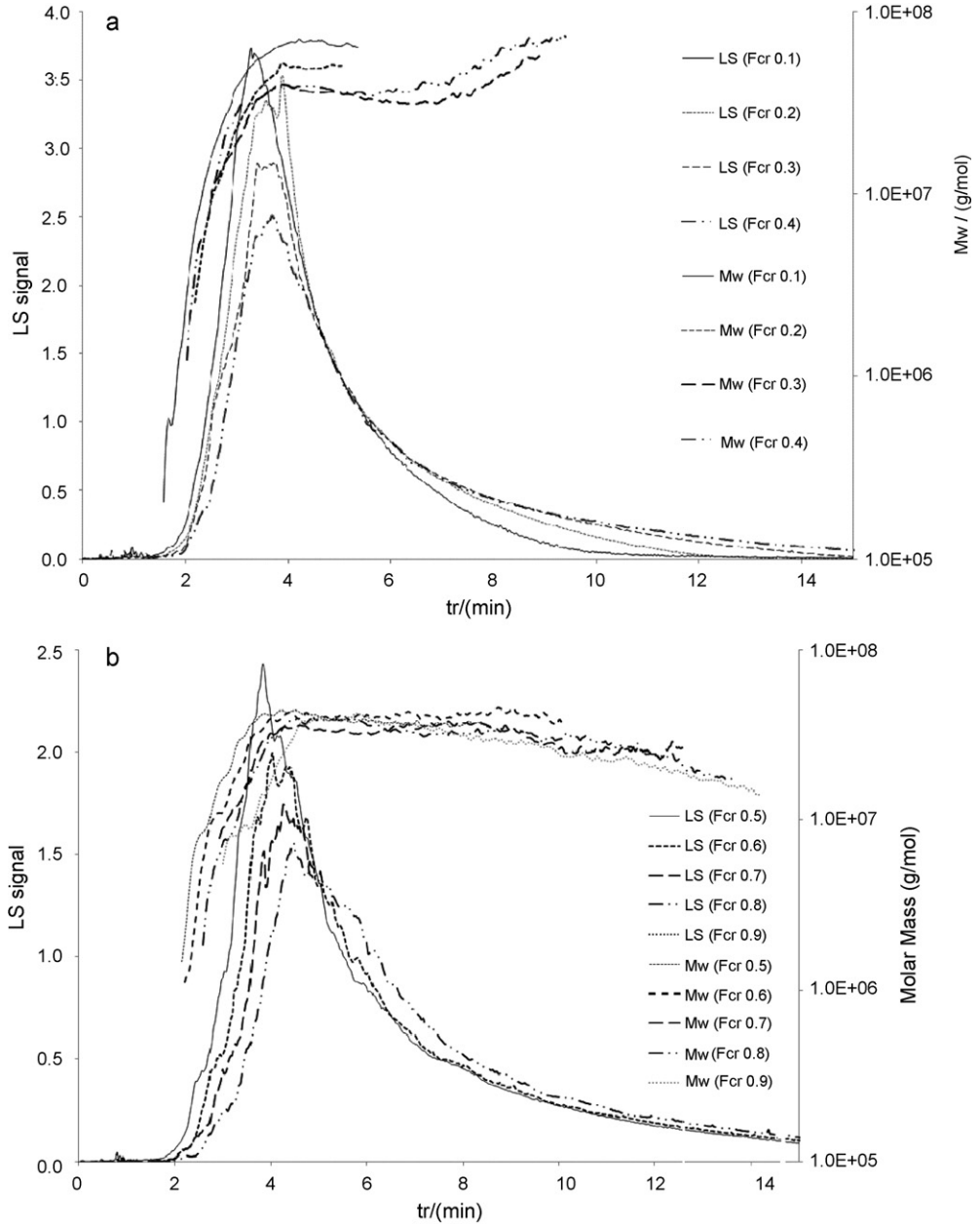

Fig. 8. (a) M_w as a function of t_r with LS peaks superimposed determined using the 3rd order Debye fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) M_w as a function of t_r with LS peaks superimposed determined using the 3rd order Debye fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

3.5. Average M_w and R_g values obtained with varying flow rates for waxy maize starch

The average M_w and R_g values of waxy maize starch obtained at a fixed F_{ch} rate of 1.0 ml/min with varying F_{cr} rates determined using Berry (1st and 2nd order) fits and 3rd order Debye fit are presented in Table 2. It should be noted that the errors generated by the ASTRA software quoted in Table 2, are not absolute and only represent the quality of the data collected. The LS data corresponding to low angles (14° to 48°) were excluded due to oversaturation (Table 2). The resultant M_w and R_g values are therefore prone to errors. The results show that M_w and R_g vary slightly with the LS fit model used. The values obtained are of the same order as reported by others using alternative dissolution procedures and measurement techniques which are summarized in Table 1.

The average M_w and R_g values of waxy maize starch determined with AF4/MALS/RI were found to be 32×10^6 g/mol and 138 nm (1st order Berry fit) and 37×10^6 g/mol and 164 nm (2nd order Berry fit), respectively. We obtained high mass recoveries ranging from 100% to 92% determined from the mass injected. A number of researchers have reported M_w values ranging from 20×10^6 g/mol to over 300×10^6 g/mol and R_g values ranging from 60 nm to over 300 nm as detailed in Table 1. The variation of the M_w and R_g values reported in the literature may arise from a number of factors including the source of waxy maize starch, the dissolution process, the analytical technique used and in the case of MALS the procedure used to process the light scattering data. Bowen et al. reported results using AF4/MALS/RI for waxy maize starch samples which were dispersed in water and treated in a pressure cell at 140 °C for 10 min with an applied pressure of 4 bar in order to dissolve (2006). A M_w value of 409×10^6 g/mol was determined which is considerably higher

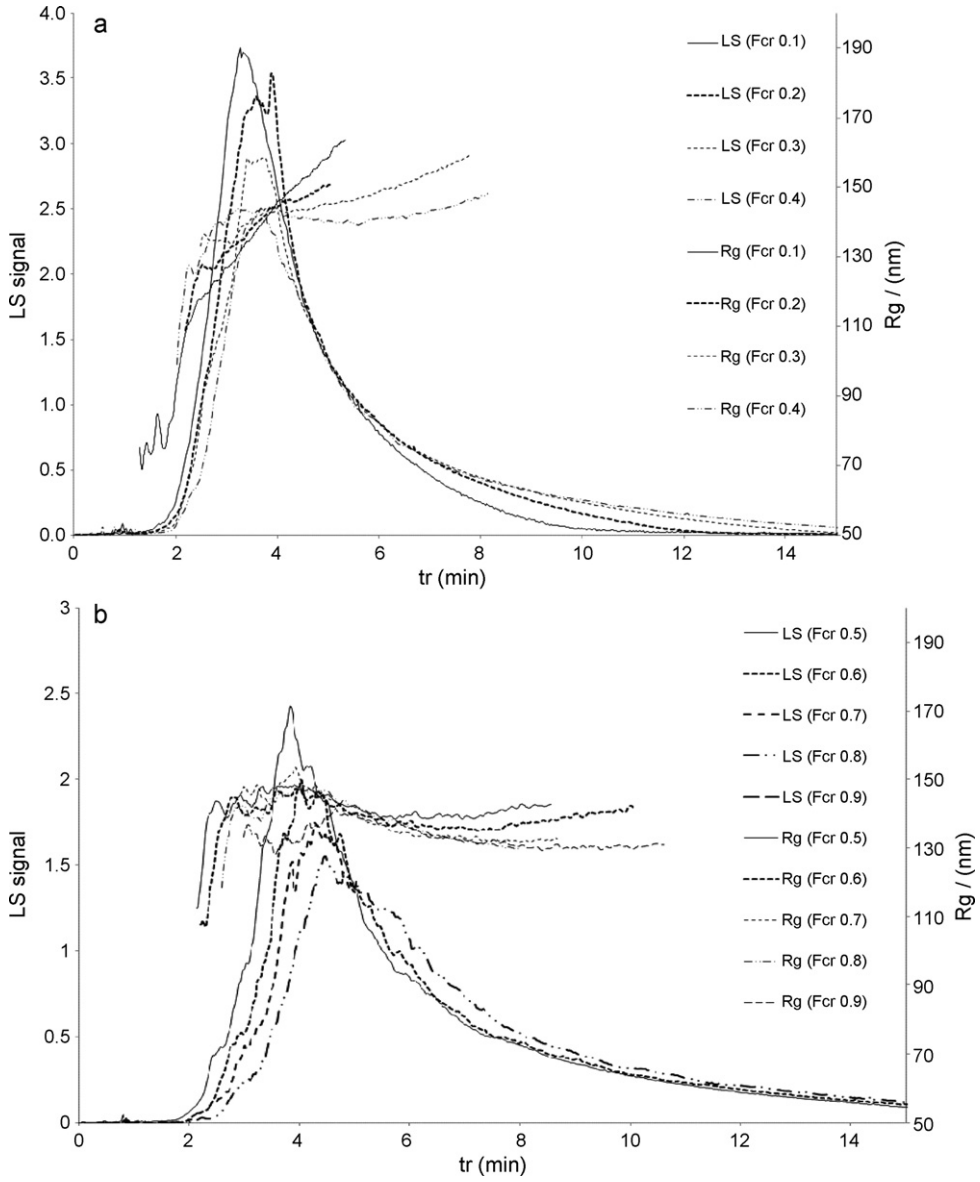

Fig. 9. (a) R_g as a function of t_r with LS peaks superimposed determined using the 1st order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) R_g as a function of t_r with LS peaks superimposed determined using the 1st order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

Table 2
Average M_w and R_g results for waxy maize starch obtained at fixed F_{ch} rate of 1.0 ml/min with varying F_{cr} rates determined using the 1st order Berry fit, the 2nd order Berry fit and the 3rd order Debye fit. The %error values generated by ASTRA software are given in brackets, which represents the quality of the data collected from LS detector and are not absolute errors.

F_{cr} (ml/min)	LS detectors excluded	1st order Berry fit		2nd order Berry fit		3rd order Debye fit		% mass recovery
		M_w (g/mol)	R_g (nm)	M_w (g/mol)	R_g (nm)	M_w (g/mol)	R_g (nm)	
0.1	14°, 26°, 35° & 43°	33×10^6 (3%)	141 (1%)	41×10^6 (6%)	167 (3%)	36×10^6 (3%)	119 (3%)	100
0.2	14°, 26°, 35° & 43°	31×10^6 (4%)	140 (2%)	41×10^6 (4%)	178 (2%)	30×10^6 (3%)	121 (3%)	100
0.3	14°, 26°, 35° & 43°	33×10^6 (4%)	146 (1%)	42×10^6 (7%)	174 (3%)	31×10^6 (2%)	120 (2%)	100
0.4	14°& 26°	34×10^6 (4%)	142 (1%)	39×10^6 (6%)	162 (3%)	32×10^6 (3%)	120 (3%)	100
0.5	14° & 26°	33×10^6 (3%)	144 (1%)	38×10^6 (6%)	165 (3%)	31×10^6 (2%)	120 (3%)	98
0.6	14° & 26°	32×10^6 (4%)	141 (1%)	37×10^6 (6%)	163 (3%)	30×10^6 (3%)	120 (3%)	98
0.7	14° & 26°	31×10^6 (4%)	140 (1%)	35×10^6 (6%)	160 (3%)	29×10^6 (3%)	118 (3%)	98
0.8	14° & 26°	31×10^6 (4%)	138 (1%)	34×10^6 (6%)	158 (3%)	29×10^6 (3%)	117 (3%)	99
0.9	14° & 26°	29×10^6 (5%)	134 (2%)	34×10^6 (7%)	161 (3%)	28×10^6 (3%)	118 (3%)	92
1.0	14°	27×10^6 (4%)	135 (2%)	28×10^6 (6%)	150 (3%)	25×10^6 (3%)	115 (4%)	88

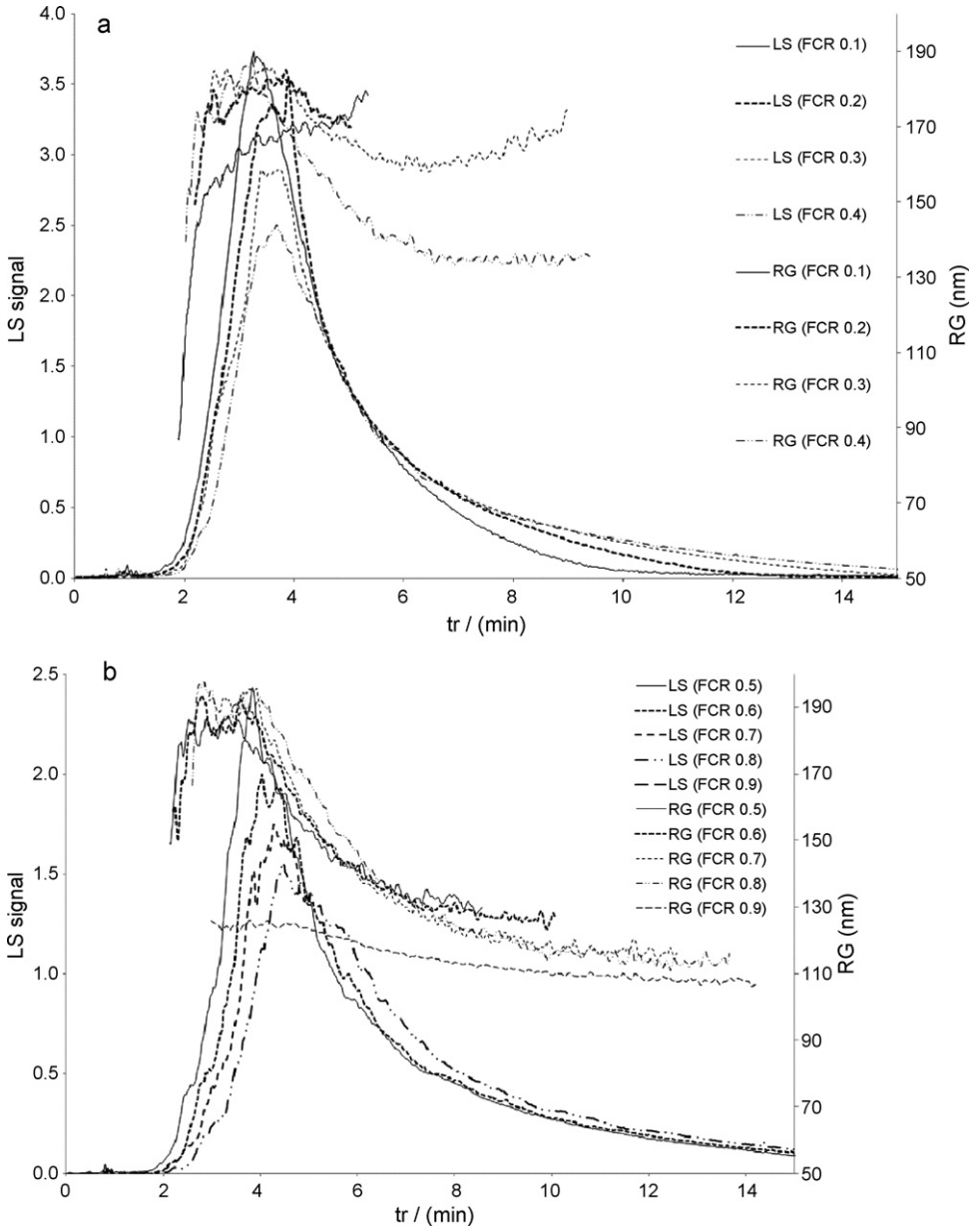

Fig. 10. (a) R_g as a function of t_r with LS peaks superimposed determined using the 2nd order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) R_g as a function of t_r with LS peaks superimposed determined using the 2nd order Berry fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

than values found in our work and by others. Rolland-Sabaté et al. also determined the M_w and R_g of waxy maize starch using a similar dissolution procedure to ours together with AF4/MALS/RI and reported values of 318×10^6 g mol^{-1} and 228.9 nm, respectively (2007). van Bruijnsvoort et al. also determined the M_w distribution of waxy maize starch using aFFFF coupled with MALS/RI detectors and also reported M_w values in excess of 100×10^6 g/mol with R_g values over 300 nm (2001). Interestingly, they noted the presence of a steric/hyperlayer effect since they observed that R_g decreased rather than increased with elution time. In these studies the starch samples were simply prepared by heating in water and it is likely that they were not fully solubilised.

3.6. Effect of varying flow rates upon M_w and R_g distributions for waxy maize starch

The M_w distribution as a function of elution time for waxy maize starch obtained at a fixed F_{ch} rate of 1.0 ml/min and varying F_{cr} rates ranging from 0.1 ml/min to 0.9 ml/min, employing three different LS models: 1st order Berry (Fig. 6a and b), 2nd order Berry (Fig. 7a and b), and 3rd order Debye fit (Fig. 8a and b). Similar molar mass profiles for waxy corn starch were obtained with all three different LS fits. At a F_{cr} rate of 0.1 ml/min, M_w determined with the 1st order Berry fit increases sharply from $\sim 1 \times 10^6$ g/mol to 50×10^6 g/mol and then gradually increases to 80×10^6 g/mol. Thereafter, it decreases to 50×10^6 g/mol. Similar behaviour is seen with a F_{cr} rate of 0.2 ml/min. At high F_{cr} rates the M_w increases rapidly from $\sim 5 \times 10^6$ g/mol to 40×10^6 g/mol and then gradually increases to $\sim 50 \times 10^6$ g/mol, after which it increases rapidly to $\sim 70 \times 10^6$ g/mol. Similar M_w distributions were also obtained at a fixed F_{ch} rate of 0.5 ml/min with varying F_{cr} rates determined using the 1st order Berry fit, shown in Fig. 14. The separation of R_g as a function of t_r at a fixed F_{ch} rate of 1.0 ml/min with variable F_{cr} rates was determined with the 1st order Berry fit (Fig. 9a and b), the 2nd order Berry fit (Fig. 10a and b), and 3rd order Debye fit (Fig. 11a and b).

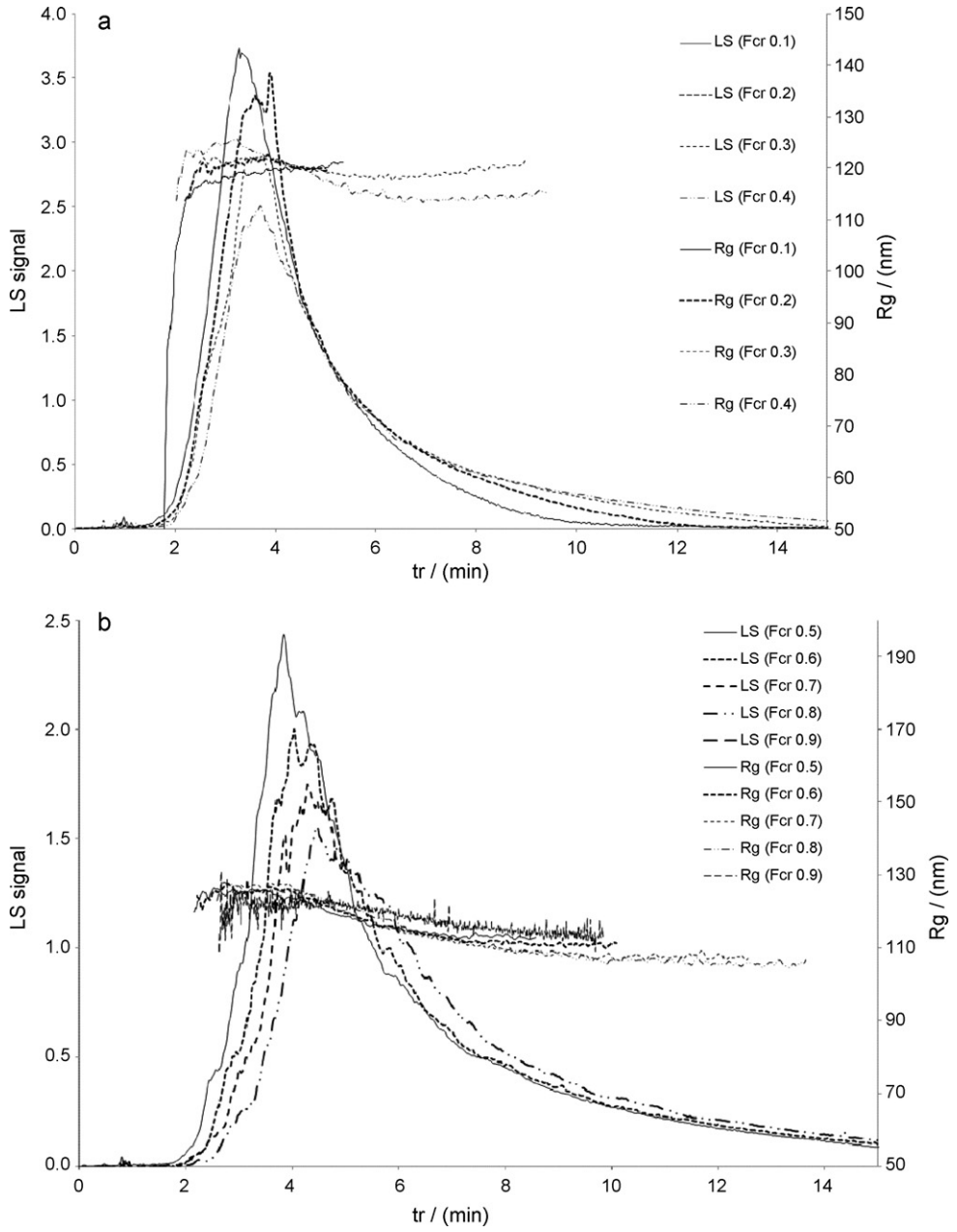

Fig. 11. (a) R_g as a function of t_r with LS peaks superimposed determined using the 3rd order Debye fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.1 ml/min to 0.4 ml/min. (b) R_g as a function of t_r with LS peaks superimposed determined using the 3rd order Debye fit obtained for waxy maize starch at a fixed F_{ch} rate of 1.0 ml/min with F_{cr} rates ranging from 0.5 ml/min to 0.9 ml/min.

At a F_{cr} rate of 0.1 ml/min for waxy maize starch using the 1st order Berry fit, R_g was found to rise sharply from 70 nm to 120 nm and then gradually increases to 190 nm, as seen in Fig. 9a. At higher F_{cr} rates of 0.2–0.4 ml/min, R_g separation is hardly observed with R_g values ranging from 120 nm to 150 nm as shown in Fig. 9a. R_g separations obtained at lower F_{cr} rates determined with the 2nd order Berry fit (Fig. 10a) are similar to the ones obtained with 1st order Berry fit. At higher F_{cr} rates (0.5 ml/min to 0.9 ml/min) the R_g values initially do not change and slowly decrease afterwards. As with the 3rd order Debye fit no R_g separation is observed shown in Fig. 11a and b. Therefore, the 1st order Berry was found to be a suitable method for determining the M_w and R_g of waxy maize starch.

3.7. *Effect of non-linear elution programmes upon the M_w and R_g distributions of waxy maize starch*

Non-linear elution, such as gradient and exponential decay programmes in which the F_{ch} rate is constant and the F_{cr} rate lowers as a function of retention time, was used to analyse waxy maize starch.

At a fixed F_{ch} rate of 0.7 ml/min, the F_{cr} rate was decreased in a gradient fashion from 0.7 ml/min to 0.05 ml/min over 20 min. The LS and RI elution peaks with the M_w distribution and R_g superimposed, as shown in Figs. 12 and 13a and b, respectively. At a fixed F_{ch} rate of 0.7 ml/min, the F_{cr} rate was decreased exponentially from 0.7 ml/min to 0.05 ml/min over 20 min. The LS and RI

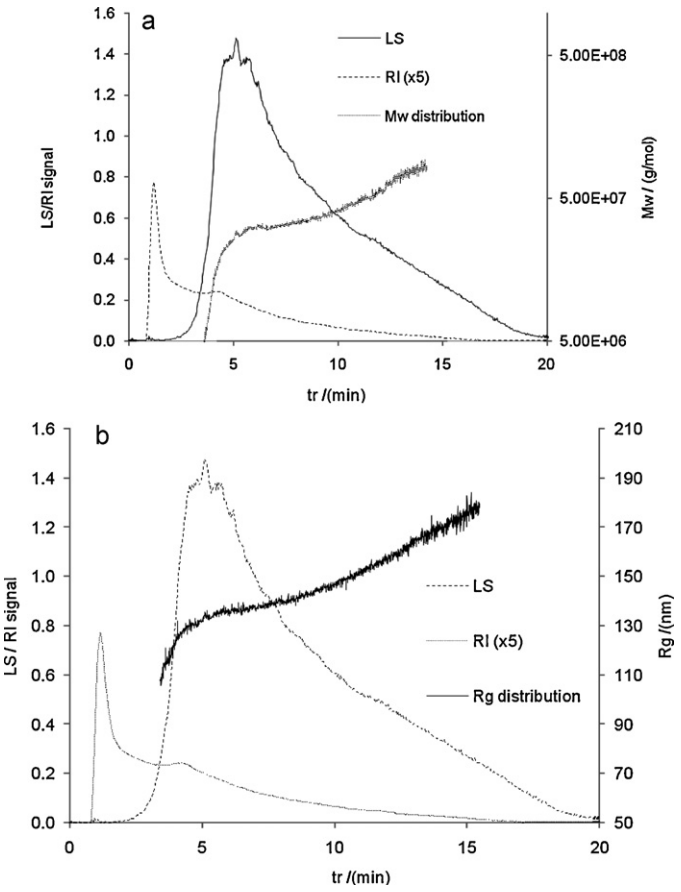

Fig. 12. (a) LS and RI peaks with the M_w distribution of waxy maize starch obtained at a fixed F_{ch} rate of 0.7 ml/min with F_{cr} rate varied from 0.7 ml/min to 0.05 ml/min in a gradient. (b) LS and RI peaks with the R_g distribution of waxy maize starch obtained at a fixed F_{ch} rate of 0.7 ml/min with F_{cr} rate varied from 0.7 ml/min to 0.05 ml/min in a gradient.

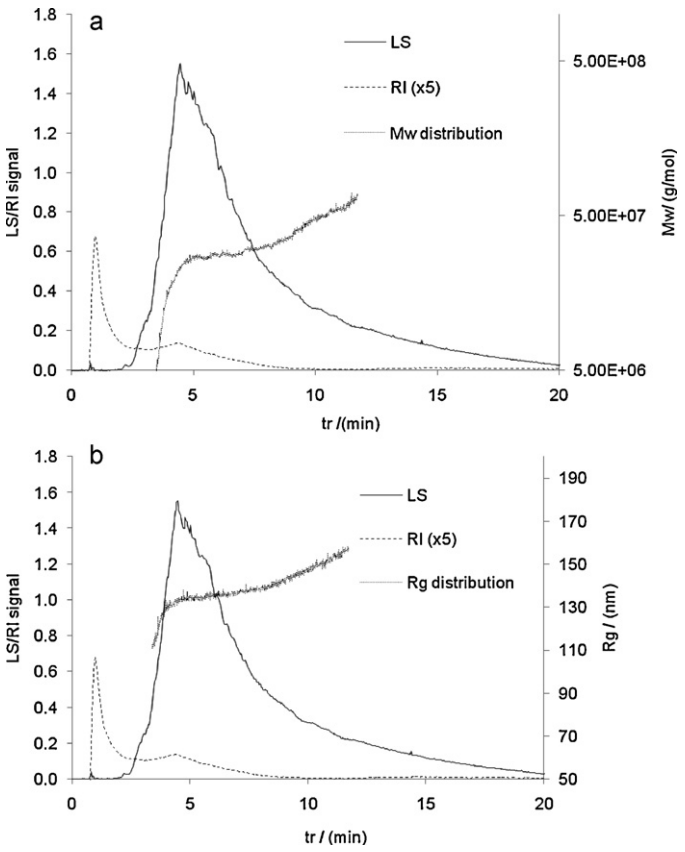

Fig. 13. (a) LS and RI peaks with the M_w distribution of waxy maize starch obtained at a fixed F_{ch} rate of 0.7 ml/min with F_{cr} rate decreased exponentially from 0.7 ml/min to 0.05 ml/min. (b) LS and RI peaks with the R_g distribution of waxy maize starch obtained at a fixed F_{ch} rate of 0.7 ml/min with the F_{cr} rate decreased exponentially from 0.7 ml/min to 0.05 ml/min.

elution peaks with the M_w distribution superimposed is shown in Fig. 13a. The R_g distribution of waxy maize starch with LS and RI peaks superimposed is shown in Fig. 13b.

The majority of the sample eluted within 15 min with the gradient or exponential elution programme. The M_w rapidly increased from 5×10^6 g/mol to 45×10^6 g/mol, and then slowly increased to 80×10^6 g/mol for both gradient and exponential decay programmes as seen in Figs. 12 and 13a, respectively. R_g of waxy maize starch increases gradually from ~100 nm to 180 nm during gradient elution, as seen in Fig. 12b. Likewise, the R_g of waxy maize starch also increases gradually from ~110 nm to 170 nm using an exponential decay elution programme as seen in Fig. 13b. The average molar mass and R_g values determined for waxy maize starch using the non-linear elution programmes are shown in Table 3. The average M_w and R_g values obtained with both gradient and exponential decay elution programmes are similar to the values obtained with linear elution programmes. Very high mass recoveries were obtained with both gradient and exponential decay elution programmes.

Table 3
Average molar mass and R_g values for waxy maize starch obtained with gradient and exponential decay programmes determined using the 1st order Berry fit. The % error values generated by ASTRA software is given in brackets and represent the quality of the LS detectors. LS data from low angles 14° and 26° were excluded from the determination of these results due to oversaturation.

Mode	M_w (g/mol)	R_g (nm)	% mass recovery
Gradient	33×10^6 (5%)	149 (2%)	99
Exponential decay	30×10^6 (5%)	146 (3%)	100

3.8. Determination of D and R_h of waxy maize starch from aFFFF

The diffusion coefficient, D and the hydrodynamic radius, R_h of waxy maize starch were calculated from AF4 elution profiles obtained for waxy maize starch. The retention time t_r at the peak maxima of LS peaks for waxy maize starch was measured and the D and R_h was determined using the Stokes–Einstein relationship (Eqs. (1) and (4)). The D and R_h of waxy maize starch at fixed F_{ch} rates of 1.0 ml/min with varied F_{cr} rates are shown in Table 4.

The average D and R_h values range from 1.4×10^{-8} cm^2/s to 8.3×10^{-8} cm^2/s and 30 nm to 178 nm, respectively. Typically, at higher F_{cr} rates D increases and R_h decreases. The large molecules with low D are retained in the channel longer than the small molecules. At F_{cr} rates ranging from 0.2 ml/min to 0.4 ml/min the

Table 4
D and R_h of waxy maize starch calculated from t_r at the peak maxima of LS peaks obtained at a fixed F_{ch} rate of 1.0 ml/min.

F_{cr} (ml/min)	t_r (min)	D (cm^2/s)	R_h (nm)
0.1	3.2	1.4×10^{-8}	178
0.2	3.4	3.3×10^{-8}	76
0.3	3.5	4.9×10^{-8}	52
0.4	3.6	6.3×10^{-8}	40
0.5	3.8	7.3×10^{-8}	35
0.6	4.0	8.2×10^{-8}	31
0.7	4.2	8.9×10^{-8}	28
0.8	4.4	8.3×10^{-8}	30

Table 5
The conformation parameter (α, β and γ) and linear regression R^2 values obtained for waxy maize starch at a fixed F_{ch} rate of 0.5 ml/min and varied F_{cr} rates.

F_{cr} (ml/min)	α	R^2	β	R^2
0.1	0.23	0.992	0.29	0.992
0.2	0.26	0.989	0.33	0.997
0.3	0.26	0.996	0.30	0.988
0.4	0.21	0.997	0.32	0.975

average D values are in the region of $\sim 5.0 \times 10^{-8}$ cm^2/s and R_h values are ~60 nm. These are similar to the D and R_h values determined with DLS of 3.35×10^{-8} cm^2/s an 76 nm, respectively. Bello-Perez et al. (1996) reported a similar R_h value of 58 nm for waxy maize starch (Table 1).

3.9. Conformation of waxy maize starch

The conformation of the waxy maize starch molecules was determined using Eqs. (8) and (9).

$$D = C_1 M_w^{-\alpha} \quad (8)$$

$$R_g = C_2 M_w^{\beta} \quad (9)$$

C_1 and C_2 are constants. Log D was plotted against the corresponding log M_w values as shown in Fig. 14a. The values for the conformation parameter, α, were determined from the slope as shown in Table 5. Similarly, the conformation parameter β was determined from the plot of log R_g versus log M_w as shown in Fig. 14b and Table 5. The values of the conformation parameters, α and β, were found to be 0.2–0.33 indicative of a compact, spherical structure. Our findings are in agreement with the values reported by Fishman and Hoagland (1994), Bello-Perez et al. (1998a, 1998b) and Aberle et al. (1994).

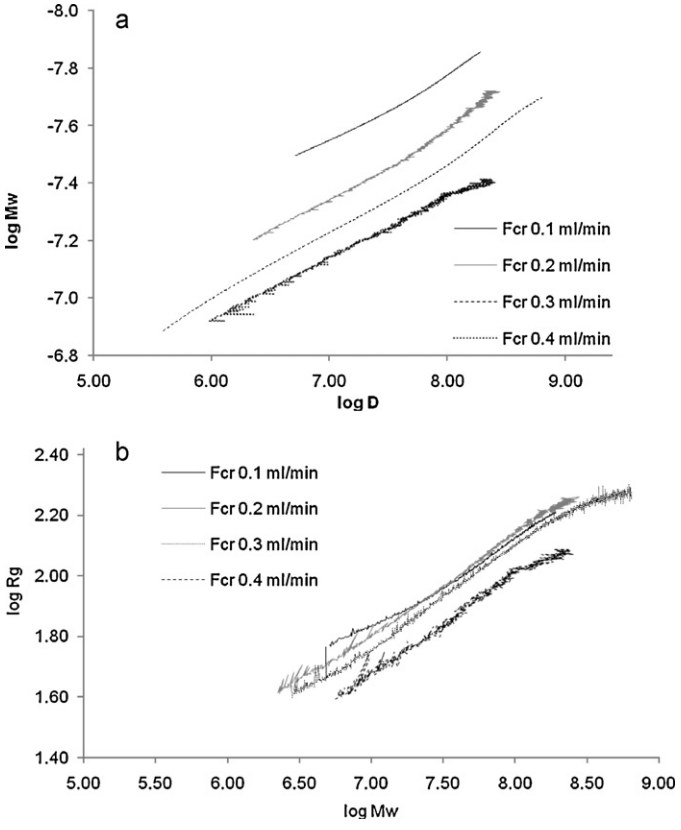

Fig. 14. (a) Log D plotted as a function of log M_w for waxy maize starch at a fixed F_{ch} rate of 0.5 ml/min and varied F_{cr} rates. (b) Log R_g plotted as a function of log M_w for waxy maize starch at a fixed F_{ch} rate of 0.5 ml/min and varied F_{cr} rates.

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