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Carbohydrate Polymers 52 (2003) 269–274

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Partial fractionation of wheat starch amylose and amylopectin using zonal ultracentrifugation

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Received 4 June 2002; revised 26 September 2002; accepted 14 October 2002

Abstract

A novel technique for partial separation of amylose and amylopectin using zonal ultracentrifugation is introduced, supported by analytical ultracentrifuge measurements. Starch was first solubilised in 90% DMSO and 5% sucrose and then layered onto a gradient forming solution of 10–30% sucrose in 90% DMSO, with a cushion made up of 36% caesium chloride and 25% sucrose in water. Solutions were then run in a preparative ultracentrifuge at a rotor speed of 14000 rpm for approximately 25 min. The results for wheat starch showed that this method produced highly purified amylopectin in both soluble and highly aggregated forms, and at least partial purification of the amylose fractions.

Keywords: Starch separation; Analytical ultracentrifugation; Zonal ultracentrifugation; Wheat

1. Introduction

Starch is a large polysaccharide, of considerable industrial importance particularly to the food, pharmaceutical, health care and paint industries (see, e.g. Englyst & Hudson, 1997) and is mainly composed of amylose and amylopectin. In, for example, cereal starches, there is approximately 25% amylose and 75% amylopectin (Shibanuma, Takeda, Hizukuri & Shibata, 1994). Amylose is the linear portion of starch with slight branching. It is a polymer of glycopyranosyl monomers linked to each other by α $(1 \rightarrow 4)$ linkages. Amylopectin has the same backbone as amylose, but it also branched by α $(1 \rightarrow 6)$ linkages (Cura & Krisman, 1990; Lasztity, 1999; Shibanuma et al., 1994). The molecular weights of amylose and amylopectin have been estimated to be about 10^5 and 10^8 Da respectively (Roger, Bello-Perez & Colonna, 1999; Roger & Colonna, 1996). The presence of intermediate fractions in starches such as amylomaize, potato and wrinkled pea starches have been reported. Such molecules are characterized by a lower molecular weight than that of amylose and have a slightly branched structure (Banks, Geddes, Greenwood & Jones, 1972; Colonna & Mercier, 1984; Lasztity, 1999). Separation of amylose and amylopectin has been a matter of interest for many years, but only limited success has been achieved (Cornell, McGrane & Rix, 1999). Previous studies on cereal starch samples using sedimentation velocity experiments have shown a sedimentation coefficient of about 4–10S for amylose and 100–400S for amylopectin (see, e.g. Lelievre, Lewis & Marsden, 1986; Fronimos, 1990; Millard, Wolf, Dintzis & Willett, 1999). Meanwhile it has been shown that the sedimentation coefficients of the starch components are highly concentration dependent (Tongdang, Bligh, Jumel & Harding, 1999).

In this study we introduce what we believe is a novel technique especially in cereal science for partial fractionation of amylose and amylopectin using ultracentrifugation. In this method separation of amylose and amylopectin has been achieved on the basis of their differences in size and sedimentation rates, when applied in a preformed density gradient of sucrose in a zonal ultracentrifuge. The need for a density gradient for a better separation result is firstly to prevent convection within the rotor, which would disrupt the bands of particles as they sediment, and secondly, to increase the relative centrifugal field, thus maintaining an almost

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0144-8617/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
PII: S0144-8617(02)00295-3

constant rate of sedimentation of the particles (Ralston, 1993). Sedimentation velocity analysis in the analytical ultracentrifuge—an already proven procedure for separating analytically amylose from amylopectin—is used as the main technique for assessing the success of the zonal separation (Banks, Greenwood & Muir, 1974). Dimethyl sulfoxide (DMSO) has been reported to be a mild reagent, which can solubilize starch with no observable degradation, which has been used to solubilize starch in this work (Cheetham & Tao, 1997; Jackson & Lincoln, 1991). Meanwhile there are several reports showing the effect of defatting of starch structure and physicochemical properties of starch (Morrison, 1981; Vasanthan & Hoover, 1992). Hence defatted starch was also used to test the effect of lipids on starch separation.

2. Materials and methods

2.1. Defatting of starch

Pure wheat starch from Sigma Chemicals Co., USA was defatted by 'Soxhlet' extraction for 24 h by using methanol (85%v/v) as the solvent (Cornell et al., 1999). The starch was then dried in the air at room temperature and its moisture content was determined.

2.2. Assay for amylose content of starch

Amylose content and the ratio of amylose and amylopectin of wheat starch were estimated using a commercial assay kit from Megazyme (Ireland) Gibson, Solah, & McCleary, 1997.

2.3. Sample preparation

The sample was prepared by dissolving 50 mg/ml of pure wheat starch and 5% sucrose (w/v) in 50 ml of 90% DMSO (v/v in distilled water) by heating in a boiling water bath and mixing on a high speed vortex mixer occasionally for 15 min to avoid formation of any gelatinous starch (Gibson et al., (1997). The sample was then cooled down at room temperature and left mixing on a roller mixer overnight. The same procedure was followed for the defatted wheat starch.

2.4. Density gradient layers in the zonal ultracentrifuge chamber

A Beckman zonal ultracentrifuge B-XIV titanium zonal rotor running at 2000 rpm was filled with a gradient of densities (540 ml) in the range of 1.2–1.3 g/ml made up of 10–30% (w/v) of sucrose in 90%DMSO. A cushion of density, ρ, approximately 1.4 g/ml made up of 36% (w/v) of caesium chloride and 24% (w/v) sucrose in distilled water was used both for displacement of air prior to loading the sample (25 ml via the central feed) at the start of the run and for displacement of the gradient for collection of the samples at the end of the run. An overlay (25 ml) of 90% DMSO was also used. After evacuation of the chamber and acceleration of the rotor to 14000 rpm, at 20 °C a run time of 25 min was employed. After deceleration of the rotor to 2000 rpm, ferrochemical oil (FC43) of density $\rho = 1.9$ g/ml (approximately) was injected into the peripheral feed of the rotor chamber to displace the fractions, which were collected in 24 tubes (25 ml in each).

2.5. Density measurements

It was important to check that the gradient formed was stable and adequately linear throughout the run. The density of each fraction was measured by weighing 1 ml of each fraction accurately using an analytical balance and plotting the density thus measured against fraction number (effectively against volume).

2.6. Determination of amylose and amylopectin fractions by iodine staining

Iodine staining was used as a rapid assay to determine the fractions containing amylose or amylopectin by the development of two distinct colours, which are a dark blue colour for the fractions containing mainly amylose and a brown colour for the fractions containing mainly amylopectin. To 1 ml of each fraction 0.35 ml of KOH (0.1 N), 0.4 of HCl (0.1 N) and 0.07 ml of KI–I_2 solution was added (Gerard, Barron, Colonna & Planchot 2001). A blank sample of 90% DMSO was used to compare the colours. The blue and the brown colour fractions were selected for further experiments.

2.7. Purification of starch components from the solution

Fractions for further purifications of starch were chosen based on the iodine staining. These fractions were heated in a boiling water bath for a few minutes, then about 50 ml of pure ethanol was added to each fraction and the suspension was mixed by inversion. Heating the fractions before adding ethanol minimised the starch aggregation during the precipitation process. After 2 h standing at room temperature, the precipitate was collected by centrifugation (2000 *g*, 10 min). The supernatant was discarded and the pellet was collected and washed with more ethanol several times, then dried in a vacuum oven at 50 °C.

2.8. Resolubilization of starch in 90%DMSO

Dried starch was first dispersed in 5 ml of 90% DMSO in screw capped tubes by mixing at low speed on a vortex mixer. The tubes were then capped and placed in a boiling

water bath. After a few minutes, the tubes were removed and vortexed at high speed, then returned to the water bath for 15 min with intermittent stirring. If gelatinous lumps of starch remained after 15 min, the stirring and heating steps were continued until they completely dispersed. The samples were then cooled down to room temperature and left mixing on a roller mixer overnight.

2.9. Sedimentation velocity experiment using the Beckman optima XL-I

The Beckman Optima XL-I was used to perform sedimentation velocity experiments in order to obtain information about the purity of the fractionated amylose and amylopectin. Purity was assessed from the sedimentation coefficient distribution profiles of the components present in the samples. The purified samples were run in the analytical ultracentrifuge model XL-I first at 8000 rpm and then at 50000 rpm both for four hours at 20 °C. As the sedimenting boundary moves towards the cell base the changes in concentration (of the sedimenting species) over the time ($dc(r)/dt$) at a given radial position r, are produced and the apparent weight average sedimentation coefficient, s^* is then calculated using the program DCDT + (Philo, 2000). The sedimentation coefficient expressed in terms of the standard density and viscosity of water at 20 °C ($s_{20,w}$) can then be generated according to (Tanford, 1961):

$$s_{20,w} = s^* \left[\frac{(1 - \bar{\nu}_{20}\rho_{20,w})}{(1 - \bar{\nu}_{T,b}\rho_{T,b})} \cdot \left(\frac{\eta_{T,b}}{\eta_{20,w}} \right) \right]$$

Where $\bar{\nu}$ is the partial specific volume of the solute, $\rho_{20,w}$ is the density of water and $\eta_{20,w}$ the viscosity of water (all at 20 °C): and $\bar{\nu}_{T,b}$ $\rho_{T,b}$ are the respective parameters in and for the solvent employed at temperature T (Ford & Graham, 1991; Harding, 1992).

The parameters used in this work are as follow: $\bar{\nu}$ of amylose in 90% DMSO at 20 °C: 0.643 ml/g (Fujii, Honda & Fujita, 1973); $\bar{\nu}$ of amylopectin in 90% DMSO at 20 °C: 0.613 ml/mg; density of water at 20 °C: 0.9982 g/ml (Fronimos, 1990) and density of 90% DMSO at 20 °C: 1.0861 g/ml (Tongdang, 2001); viscosity of water at 20 °C: 1.004 centipoises; viscosity of 90%DMSO at 20 °C: 4.0 centipoises (Van Holde, Johnson & Ho, 1998).

2.10. Detection of amylopectin in the amylose containing fractions using concanavalin A ('Con A')

Under defined conditions of temperature, ionic strength and pH, the lectin Con A specifically complexes branched polysaccharides based on α-D-glucopyranosyl (e.g. amylopectin) or α-D-mannopyrannosyl units at multiple non-reducing end groups with the formation of a precipitation. Therefore the Con A was used to investigate the purity of the amylose containing fractions. In this experiment starch solution in 90% DMSO was dissolved in sodium acetate solution (200 mM, pH 6.4), and specifically amylopectin precipitated by addition of Con A and then centrifugation at 10000 g for 10 min (Gibson et al., 1997).

2.11. The ratio of amylose /amylopectin

The concentration of each component in the fractions was measured using the DCDT + algorithm by the following method: the scans obtained from sedimentation velocity experiments in which the boundary had moved almost to the middle of the cell were selected. Then the sedimentation profiles of each component, which showed the apparent sedimentation coefficient distribution; $g^*(s)$ against sedimentation coefficient; $s_{20,w}$ was obtained. The area under a peak represented the loading concentration of the fraction (Philo, 2000).

3. Discussion

Density measurements confirm the linearity and stability of the gradient layers (Fig. 1). Therefore the gradient layers within the zonal rotor had been performed successfully. The iodine staining showed that mainly amylose was fractionated in the first four fractions (a blue colour developed in these fractions by adding iodine). Furthermore the results of sedimentation velocity experiments at 50000 rpm (Fig. 2) showed the presence of a macromolecule with a sedimentation coefficient value about 6–8S, which can be related to separated amylose in these fractions. Although at 8000 rpm a component with a sedimentation coefficient about 70–150S was also present in these fractions (Fig. 3). By addition of Con A to the first four fractions a thin layer of precipitate formed, which revealed that branched macromolecules were also present in the first fractions. These might be either a low sedimentation coefficient amylopectin or branched amylose. For fractions 5, 6, 23, and 24 iodine staining showed a brown colour. Therefore it can be concluded that amylopectin was the major component in these fractions. From the results of sedimentation velocity experiments of these fractions,

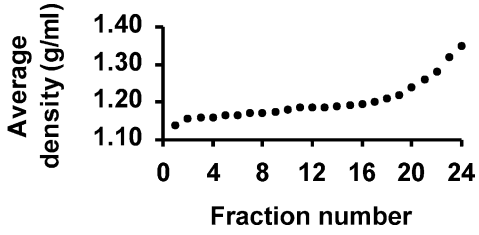

Fig. 1. The average densities of the zonal fractions as a function of fraction number.

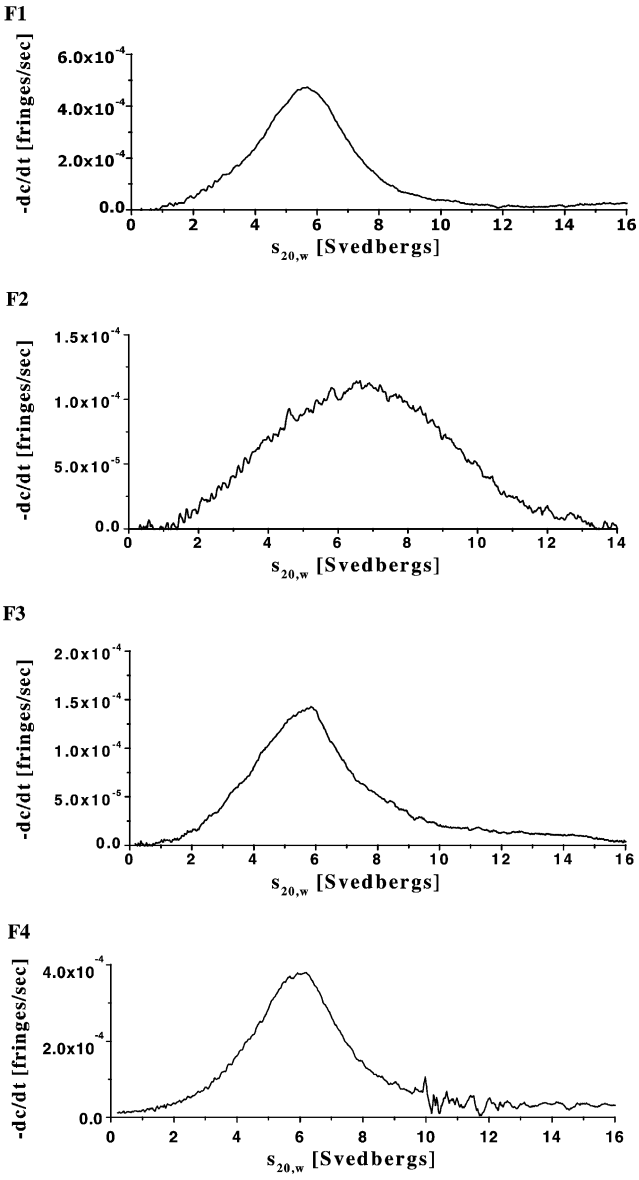

Fig. 2. Analytical ultracentrifuge sedimentation velocity diagrams for the first four fractions at 50000 rpm rich in amylose. (F = fraction number from the zonal ultracentrifuge experiment), 20.0 °C. Optical records recorded using Rayleigh interference optics, and converted into a time derivative concentration distribution as a function of the apparent sedimentation coefficient s^* using the DCDT + programme (see text).

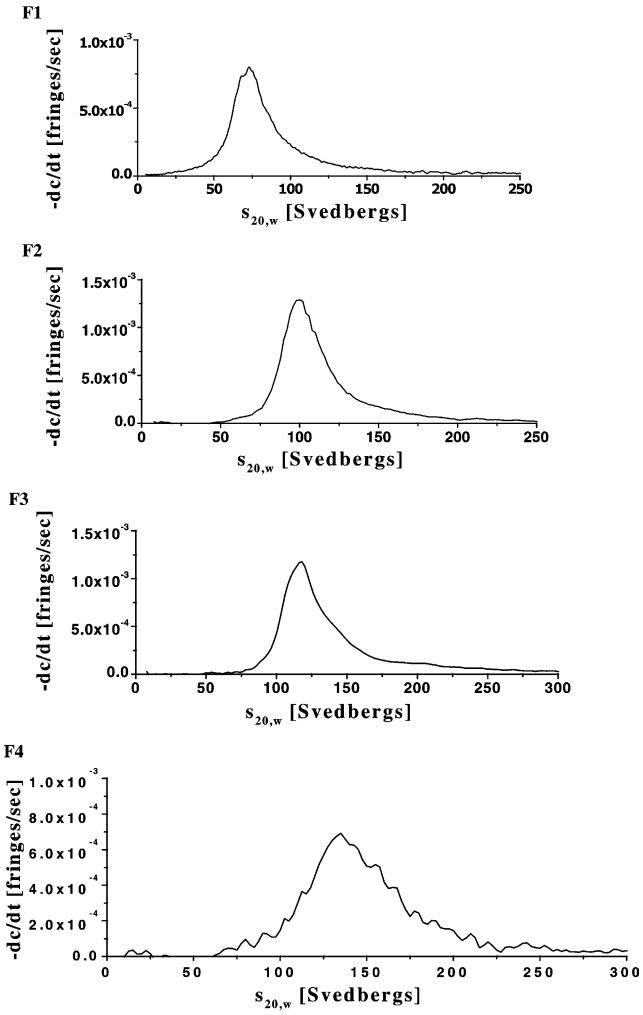

Fig. 3. Analytical ultracentrifuge sedimentation velocity diagrams for the first four fractions in the speed of 8000 rpm showing an intermediate s component. (F = fraction number). Other details, as Fig. 2.

no peak was observed at 50000 rpm (Fig. 4) while a peak with a sedimentation coefficient about 150–350S was observed at 8000 rpm (Fig. 5) confirming the iodine staining results and the successful separation of amylopectin from amylose in these fractions.

The ratio of amylose/amylopectin of fractionated wheat starch obtained by the DCDT + algorithm for the first four fractions was approximately 1.5, 0.8, 0.6, and 0.4 respectively. These values show that the ratio of amylose and amylopectin decreased from fraction one to fraction four. Moreover these values are significantly higher than that of non-fractionated wheat starch, which were about 0.33% using the Con A method. Therefore fractions 1–4 were amylose-rich fractions while fractions 5, 6, 22, and 23 seem to be free from amylose as the ratio of amylose/amylopectin value was effectively zero for these fractions.

The results for the defatted wheat starch sample (Table 1) were very similar to the results for non-defatted wheat starch. Although it has been reported that defatting of starch can change the structural and functional properties of starch (Vasanthan and Hoover, 1992) it seems that by defatting the wheat starch, no significant change in fractionation resulted.

In conclusion, we have found that the zonal ultracentrifugation can separate amylose and amylopectin with a considerable degree of success, as confirmed by the analytical ultracentrifuge and iodine staining experiment. This method produces highly purified amylopectin, in the form of both soluble (fractions 5

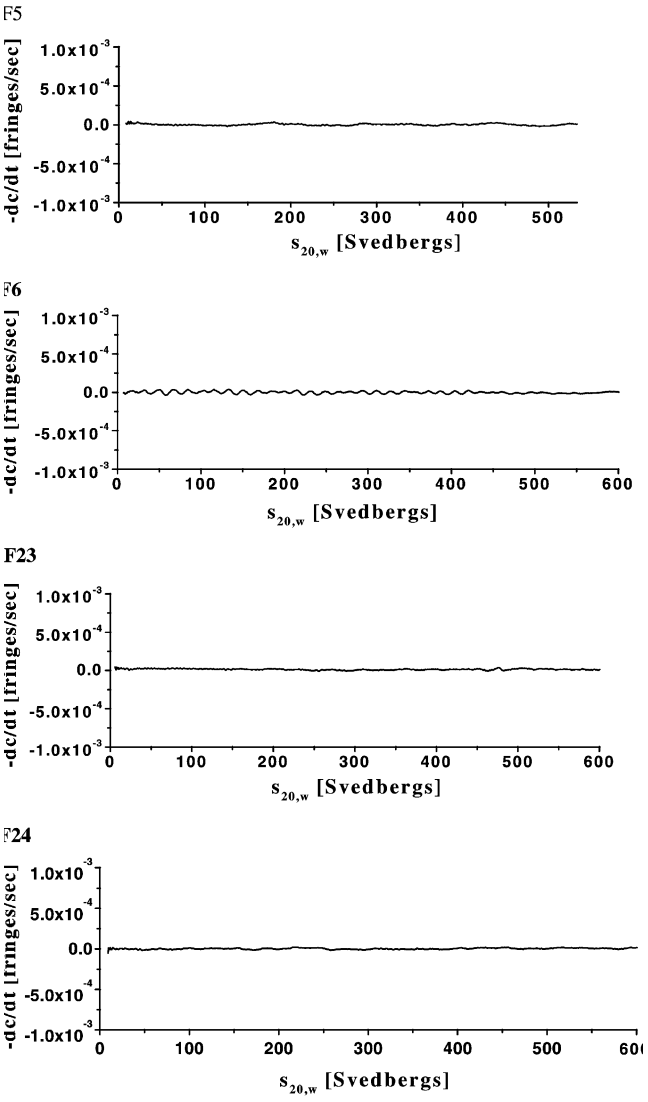

Fig. 4. Analytical ultracentrifuge sedimentation velocity diagrams for the amylopectin containing fractions (5, 6, 23, and 24) at 50000 rpm. (F = fraction number). Other details, as Fig. 2.

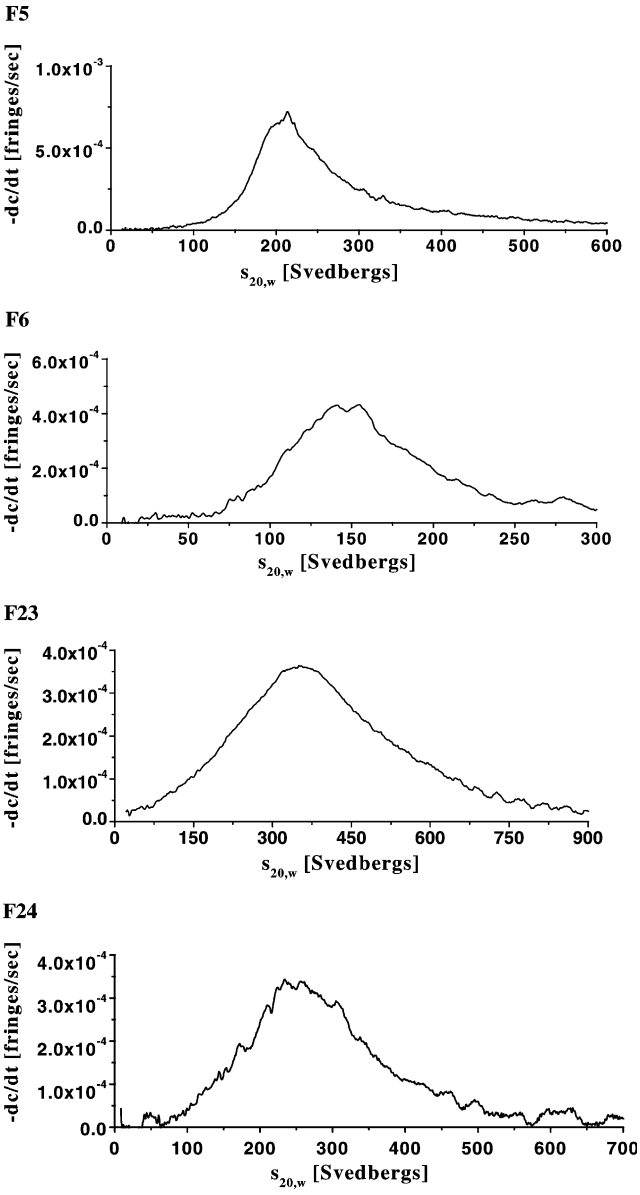

Fig. 5. Analytical ultracentrifuge sedimentation velocity diagrams for the amylopectin containing fractions (5, 6, 23, and 24) at 8000 rpm (F = fraction number). Other details, as Fig. 2. This shows the absence of the amylose and the intermediate s component.

and 6) and highly aggregated (fractions 23 and 24) forms. The amylose fractions appeared to contain, in addition to low sedimentation coefficient amylose (6–8S) some faster sedimenting material, which further study may show to be either branched amylose or an unusually low sedimentation coefficient form of amylopectin. The present work defines a rapid and a novel technique, which provides a relatively large scale yield of the principal components of starch. This technique has additionally served to isolate a fraction with a sedimentation coefficient intermediate between those of the amylose and the amylopectin fractions. The relationship of this 'intermediate s' fraction to the latter two major fractions will require further investigation.

Table 1
Summary of zonal fractionation results for defatted wheat starch

Fractions	Iodine test results	$s_{20,w}$at 50,000 rpm[a]	$s_{20,w}$ at 8000 rpm[a]	Amylose/ Amylopectin[b]
1	Blue	7S	100S	1.4
2	Blue	6S	120S	0.5
3	Blue	7S	150S	0.7
4	Blue	8S	150S	0.5
5	Brown	No peak	300S	0.0
6	Brown	No peak	200S	0.0
22	Brown	No peak	400S	0.0
23	Brown	No peak	300S	0.0

[a] Peak value from Gaussian fit.
[b] Weight concentration ratio.

Acknowledgements

M.M. wishes to thank the Ministry of Science Research and Technology of the Islamic Republic of Iran for their support during her PhD.

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