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Molecular fractionation of starch by density-gradient ultracentrifugation

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Abstract

Amylose and amylopectin in corn and potato starches were fractionated by centrifugation at 124,000*g* for 3–72 h at 40 °C in a gradient media, Nycodenz, based on their sedimentation rate differences. The fractions were collected from a centrifuge tube, and then analyzed by the phenol–sulfuric acid method and iodine-binding test. Amylopectin, a large and highly branched starch molecule, migrated faster than amylose and quickly reached its isopycnic point with a buoyant density of about 1.25 g/mL, exhibiting a sharp and stable carbohydrate peak. Amylose, which is a relatively small and linear molecule, however, migrated slowly in a broad density range and continued moving to higher density regions, eventually overlapping with amylopectin peak as the centrifugation continued. This could indicate that the buoyant density of amylose is similar to that of amylopectin. Under centrifugal conditions of 3 h and 124,000*g*, amylose and amylopectin molecules were clearly separated, and the presence of intermediate starch molecules (11.5 and 7.7% for corn and potato starch, respectively) was also observed between amylose and amylopectin fractions. The amylose content of corn and potato starches was 22.6 and 21.1%, respectively, based on the total carbohydrate analysis after the ultracentrifugation for 3 h. In alkaline gradients (pH 11 or 12.5), the sedimentation rate of starch molecules and the buoyant density of amylopectin were reduced, possibly due to the structural changes induced by alkali.

Keywords: Ultracentrifugation; Density-gradient; Nycodenz; Starch

1. Introduction

Centrifugation has been used as a common technique for fractionating or analyzing various components of a mixture. Even though other advanced analytical or preparative techniques, including high-performance liquid chromatography, have been developed, ultracentrifugation is still a useful tool in polymer science with its precise measurement and diverse applications. Preparative ultracentrifugation is mostly used for isolating particles or macromolecules, whereas analytical ultracentrifugation gives detailed information on the size and shape by observing behavior of the separating objects in a centrifugal field.

In biological science, ultracentrifugation has been applied mainly to protein or nucleic acid analysis because these biopolymers are relatively monodisperse and easy to characterize. In contrast, polysaccharides are difficult to fractionate or characterize by ultracentrifugation due to their conformational and molecular heterogeneities, and furthermore, the lack of a chromophore in most polysaccharides makes it impossible to characterize these by analytical ultracentrifugation with UV–Vis absorbance. Therefore, most ultracentrifugation studies on polysaccharides have been limited to data acquired from an analytical ultracentrifuge equipped with Rayleigh interference optics to measure the sedimentation coefficient,[1,2] which can be combined with the diffusion coefficient to evaluate the weight-average molecular weight and other conformational information.[3–5]

Starch is a mixture of two major polysaccharides, amylose and amylopectin. Amylose is a relatively linear glucan in which D-glucopyranose units are linked mainly by α-(1→4) linkages, whereas amylopectin, the largest natural macromolecule in the world, is highly

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branched with α-(1→6) linkages on α-(1→4) glucosyl backbone. Based on their differences of molecular size and structure, both starch molecules are readily fractionated by using gel-permeation chromatography or by selective alcohol complexation, followed by precipitation. Starch has been also studied, mainly by analytical ultracentrifugation, to measure the sedimentation coefficient of its components, amylopectin and amylose, and then, to calculate the molecular weight.[5–8] However, it has been reported that the accurate molecular weight of unseparated starch components could not be obtained using sedimentation coefficients because of unreliable values for the diffusion coefficient or an unclear swollen factor of specific volume.[9] In case of preparative ultracentrifugation, it has been used only for preparation of starch components using differential precipitation, by which amylose was purified by discarding the amylopectin pellet.[10,11]

Density-gradient ultracentrifugation, one of the preparative and analytical techniques that makes use of centrifugal force, performs the centrifugal sedimentation in a specific medium, a density-gradient, in which the density gradually increases along the centrifuge tube.[12] One of the advantages in density-gradient ultracentrifugation is that particles stabilized in a band can be readily fractionated and analyzed without disturbance. In this density gradient, the particles of different sizes, shapes and densities move as separate zones with different sedimentation rates (rate–zonal separation). By sufficient centrifugation, the particles move until their densities are same as that of surrounding medium, and thus the particles are separated according only to their densities (isopycnic separation). The density of the gradient where the particles band together is called their buoyant density, and this position in the gradient is called the isopycnic or quasi-equilibrium point.[13]

In the present study, corn and potato starch molecules were fractionated by density-gradient ultracentrifugation, using a general preparative ultracentrifuge of the type installed in many of laboratories. Starch molecules were analyzed based on isopycnic separation, and the time-dependant movement of molecules to their isopycnic points was observed and discussed in relation with their molecular structures.

2. Experimental

2.1. Materials

Corn starch was purchased from Sigma Chemical Co. (St. Louis, MO), and potato starch was provided by Handuk Avebe, Inc. (Seoul, Korea). Nycodenz (5-(*N*-2,3 - dihydroxypropylacetamido) - 2,4,6 - triiodo - *N*,*N*′-bis(2,3-propyl)-dihydroxyisophthalamide) was purchased from Axis-Shield (Nycomed), Inc. (Oslo, Norway).

2.2. Preparation of gradient media

Nycodenz is an inert material that does not react with starch molecules and little affects the total carbohydrate assay with phenol–H_2SO_4.[14,15] Furthermore, Nycodenz solution is not so viscous as the sucrose gradient, which has been commonly used for proteins or nucleic acids.

Two Nycodenz solutions (10 and 60% in deionized water) were filtered through a 5-μm PTFE membrane filter, sterilized by autoclaving (121 °C, 30 min), and then degassed by a vacuum pump while stirring. The concentrations were readjusted by adding deionized water based on the refractive index following the Eq. (1) as below:[14]

$$\text{Concentration } (\%) = 607.75\, n_{20^\circ} - 810.13 \quad (1)$$

The relation between density and refractive index (n_{20°) of the Nycodenz solutions is in the Eq. (2):

$$\text{Density } (\text{g/mL}) = 3.242\, n_{20^\circ} - 3.323 \quad (2)$$

Since the Nycodenz solutions were slightly acidic, they were neutralized with 2 N NaOH. Alternatively, the Nycodenz solution was adjusted to pH 11 or 12.5 with 2 N NaOH to examine the alkaline effect on fractionation. The pH change did not make any noticeable influence on the refractive index of the gradient solutions.

Polyallomer tubes (13.2 mL) for a SW41Ti rotor (Beckman-Coulter, Inc., Fullerton, CA) were used for ultracentrifugation, and the density gradient was made with an instrument (Gradient Mate, Biocomp Instruments, Inc., Fredericton, NB, Canada). Both Nycodenz solutions (10 and 60%, from top to bottom) were layered in an equal volume (about 5.5 mL each) in a tube, which was subsequently tilted at an angle of 55°, and rotated at 40 °C, 30 rpm for 6 min, and then 80° at 40 °C, 10 rpm for 1 min 10 s, according to the procedure of Coombs and Watts.[16]

2.3. Preparation of starch solutions

Corn or potato starch was purified by using 90% dimethyl sulfoxide (Me_2SO) and absolute EtOH, and starch sample solutions for ultracentrifugation were prepared following the procedure of Hizukuri and coworkers[17] The purified starch (20 mg, dry basis) was soaked in 75% EtOH (100 μL), and then water (200 μL) and 2 N NaOH (200 μL) were added successively. The starch dispersion was vortexed for 20 min for complete dissolution, and then diluted by adding 700 μL of water. After neutralization with 2 N HCl (200 μL), the solution was diluted with water to a total volume of 2

mL and then filtered through a 3-μm nitrocellulose filter (Millipore Corporation, Bedford, MA) prior to centrifugation.

2.4. Ultracentrifugation and fractionation

An aliquot of the starch solution (2 mg in 200 μL) was loaded on the gradient and centrifuged in a prewarmed (40 °C) SW41Ti rotor using an ultracentrifuge (XL-100K, Beckman-Coulter Inc., Fullerton, CA) at 124,000*g* (31,700 rpm) and 40 °C. After the centrifugation, tubes were carefully taken from rotor and fractionated (140 μL each × 70) by a piston gradient fractionator (Biocomp Instruments, Inc., Fredericton, NB, Canada).[18] The piston was programmed to descend in the tube at the speed of 0.1 mm/s. The gradient residue in the concave at the bottom of tube was also collected for the analysis of any precipitation.

2.5. Analysis of fractions

Each fraction (50 μL) was mixed with water (150 μL) for total carbohydrate analysis by the phenol–H_2SO_4 method.[19] A blank test with the gradient media containing no starch was done for baseline correction. Another aliquot (50 μL) was used for iodine staining by adding 0.1N acetate buffer (100 μL, pH 4.8), water (830 μL) and iodine solution (20 μL) (0.1% I_2 + 1% KI), following the procedure of Takeda and coworkers.[20] After standing 20 min at room temperature, the absorbance at 680 nm and λ_{max} of the iodine complex were measured with a spectrophotometer (Varian Cary 500, Palo Alto, CA). The iodine-binding capacity of starch in each fraction was calculated by dividing the absorbance at 680 nm by total carbohydrate content. The rest of the fraction (40 μL) was used for measuring the refractive index with an Abbe refractometer (DR-A1, Atago Co., Tokyo, Japan) and calculating the density of each fraction based on Eq. (2).

3. Results and discussion

By centrifuging corn and potato starch samples in a density-gradient for 3 h, two distinct total carbohydrate peaks were observed (Fig. 1): a broad peak in the early fractions (top in tube), and a sharp peak in the late fractions (bottom in tube). The absorbance at 680 nm by iodine staining also gave two peaks at the identical locations, but the ratio of the absorbance to the total carbohydrate content was different between the two peaks. The ratio indicated that the broad peak in the early fractions was amylose, and the sharp one in the later fractions was amylopectin due to the difference in the iodine-binding property. Amylopectin sedimented faster than amylose because of its higher sedimentation coefficient resulting from the larger molecular weight and smaller friction from its spear-like shape, whereas amylose, having relatively smaller molecular weight and greater linearity, was expected to migrate much more slowly.

Between corn and potato starches, several differences in the centrifugation profile were observed (Fig. 1). There was an intermediate peak for total carbohydrate, ranging broadly between amylose and amylopectin, which was more clearly observed for potato starch. The ratio of absorbance at 680 nm to total carbohydrate content for the fraction was between those of amylose and amylopectin, indicating that the chain structure might be also of intermediate molecular weight. The amount of the intermediate-sized starch molecules was 7.74% for potato starch and 11.46% for corn starch, based on total carbohydrate content. The intermediate fraction has been found in some other starches such as maize mutants,[21] wrinkled pea[22] and oat[23] starches, with an intermediate structure between amylose and amylopectin. Also some researchers[24,25] have reported the presence of an intermediate fraction in potato starch that was detected during amylose isolation from potato starch, but it is noteworthy that the intermediate starch molecules were observed as a distinctive peak between the amylose and amylopectin fractions. In corn

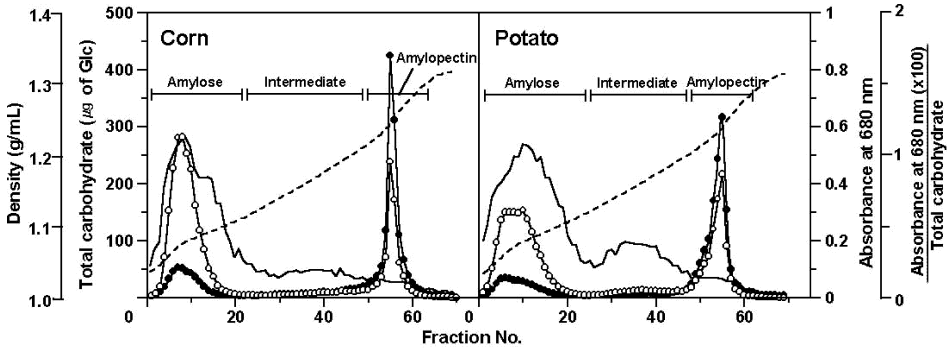

Fig. 1. Ultracentrifugation profile of corn and potato starches at 124,000*g*, 40 °C, pH 7 and 3 h (----, density; —●—, total carbohydrate content; —○—, absorbance at 680 nm; —, absorbance at 680 nm (× 100)/total carbohydrate content).

Table 1
Characterization of corn and potato starch molecules fractionated by ultracentrifugation (124,000*g*, 40 °C, and 3 h)

	Corn			Potato		
	Amylose	Intermediate	Amylopectin	Amylose	Intermediate	Amylopectin
Content (%)	22.55 ± 0.17	11.46 ± 1.10	65.74 ± 1.22	21.14 ± 0.63	7.74 ± 0.75	71.13 ± 0.85
Buoyant density (g/mL) [a]	1.070 ± 0.007	1.163 ± 0.021	1.258 ± 0.002	1.077 ± 0.009	1.149 ± 0.019	1.248 ± 0.003
Λmax	610–653	nd [b]	574–579	610–666	nd [b]	557–567
Absorbance at 680 nm (× 100)/total carbohydrate content (μg of Glc)	0.90–1.12	0.18–0.23	0.09–0.13	0.90–1.08	0.26–0.40	0.12–0.14

[a] Densities of amylose or intermediate molecules were determined from the peak density in total carbohydrate profile after 3 h of centrifugation.
[b] nd, not determined.

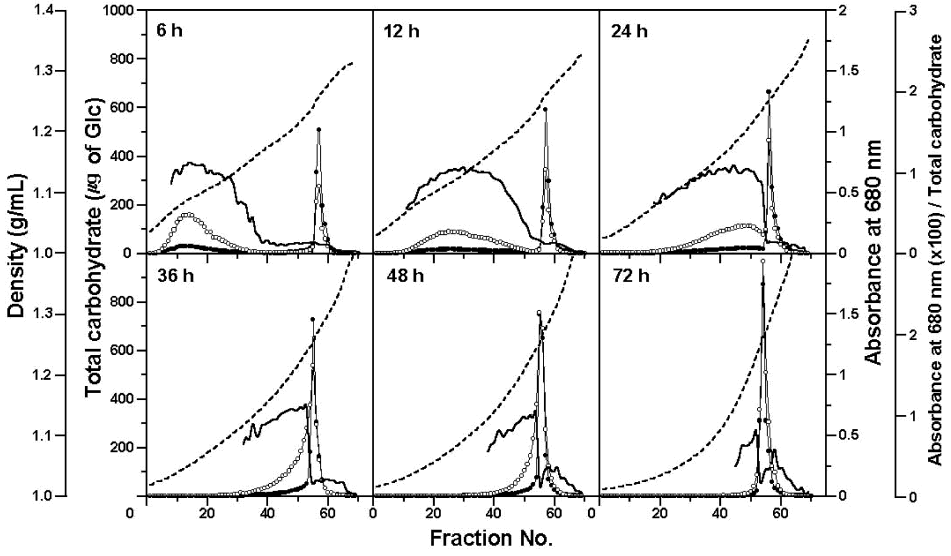

Fig. 2. Ultracentrifugation profile of corn starch at different centrifugation times (6, 12, 24, 36, 48 and 72 h) at 124,000*g*, 40 °C and pH 7 (----, density; —●—, total carbohydrate content; —○—, absorbance at 680 nm; and —, absorbance at 680 nm (× 100)/total carbohydrate content).

starch, however, the ratio of absorbance to total carbohydrate for the intermediate fraction was smaller than that for the intermediate fraction of potato starch. This might suggest that molecular structure of the intermediate fraction between amylose and amylopectin in corn starch was closer to that of amylopectin. The relative percentages of amylose, intermediate and amylopectin in corn and potato starches were measured based on total carbohydrate content from the 3 h ultracentrifugation profile (Table 1). The amylose content of corn and potato starches measured in this experiment was 22.55 and 21.14%, respectively, in accordance with the results reported elsewhere.[26,27]

The λ_{max} of the iodine–starch complex showed a distribution of 610–653 and 574–579 nm for corn amylose and amylopectin fractions, respectively, whereas 610–666 and 557–567 nm in potato amylose and amylopectin fractions were observed, respectively (Table 1). The λ_{max} of amylose fraction for both starches varied in a wide range (43–56 nm), whereas that of amylopectin fractions was in a narrow range (< 10 nm). This wide variation in λ_{max} for amylose fractions resulted from structural heterogeneity, possibly both in degree of polymerization and in degree of branching, as observed also by gel-permeation chromatography.[26,28] The λ_{max} values of amylose and amylopectin, which have been chemically separated from corn and potato starches,[27,29] were not significantly different from those observed in this experiment. The minor differences might arise from the influence of the gradient medium.

As centrifugation time increased, there were changes in the ultracentrifugation profile (Figs. 2 and 3). Amylopectin arrived its isopycnic point before 3 h of cen-

trifugation, and remained as a sharp peak, whereas the amylose peak continually migrated with centrifugation time. In the isopycnic separation, the density of gradient where the molecules stopped sedimenting is their buoyant density, so the buoyant density of corn and potato amylopectins were 1.258 and 1.248 g/mL, respectively (Table 1). These values were slightly lower than the buoyant density of glycogen (1.29 g/mL)[15] or some other polysaccharides (1.28 g/mL)[12] in Nycodenz solution, and the low density might be from the temperature used in the present experiment (40 °C). The difference in density between two amylopectins resulted from their structural differences, possibly in chain length and degree of branching, which could be also suggested by the different λ_{max} value. The presence of phosphate esters covalently linked to potato amylopectin[30] might be another factor influencing the density in solution. The esters increase the hydrophilic nature of the amylopectin chains, and may inhibit the chain–chain associations, resulting in a density decrease in the gradient.

In contrast to the fast isopycnic banding of amylopectin, amylose dispersed into broader density range and gradually moved to higher density region as the centrifugation continued (Figs. 2 and 3). The buoyant density values of amylose and the intermediate fractions, shown in Table 1, were measured from the peak positions of the total carbohydrate curve after 3 h of centrifugation, although they were not the real buoyant densities of amylose and the intermediate fractions. The movement of amylose continued up until 72 h of ultracentrifugation, and its peak finally overlapped with the amylopectin peak and disappeared, although there were a few amylose molecules still moving which could be observed by the high absorbance/carbohydrate ratio before the amylopectin peak (72 h in Figs. 2 and 3). The overlapping of the two fractions may indicate that the isopycnic density of amylose may not be greatly different from that of amylopectin. Some researchers have reported that the densities of amylose and amylopectin, calculated from partial specific volume in water or DMSO, were similar (1.6–1.7 g/mL).[6,8,9] The high water activity of Nycodenz reduced the buoyant density of molecules,[15] and there were reports that the density of polysaccharides was around 1.6 g/mL in CsCl or Cs_2SO_4 gradient having lower water activity.[2,12] From the comparison of ultracentrifugation profiles between corn and potato amyloses (Figs. 2 and 3), potato amylose sedimented faster than corn amylose, and this could be explained by the relatively larger molecular size and the more highly branched structure of potato amylose.[27,31]

Since the Nycodenz solute was also affected by centrifugal force, and therefore, the density distribution of the gradient changed with centrifugation time, the total carbohydrate profile of starch was replotted against the buoyant density of the gradient for comparison (Fig. 4). It was clearly shown that the amylose fractions continued sedimenting and disappeared behind the amylopectin peak as the centrifugation continued. However, it could be observed that the range of the amylopectin peak gradually increased to the higher density region as centrifugation time increased (Fig. 4). The incident increase in the absorbance/carbohydrate ratio of fractions after amylopectin (48 and 72 h of Figs. 2 and 3) indicated that this unusual increase resulted from amylose molecules. Some amylose molecules might have

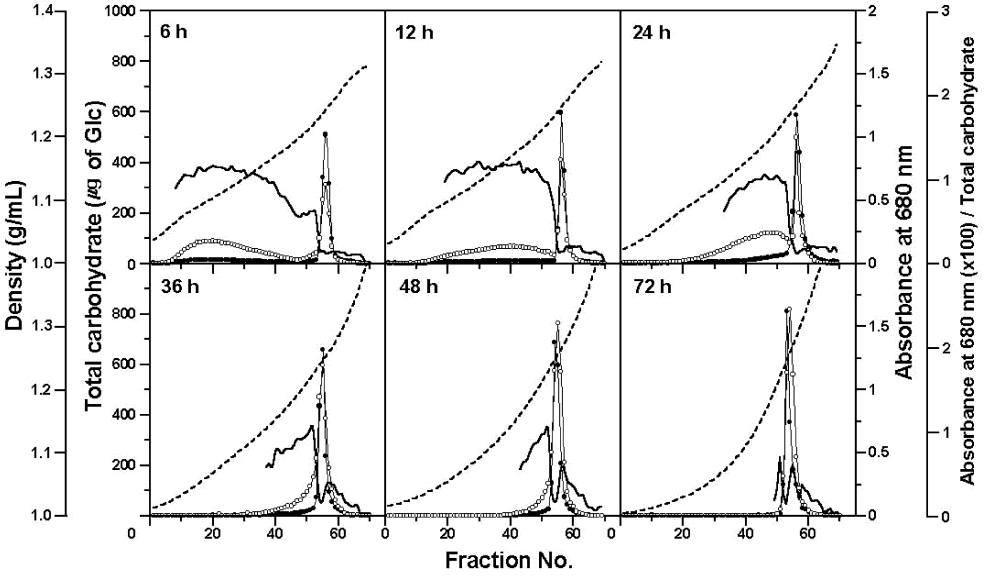

Fig. 3. Ultracentrifugation profile of potato starch at different centrifugation times (6, 12, 24, 36, 48 and 72 h) at 124,000*g*, 40 °C and pH 7 (----, density; —●—, total carbohydrate content; —○—, absorbance at 680 nm; and —, absorbance at 680 nm (× 100)/total carbohydrate content).

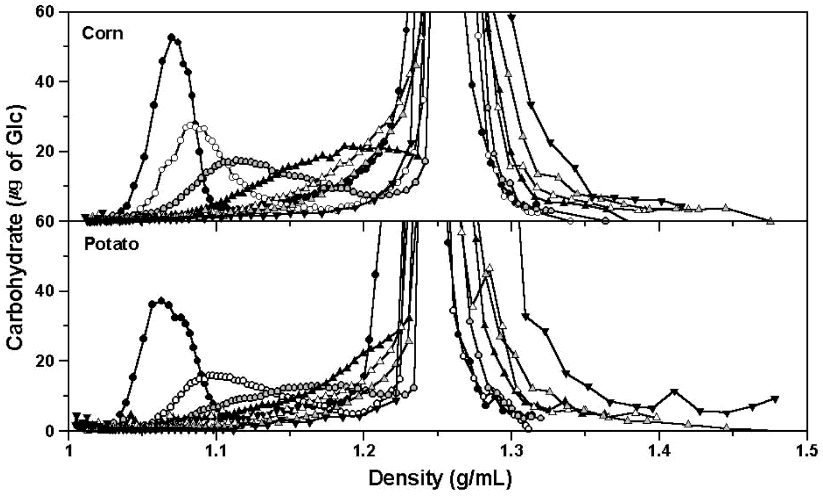

Fig. 4. Changes in ultracentrifugation profile of corn and potato starches in density-base at different centrifugation times at 124,000*g*, 40 °C, and pH 7 (—●—, 3 h; —○—, 6 h; —▲—, 12 h; —△—, 24 h; —■—, 36 h; —□—, 48 h and —▼—, 72 h).

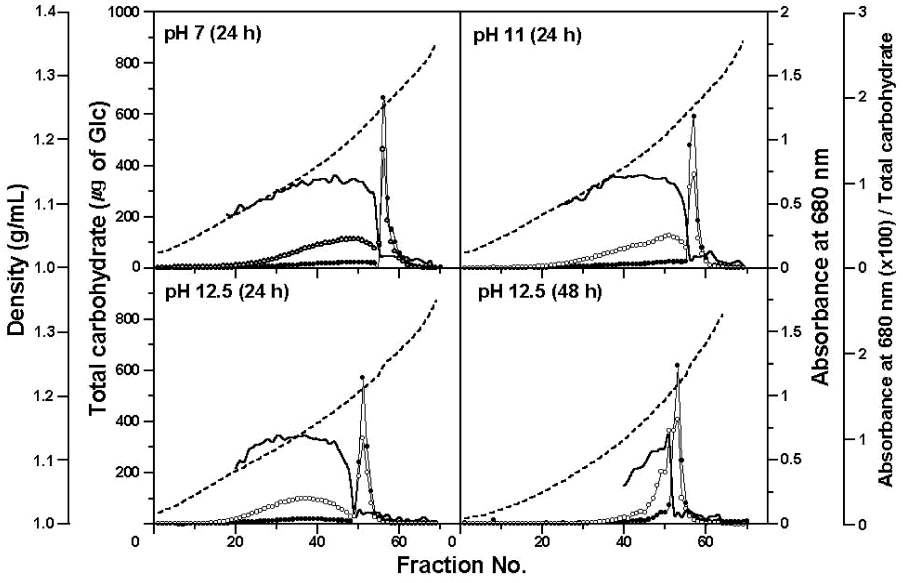

Fig. 5. Ultracentrifugation profile of corn starch at different medium pHs (7, 11 and 12.5) by centrifugation for 24 or 48 h at 124,000*g* and 40 °C (----, density; —●—, total carbohydrate content; —○—, absorbance at 680 nm; and —, absorbance at 680 nm (× 100)/total carbohydrate content).

higher buoyant density than amylopectin. However, it was expected that the minor amylose chains were aggregated due to the gradual increase in regional concentration and the extended centrifugation, increasing their density slightly, although more studies need to be carried out to determine the precise mechanism. The broadening of the amylose peak as shown in Fig. 4 was expected to be induced by the different sedimentation velocity among the front, middle and back portion of the amylose fraction, according to the structural heterogeneity, possibly, in molecular weight, degree of branching or chain length.

An alkaline density-gradient was performed to examine alkaline effect on the sedimentation and to retard the possible aggregation of amylose. When corn starch was centrifuged at pH 11 for 24 h, there was almost no change in centrifugation profile, compared to the neutral conditions (Fig. 5). On the other hand, when the pH increased to 12.5, the amylopectin peaks shifted to the lower density region (approx 1.22 g/mL), and the sedimentation rate of the amylose peak was slightly reduced. It would appear that the alkaline pH affected the hydration of starch molecules, as well as ionized the hydroxyl group in the reducing end, and thus caused the density change of starch molecules. A similar phenomenon was reported in which an alkaline gradient changed the hydration of protein and therefore resulted in a decrease of buoyant density.[15] Also, the conforma-

tional change induced by the alkaline conditions, possibly to a more extended structure, could reduced the sedimentation rate, but no alkaline-induced effect on the possible amylose aggregation could be observed. There might be a degradation of starch molecules under such high pH conditions over a long period time as reported in many researches, even though minor structural degradation might not significantly affect the density profile of starch molecules.

4. Conclusions

Density-gradient ultracentrifugation using a preparative ultracentrifuge was investigated for analyzing and separating starch molecules. By a short ultracentrifugation (3 h), amylopectin and amylose could be clearly separated by the difference in their sedimentation rates, but amylose migrated to the density region of amylopectin as centrifugation time increased, due to the similar buoyant densities of the two molecules. In addition, an intermediate starch fraction could be fractionated in a density region between amylose and amylopectin in the short time of centrifugation. Density-gradient ultracentrifugation could provide an effective tool for quantification or preparative separation of starch molecules without loss by adsorption or degradation that commonly occurs in conventional column chromatography. In addition, by comparing the buoyant density of amylopectin (1.25–1.26 g/mL), the structural differences of amylopectin from different sources can be estimated.

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