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EXAMINATION OF THE PURITY AND STRUCTURE OF AMYLOSE BY GEL-PERMEATION CHROMATOGRAPHY

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ABSTRACT

Gel-permeation chromatography on TOYOPEARL HW-75F is a sensitive and useful method for examining the purity of amylose specimens. The required recrystallisation frequency for preparing amylose free from amylopectin depended on the starch source. The iodine affinities of chromatographically pure amylose specimens were 20–20.5 g/100 g. The pure kuzu, tapioca, and potato amyloses had $\overline{\text{d.p.}}$ values of 1540, 2660, and 4920, with 5, 8, and 7 chains on average, respectively. Wheat amylose obtained by aqueous leaching at 70° was a short-chain molecule ($\overline{\text{d.p.}}$, 570) with 2 chains on average. These amylose specimens consisted of molecules differing in molecular weight and chain length. The branched molecules showed broad distributions of molecular weight. *Pseudomonas* isoamylase liberated short (maltotetraose) to long (probably d.p. >100) chains from potato amylose.

INTRODUCTION

Most amylose samples possess some branches attached by α-(1→6) linkages, and their fine structures appear to be characteristic of their origins[1–6]. Our current interest in the fine structures is related to the distinctive properties of various kinds of starch. One problem in such studies is the separation of intact amylose free from amylopectin and evaluation of its purity, which is usually examined by iodine-affinity determination[3,5]. However, this analysis is not sensitive enough to detect a small proportion of contaminating amylopectin[3,5]. Since the contamination, even if it is very small, leads to serious errors in structural analyses, a sensitive method for its detection is desired.

We have found gel-permeation chromatography on TOYOPEARL HW-75F to be a sensitive and useful method for determining the purity of amylose specimens and also for demonstrating heterogeneity in terms of molecular weight and branched structure.

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MATERIALS AND METHODS

Materials. — Starch samples of kuzu (*Pueraria hirusuta* Matsum), wheat, and tapioca were products of Hirohachido-shoten (Kagoshima), Sanwa Denpun Kogyo Co. (Nara), and Nichiden Kagaku Co. (Osaka), respectively. Potato (Eniwa variety) starch was a gift from Dr. S. Yoshioka (Hokkaido Agricultural Experimental Station). Lily starch was prepared from lily (Maximovicz's lily) bulbs by the method described[7].

Amylose samples of lily, kuzu, tapioca, and potato were fractionated by the method of Lansky *et al.*[8] with minor modifications[4], and purified by recrystallisation 3–6 times from hot, aqueous 10% 1-butanol by cooling in an atmosphere of nitrogen. Wheat amylose was prepared by aqueous leaching of the granules at 70° for 2 h in 6.4mM phosphate buffer (pH 6.8) under a nitrogen atmosphere. The amylose was precipitated by the addition of 0.1 vol. of 1-butanol and recrystallised twice.

Beta-amylase was prepared[9] from sweet potato, and the final crystalline product was recrystallised from aqueous ammonium sulfate to improve the stability during storage. Crystalline *Pseudomonas* isoamylase[10], free of alpha-amylase (no decrease of blue value of amylose incubated with excess amount of the enzyme for several hours), was a gift from Dr. T. Harada. TOYOPEARL HW-75F was obtained from Toyo Soda Manuf. Co. Ltd. (Tokyo).

Methods. — Iodine affinity was determined at 30° by the amperometric titration procedure of Larson *et al.*[11]. The blue value was determined by the method described elsewhere[7]. The average degree of polymerisation ($\overline{\text{d.p.}}$) of amylose was determined by the modified Park–Johnson method[4]. The average chain-length ($\overline{\text{c.l.}}$) was determined by the rapid Smith-degradation method[4] with minor modifications. The amylose solution was adjusted to pH 5.0 with M HCl after addition of M acetate buffer (pH 5.0), to give a final concentration of 20mM (under the previous conditions, the pH of the amylose solution was adjusted to 6.5–7.0 and no buffer was used). The adjustment to pH 5.0 improves the reproducibility and accuracy; thus, it decreased the standard deviation of $\overline{\text{c.l.}}$ from ~10% to 5% of the mean of $\overline{\text{c.l.}}$, although the reason is unknown. The number of chains per molecule is $\overline{\text{d.p.}}/\overline{\text{c.l.}}$. The beta-amylolysis limit was determined by hydrolysing the specimens at 37° for 4 h in 20mM acetate buffer (pH 4.8) with 25 U (μmol of maltose released per min, at 37° and pH 4.8) of beta-amylase per mg of substrate. The hydrolysis reached an upper-limit value after 30-min incubation and it was maintained for 6 h. The concurrent hydrolysis with beta-amylase and pullulanase was done by the procedure described previously[4]. Reducing sugar was determined by the methods of Somogyi[12] and Nelson[13]. Phosphate was determined as inorganic phosphate by the method of Itaya and Ui[14] after treatment with hot perchloric acid[15]. The limiting viscosity number [η] was determined in M KOH with an Ostwald viscometer at 22.5°.

Gel filtration of amylose was performed as follows. The amylose solution (10 mg in 2 mL), prepared as described previously[4], was applied to a column (2.6 ×

100 cm) packed with TOYOPEARL HW-75F and eluted with 50mM NaCl. The flow rate was maintained at 40 mL/h, and fractions of 5 mL were collected. The column was kept at 45° by circulating water. Total carbohydrate was assayed by the anthrone–sulfuric acid method[16]. The carbohydrate recovery was >97% in the chromatography. The void volume of the column was estimated from the elution volume for potato amylopectin.

Paper chromatography (p.c.) of potato amylose debranched with *Pseudomonas* isoamylase was performed as follows. Potato amylose (150 mg in 15 mL) was incubated at 45° for 2.5 h in 20mM acetate buffer (pH 3.5) with 16.5 U (μmol of reducing sugar equivalent to glucose per min, at 40° and pH 3.5) of the isoamylase. The reaction was terminated by heating for 1.5 min in a boiling water bath, ethanol (30 mL) was added, and the mixture was kept overnight in a refrigerator. The resulting precipitate was removed by centrifugation and the supernatant solution (containing oligosaccharides) was concentrated to 10 mL at 35–40° *in vacuo*. A mixture (5.25 g) of dry Amberlite IR-100 (H^+) and Amberlite IR-45 (HO^-) resins in the weight ratio of 2:5 was added, the supernatant was lyophilised, and a solution of the residue (~190 μg) in water (30 μL) was subjected to ascending p.c. five times on Toyo Roshi No. 51 paper with aqueous 70% 1-propanol at 45°. Maltosaccharides were detected with alkaline silver nitrate[17] after digestion with glucoamylase[18].

RESULTS AND DISCUSSION

Fig. 1 shows the gel-filtration patterns of lily-amylose samples, I (LAM-I) and II (LAM-II), which were recrystallised 3 and 6 times, respectively, from hot,

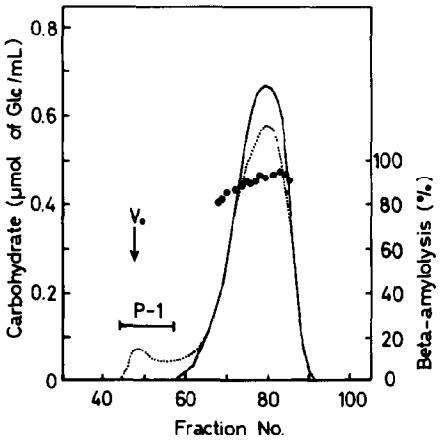

Fig. 1. Gel filtration on TOYOPEARL HW-75F of lily amylose: samples I (LAM-I) and II (LAM-II) recrystallised 3 and 6 times, respectively. The column (2.6 × 100 cm) was eluted with 50mM NaCl under the conditions described in the text: ····· and ——, carbohydrate concentrations of LAM-I and LAM-II, respectively; ●, beta-amylolysis limit of LAM-II.

aqueous 10% 1-butanol. The iodine affinities of LAM-I and -II were 18.0 and 20.0 g/100 g, respectively, and the properties of LAM-II were reported previously[7]. LAM-II showed a single peak, whereas LAM-I showed a main peak and a minor peak was eluted in the void volume of the column. A similar minor peak eluted in the void volume was found by other workers also. Ghiasi and Hoseney[19] suggested that the peak observed for wheat amylose (iodine affinity, 18.7 g/100 g) was insoluble amylose, whereas Würsch *et al.*[20] considered that it was amylopectin adulterating a mango-amylose sample. To identify the minor peak (P-1) of LAM-I, the appropriate fractions were combined and lyophilised. P-1 (6.9% of LAM-I) was refluxed with water-saturated 1-butanol to remove lipid that interfered with some analyses. The treated P-1 had a $\overline{\text{c.l.}}$ of 23.4, a blue value of 0.224, and a beta-amylolysis limit of 56.0%. These properties accord with those of lily amylopectin[7]. This indicates that P-1 is amylopectin, but the increase in the blue value of P-1 on refluxing (before refluxing, 0.048) suggests the presence of a very small proportion of an amylose–lipid complex. A chromatogram of lily amylopectin on the same column showed that the major part was eluted in the void volume but that the minor part penetrated into the gel particles (Fig. 2), indicating a broad distribution of the molecules including a small proportion of much smaller molecules. LAM-II, recrystallised 6 times, had no carbohydrate fraction in the void volume (Fig. 1) and appeared to be free from amylopectin, since the repeated recrystallisation possibly also excluded the low-molecular-weight fraction of amylopectin. The P-1 fractions were also found in the 3-times recrystallised amylose samples from tapioca and sweet-potato starches, although the amounts were less than that of LAM-I. However, potato amylose recrystallised twice was free from the P-1 fraction. Thus, the removal of amylopectin by recrystallisation depends on the origin of the starch. The purity of an amylose sample is usually examined by determination of the iodine affinity, and recrystallisation is repeated until a value of 19–20 g/100 g is

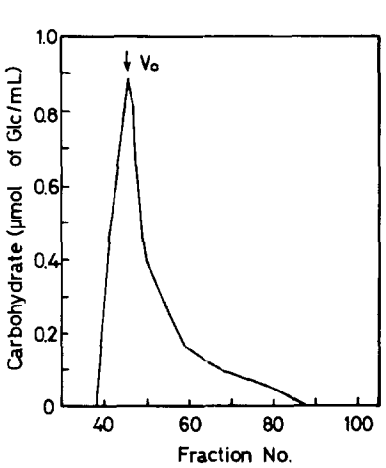

Fig. 2. Gel filtration of lily amylopectin on TOYOPEARL HW-75F.

reached. This technique is useful but not sensitive enough to detect a few percent of contaminating amylopectin[3,5]. However, the gel filtration of LAM-I (10 mg) on TOYOPEARL HW-75F clearly showed the presence of a small amount (6.9% as P-1) of contaminating amylopectin, and the sensitivity of the detection increased with an increase of the load (up to 100 mg) on the column. Thus, gel-permeation chromatography is a sensitive and useful method for assessing the purity of amylose samples.

Samples of kuzu, tapioca, and potato amylose, prepared by aqueous dispersion of starches and subsequent recrystallisation 5, 6, and 4 times, respectively, and a sample of wheat amylose, prepared by aqueous leaching at 70° and twice recrystallised, contained no P-1 fractions and were free from amylopectin (Fig. 3). The properties of these pure amyloses are listed in Table I; they had iodine affinities of 20 and 20.5 g/100 g, and blue values of 1.37–1.47. A very small amount of phosphate was detected in these samples, suggesting the presence of phosphate ester as in amylopectin[21–25]. Arbuckle and Greenwood[26] obtained wheat amylose having a limiting viscosity number $[\eta]$ of 145 mL/g and a beta-amylolysis limit of 98% by aqueous leaching at 70° and suggested that the leaching enabled short-chain, essentiall linear, amylose to diffuse out. This $[\eta]$ value corresponds to a $\overline{\text{d.p.}}$ of 1070 according to the equation[27] $\overline{\text{d.p.}} = 7.5\,[\eta]$, which was based on the $\overline{\text{d.p.}}$ derived from measurement of osmotic pressure. In this study, we obtained wheat amylose having a $[\eta]$ value of 80 mL/g and a beta-amylolysis limit of 88%. The determination of the reducing residue of the amylose gave a $\overline{\text{d.p.}}$ of 570, similar to that calculated from the limiting viscosity number. These findings and the determination of the non-reducing terminal residue indicate that our wheat amylose is a smaller molecule and has two chains on average. The $\overline{\text{d.p.}}$ values of kuzu, tapioca, and potato amyloses obtained by aqueous dispersion of starch granules were 1540, 2660, and 4920, respectively. Lily amylose had[7] a $\overline{\text{d.p.}}$ of 2310 and a $[\eta]$ value of 312 mL/g. These $\overline{\text{d.p.}}$ values, except that for potato amylose, were very similar to those calculated from the limiting viscosity numbers. This implies the agreement of the number-average molecular weight measured by osmotic pressure with that obtained by determination of the reducing residue. Kuzu amylose had the smallest molecular weight of these four amyloses and the value accorded with that reported[4], indicating that the fractionation and recrystallisation procedures are reproducible. Tapioca amylose had a $\overline{\text{d.p.}}$ value lower than that (3390) of the previous specimen recrystallised 4 times[4]. This may be due to the difference between the origins, as observed for potato amylose[4]. The $\overline{\text{d.p.}}$ of potato amylose from an Eniwa variety was similar to that for another (Kenebec)[4] and was a little higher than that given by Greenwood[6]. The $\overline{\text{c.l.}}$ values of kuzu and tapioca amyloses were 320 and 340, respectively, which were half that (670) of potato amylose and lower than that (470) of lily amylose[7]. The $\overline{\text{d.p.}}$ and $\overline{\text{c.l.}}$ values indicated that the amylose samples are slightly branched molecules with 5–8 chains on average. All of the chains in the amylose specimens appear to be linked by α-(1→6) linkages, since concurrent hydrolysis with beta-amylase and pullulanase gave 98–100% conversion into maltose.

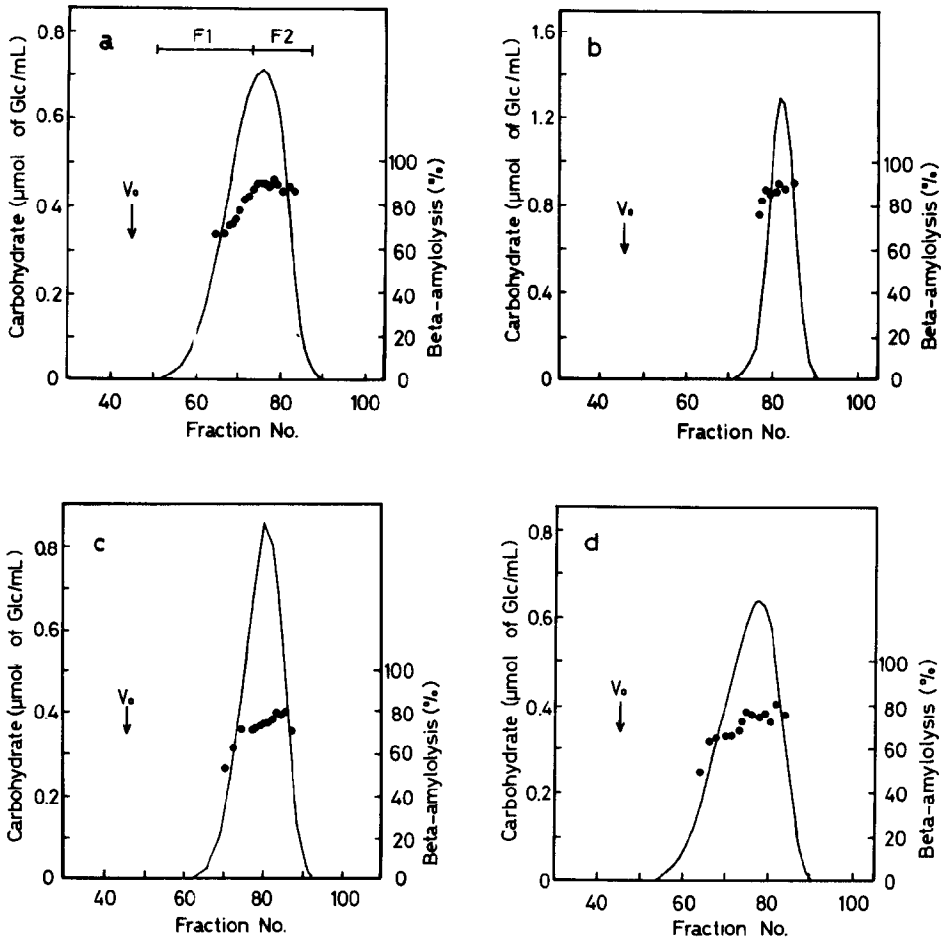

Fig. 3. Gel filtration on TOYOPEARL HW-75F of potato (a), wheat (b), kuzu (c), and tapioca (d) amyloses: ——, carbohydrate concentration; ●, beta-amylolysis limit.

TABLE I

PROPERTIES OF AMYLOSE SAMPLES

	Wheat	*Kuzu*	*Tapioca*	*Potato*
Iodine affinity (I_2, g/100 g)	20.5	20.0	20.0	20.5
Blue value	1.37	1.44	1.47	1.47
Limiting viscosity number (mL/g)	80	202	384	384
Phosphorus (p.p.m.)	5	9	7	3
Beta-amylolysis limit (%)	88	75	75	80
$\overline{D.p.}$	570	1540	2660	4920
$\overline{C.I.}$	300	320	340	670
Number of chains per molecule	1.9	4.8	7.8	7.3

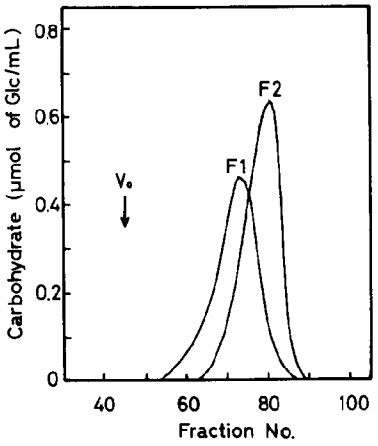

Fig. 4. Gel filtration of F1 and F2 on TOYOPEARL HW-75F.

Fig. 3a shows that potato amylose (beta-amylolysis limit, 80%; Table I) has a broad elution profile, and the beta-amylolysis limits of the fractions increase from ~65% to 90% as elution proceeds. The fractions were hydrolysed to an extent of 97–101% by the combined action of beta-amylase and pullulanase. These observations indicate that the amylose molecules are heterogeneous with regard to fine structure. To confirm the heterogeneity, the carbohydrate eluate was divided into two subfractions, F1 and F2, corresponding to up to 90% and ~90% of the beta-amylolysis limit, respectively (Fig. 3a). These subfractions were precipitated with 1-butanol, and the precipitates were washed with ethanol and ether and then dried *in vacuo*; Table II shows the properties of F1 and F2. The $\overline{\text{d.p.}}$ values of F1 and F2 were 6220 and 3360, respectively, and the $\overline{\text{c.l.}}$ values were 930 and 530, respectively. However, the number of chains per molecule of the two subfractions were similar. The beta-amylolysis limits of F1 and F2 were 77 and 90%, respectively, and the elution patterns on TOYOPEARL HW-75F (Fig. 4) showed that F1 was the faster-moving and that both fractions were eluted at the positions expected from the gel filtration of the whole amylose (Fig. 3a). These findings suggest that no re-

TABLE II

PROPERTIES OF F1 AND F2

	F1	*F2*
Weight (%)	41	59
Blue value	1.44	1.47
Beta-amylolysis (%)	77	90
$\overline{\text{D.p.}}$	6220	3360
$\overline{\text{C.I.}}$	930	530
Number of chains per molecule	6.7	6.3

trogradation occurred during gel filtration, although amylose has a high tendency to undergo retrogradation in aqueous solutions. Thus, it is concluded that the amylose is heterogeneous as to molecular weight and branched structure.

The heterogeneity of amylose has been demonstrated by aqueous leaching of starch granules[28–31] and subfractionation of amylose[32–37], and we have now used gel filtration. Wheat amylose prepared by aqueous leaching showed a narrow distribution of molecular weight, whereas the specimens obtained by aqueous dispersion from lily, kuzu, and tapioca starches had rather broad distributions of molecular weight, judging from their elution profiles (Figs. 1 and 3b–d). For these amylose specimens fractionated by gel filtration, it was shown that the beta-amylolysis limits increased with decrease in molecular size, as observed for potato amylose. However, no fractions were completely hydrolysed and the maximum beta-amylolysis limits for lily, wheat, kuzu, tapioca, and potato amyloses were ~95, 90, 80, 78, and 90%, respectively. These results imply that most of the amylose molecules are branched, with various chain lengths and broad distributions of molecular weight, and that a minor proportion of the molecules are truly linear.

When potato amylose was debranched with *Pseudomonas* isoamylase[4], its d.p. decreased from 4920 to 2290 in 30 min and no further decrease was observed on prolonged incubation. The beta-amylolysis limit increased concomitantly from 80 to 87%. This is far from complete debranching as observed previously[4], and 18% of the branch linkages were cleaved by the enzyme (the value was calculated with the equation described elsewhere[4]). The amylose was incubated for 2.5 h with the enzyme and then subjected to gel filtration on TOYOPEARL HW-75F after heating for 1.5 min in a boiling water bath to inactivate the enzyme. Fig. 5 shows

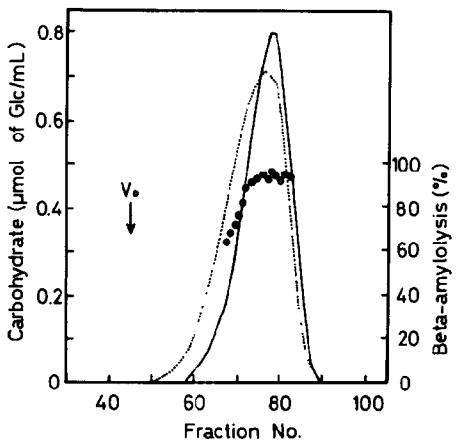

Fig. 5. Gel filtration of isoamylase-treated potato amylose on TOYOPEARL HW-75F. Potato amylose (50 mg in 5 mL) was incubated at 45° with 5.5 U of *Pseudomonas* isoamylase in 20mM acetate buffer (pH 3.5) for 2.5 h. An aliquot (1 mL) was applied to the column: —— and ●, carbohydrate concentration and beta-amylolysis limit, respectively, of the isoamylase-treated amylose; ·····, carbohydrate concentration of the native amylose.

the elution profile, and the beta-amylolysis limits of the fractions. The same profile was observed for the unheated sample. The maximum beta-amylolysis of the fraction was ~93%, which is a little higher than that (90%) of the original amylose. Comparison of the elution pattern of the isoamylase-treated amylose with that of the original amylose showed that the fraction of higher molecular weight had decreased and that the fraction of lower molecular weight had increased, implying the presence of long-chain branches. This is in keeping with the suggestion made by Banks and Greenwood[38], and Ghiasi and Hoseney[19], from hydrodynamic and gel-filtration experiments, respectively. A paper chromatogram of the isoamylase-treated amylose indicated the presence of very short (for example, maltotetraosyl and maltopentaosyl) branches, whereas that of the original amylose treated by the same procedure, except debranching, showed no such maltosaccharides. These maltosaccharides originated from the amylose and not from amylopectin, which might still have remained in the specimen, since maltohexaose was the smallest maltosaccharide detected from amylopectin under the conditions. Thus, it is concluded that potato amylose has branches with a wide distribution of chain length, from maltotetraose to long-chain (d.p. >100) branches.

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