Carbohydrate Research, 227 (1992) 113–120
Elsevier Science Publishers B.V., Amsterdam

Examination of the structure of amylose by tritium labelling of the reducing terminal*

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(Received May 16th, 1991; accepted September 14th, 1991)

ABSTRACT

The tritium labelling of the reducing terminal of amylose by treatment with sodium [^{3}H]borohydride has been used to determine the number-average d.p. ($\overline{d.p.}_n$), the molar fraction of branched molecules, and the distribution of molecules on a molar basis. Maize amylose comprised components having $\overline{d.p.}_n$ 5200, 3030, and 880, with the smallest component preponderating (81%). Isoamylolysis before and after the labelling indicated that the branched molecules contained C chains with d.p. 200–710 and long side-chains with d.p. probably up to 5000.

INTRODUCTION

Labelling of the reducing terminal of amylose by reduction with sodium [^{3}H]borohydride has potential for the structural analysis of amylose because of the specificity and sensitivity. Moreover, it should enable determination of the number-average degree of polymerisation ($\overline{d.p.}_n$) and the corresponding molar distribution by gel-permeation chromatography and, in combination with beta-amylolysis, enable determination of molar fractions of linear and branched molecules. Isoamylolysis before and after the labelling should reveal the molar distributions of the side and main chains of the branched molecules by gel-permeation chromatography without isolation of these molecules. We now report a modification of the method of Richards and Whelan[1], and its application to maize amylose.

EXPERIMENTAL

Materials. — Amyloses were fractionated from defatted starches[2] by the method of Lansky *et al.*[3] and were purified[2] by ultracentrifugation and repeated recrystallisation from aqueous 10% 1-butanol. The specimens were free of amylopectin[4]. Sodium [^{3}H]borohydride (18.1 GBq/mmol) was purchased from Daiichi Chemical Industries Co. Ltd. (Tokyo). The standard amylose, $\overline{d.p.}_n$ 278 (determined by the colorimetric methods[5]), was that prepared[6] previously. Methyl sulfoxide (Me_2SO) of gas-chromatog-

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0008-6215/92/$ 05.00

raphy grade and other chemicals of the highest grade were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo). Toyopearl HW-60F and HW-65F were the products of Tosoh Co. Ltd. (Tokyo). Sweet-potato beta-amylase[7] was recrystallised from aqueous ammonium sulfate. *Pseudomonas* isoamylase was obtained from Hayashibara Biochemical Research Laboratories Inc. (Okayama).

Determination of the reducing terminal of amylose by tritium labelling. — Amylose (~ 10 mg) was dissolved in Me_2SO (50 μL) by heating in a boiling water bath. The solution was diluted to 1 mL with water and kept at 45° before use. An aliquot (100 μL) of the solution was incubated with 0.2M [^{3}H]borohydride–Me_2SO (10 μL, 356 MBq/mmol) at 45° for 1 h. 5.15M Acetic acid (10 μL) was added, and an aliquot (100 μL) of the solution was transferred to a glass-fibre filter with aqueous 95% ethanol–50mM LiCl–0.1M HCl (−15°, at least 10 mL/filter), aqueous 95% ethanol (−15°), and acetone with stirring for 15, 10, and 5 min, respectively. The filter was dried under an infrared lamp. The radioactivity of the filter was determined in 5% 2,5-diphenyloxazole in toluene by a liquid scintillation spectrometer. The standard amylose (0.11 μmol/mL) and blank (5% Me_2SO) solutions were run simultaneously. The reducing terminal of the amylose (μmol/mL) is given by

$$(0.11\ \mu\text{mol/mL}) \times (\text{c.p.m.}_{\text{amylose}} - \text{c.p.m.}_{\text{blank}})/(\text{c.p.m.}_{\text{standard}} - \text{c.p.m.}_{\text{blank}}).$$

Determination of the molar fraction of branched molecules in amylose. — Amylose (10 mg) was treated with 0.2M [^{3}H]borohydride as described above. After the addition of 5.15M acetic acid (100 μL), the solution was neutralised with 5M NaOH (~ 100 μL). To an aliquot (130 μL) of the solution was added M acetate buffer (5 μL, pH 4.8) (for total molecules) or beta-amylase solution [5 μL, 5000 U/mL of M acetate buffer (pH 4.8)] (for branched molecules). The solutions were incubated at 37° for 3 h, an aliquot (100 μL) of each solution was put onto a glass-fibre filter and washed, and the radioactivity was determined as described above. The molar fraction of branched molecules (MF_B) is given by

$$(\text{c.p.m.}_{\text{branched}} - \text{c.p.m.}_{\text{blank}})/(\text{c.p.m.}_{\text{total}} - \text{c.p.m.}_{\text{blank}}).$$

Preparation and isoamylolysis of reduced tritiated amylose and reduced amylose. — Amylose (30 mg) was treated as described above and to the neutralised solution was added 0.1 vol. of 1-butanol to precipitate the reduced and tritiated amylose. After keeping for 1 h in an ice bath, the precipitate was collected by centrifugation (3000 r.p.m., for 15 min), washed by centrifugation with aqueous 10% 1-butanol, and dissolved in water (1.5 mL). Reduced amylose was prepared in the same manner. The reduced and tritiated amylose (1.5 mL, 20 mg/mL) was incubated[5] with isoamylase (3 μL, 1000 U/mL) in 50mM acetate buffer (pH 3.5) for 2.5 h at 45°, and the reaction was terminated by heating.

Reduced amylose was treated with isoamylase as described above. The isoamylolysate (3 mL, 10 mg/mL) was incubated with 0.2M [^{3}H]borohydride–Me_2SO (300 μL, 356 MBq/mmol) and the mixture was acidified and neutralised as described above. The precipitate (tritiated long side-chains) formed by the addition of 1-butanol was collected by centrifugation, washed with aqueous 10% 1-butanol, and dissolved in water (1.5 mL). The supernatant solution containing the tritiated short side-chains was discarded

because the gel-permeation chromatography described below could not separate them from a radioactive, involatile impurity[1] in the [^{3}H]borohydride.

Gel-permeation chromatography. — The sample solution (1 mL, ~ 20 mg/mL) was filtered (0.45 μm) and applied to a column (2.2 × 39 cm) of Toyopearl HW-65F and HW-60F (1:1 by weight) at 45°, and eluted with water at 20 mL/h (2-mL fractions). An aliquot (1 mL) of each fraction was transferred to a glass-fibre filter and the radioactivity was determined without washing. The reducing terminal of the fractions was calculated using the radioactivity incorporated into standard amylose.

Analytical methods. — Carbohydrate was determined by the phenol–sulfuric acid method[8]. The reducing terminal of amyloses was also determined by the modified Park–Johnson method[5].

RESULTS AND DISCUSSION

Determination of the reducing terminal of amylose by reduction with [^{3}H]borohydride. — The tritium labelling method used by Richards and Whelan[1] to determine the $\overline{\text{d.p.}}_n$ of hydrolysed amylose ($\overline{\text{d.p.}}_n < 62$) involved reduction with [^{3}H]borohydride followed by absorbing[9] the product onto a cellulose filter and counting of the radioactivity. However, the method gave a high blank even when [^{3}H]borohydride with high specific radioactivity (356 MBq/mmol) was used. Therefore, a modified method was devised.

Of several kinds of filters commercially available (Whatman 3MM, GC-50, GF/F, GA-200, and QA-80), Whatman GC-50 (glass-fibre filter) gave a low blank (~ 90 c.p.m.), whereas 3MM (cellulose)[1,9] and QA-80 (silica) gave high blanks (1000–1200 c.p.m.) due to adsorption of a radioactive impurity and, with the former, tritiation of cellulose. The addition of 50mM LiCl to the washing liquid (aqueous 95% ethanol containing 0.1M HCl)[1] and its precooling (−15°) were required to retain tritiated amylose on the filter. A short-chain amylose ($\overline{\text{d.p.}}_n$ ~ 50) was removed partially and maltose was removed completely from the filter by washing.

Amylose was dissolved in methyl sulfoxide instead of sodium hydroxide[1] for the subsequent enzymic reaction. To the amylose solution (10 mg/mL) was added 0.1 vol. of 0.05–0.4M [^{3}H]borohydride, the mixture was incubated at 45° for 1 h, and the radioactivity incorporated into the amylose was determined. At 0.1M [^{3}H]borohydride, the incorporation reached a maximum level and no reducing power was detected by the modified Park–Johnson method[5]; a concentration of 0.2M was used routinely. The radioactivity incorporated was proportional to the amylose concentration up to ~ 0.11 μmol/mL.

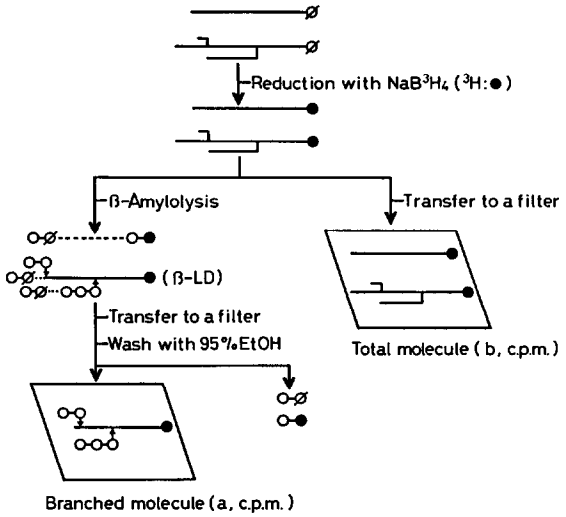
Determination of $\overline{\text{d.p.}}_n$ of amyloses by the labelling method. — The $\overline{\text{d.p.}}_n$ values, determined by the labelling and colorimetric[5] methods, were in reasonable agreement except for those of maize amylose, and are shown in Table I. The $\overline{\text{d.p.}}_n$ of maize amylose determined by each method was reproducible, but that for the labelling was lower for reasons that are unknown at present. The labelling method gave a lower deviation in $\overline{\text{d.p.}}_n$, except for potato amylose.

TABLE I

$\overline{D.p._n}$ of some amyloses

Amylose	$\overline{D.p._n}$[a]		*Ratio A:B*
	Labelling method (A)	*Colorimetric method*[5] *(B)*	
Rice[b]	1010 ± 35 (4)	930 ± 50 (3)	1.09
Rice[c]	930 ± 10 (3)	1020 ± 25 (3)	0.91
Maize	730 ± 10 (2)	930 ± 30 (3)	0.79
Amylomaize	640 ± 20 (4)	680 ± 30 (6)	0.94
Wheat	1030 ± 30 (4)	980 ± 45 (6)	1.05
Potato	4450 ± 620 (4)	4700 ± 540 (6)	0.95

[a] Mean ± standard deviation (number of experiments). [b] Japonica Nihonbare. [c] Indica IR64.



Scheme 1. Procedure for the determination of the molar fraction (a/b) of the branched molecules in amylose.

Determination of the molar fraction of branched molecules in amylose. — The conventional method[10] involves the isolation of a substantial amount of beta-limit dextrin (β-LD, ~50 mg; 250 mg as amylose) and analysis of the reducing and non-reducing terminals of the β-LD and the parent amylose. The new method (Scheme 1) required a small amount of amylose (10 mg) and was simple and rapid. Beta-amylolysis of the reduced and tritiated amylose produced labelled β-LD and small oligosaccharides (*i.e.*, maltitol) from branched and linear molecules, respectively. The beta-amylolysate was transferred onto the filter where the labelled β-LD was retained whilst the reduced oligosaccharides were removed by washing. The molar fraction of branched molecules in the amylose was calculated as the ratio of the radioactivities of the labelled β-LD to the labelled amylose.

TABLE II

Molar fractions of branched molecules in amyloses and beta-limit dextrin

Specimen	*Molar fraction of branched molecule* [a]	
	Labelling method	*Conventional method* [b]
Amylose		
Rice[c]	0.22 ±0.02 (2)	0.25 ±0.06 (3)
Rice[d]	0.25 ±0.01 (2)	0.25 ±0.04 (3)
Maize	0.30 ±0.01 (2)	0.44 ±0.15 (3)
Potato	0.38 ±0.03 (2)	
Potato(E)[e]	0.07 ±0.02 (2)	
Kuzu(E)[e]	0.12(1)	
Beta-limit dextrin		
Rice[d]	0.98 ±0.04 (4)	

[a] Mean ± standard deviation (number of experiments). [b] Calculated from the average number of chains ($\overline{Nc}$) of amylose and its β-LD by the equation: molar fraction of branched molecule = $(\overline{Nc}_{amylose} - 1)/(\overline{Nc}_{\beta\text{-}LD} - 1)$. [c] Japonica Nihonbare. [d] Indica IR64. [e] Prepared by the precipitant–enzymic method[6].

Table II shows molar fractions of branched molecules (MF_B) in amyloses and a β-LD. The values for rice amyloses agreed with those obtained by the conventional method[10], but that (0.30) for maize was lower (0.44) possibly due, at least in part, to the washing out of a small, labelled β-LD. However, the apparent $d.p._w$ distribution (460–15 000) of maize β-LD was similar to that of the parent amylose[11]. Potato amylose had MF_B 0.38, similar to that for tapioca (0.42) and lower than that for sweet potato (0.70)[10]. Potato(E) and kuzu(E) amyloses, which were isolated[6] from the alpha-amylase digest of gelatinised starch of which the amylose formed a complex with 1-butanol, gave MF_B values of 0.07 and 0.12. These amyloses ($\overline{d.p._n} \sim 260$) had been thought to comprise linear molecules by the fact that they were completely or almost completely degraded with beta-amylase [potato(E) 99% and kuzu(E) 95%][6], but the results indicate the presence of branched molecules. Thus, the new method is applicable to amyloses that contain a small proportion of branched molecules, whereas the conventional method is not since these specimens yield a very small amount of β-LD for the terminal analyses. A β-LD preparation was confirmed to contain no linear molecules by the MF_B value.

Analyses of the structure of maize amylose by the labelling method. — Fig. 1 shows the molar distribution of amylose molecules by gel-permeation chromatography of the reduced and tritiated amylose of maize on Toyopearl HW-60F and HW-65F. The apparent $d.p._w$ distribution was reported[11] to be 330–13 700 for the sub-fractions, 10% by weight, of the largest and smallest molecules; however, the apparent $d.p._n$ distribution was narrow, 360–6900 (Fig. 1). The chromatogram suggested that, on a molar basis, maize amylose comprised three components, namely, a major component with the peak top at d.p. 710 and two minor components, whereas, on a weight basis, there was a single, broad peak with d.p. 1580 at the peak top. The reduced and tritiated amylose was fractionated into three sub-fractions, F1/3 in order of elution (Fig. 1) (6,

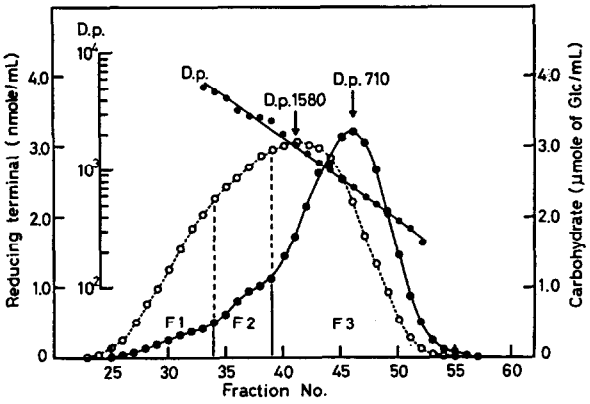


Fig. 1. Gel-permeation chromatogram and d.p. values of the reduced tritiated amylose of maize on Toyopearl HW-65F and HW-60F; ●, reducing terminal; o, carbohydrate.

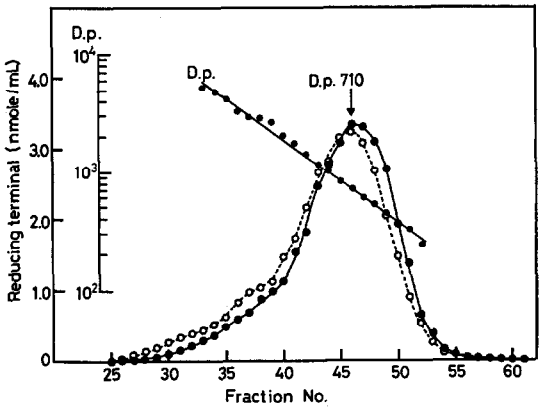


Fig. 2. Gel-permeation chromatogram and d.p. values of the reduced tritiated chains (●) in the isoamylolysate of the reduced tritiated amylose of maize on Toyopearl HW-65F and HW-60F. For reference, the chromatogram (o) and d.p. of the reduced and tritiated amylose (Fig. 1) are included.

TABLE III

Properties of F1–F3 of the reduced and tritiated amylose of maize

Property	*F1*	*F2*	*F3*
Proportion, % of total			
Mole	6	13	81
Weight	23	27	50
$\overline{D.p.}_n$	5200	3030	880

13, and 81% by mole, and 23, 27, and 50% by weight, respectively), and their properties are summarised in Table III. Their $\overline{d.p.}_n$ values were 5200, 3030, and 880, respectively. Thus, in maize amylose, the major component had the lowest molecular weight ($\overline{d.p.}_n$ 880) in agreement with previous results[12].

The elution profile (Fig. 2) of the isoamylolysate of the reduced and tritiated amylose on gel-permeation chromatography showed the molar distribution of the sum of the C chains (*i.e.*, the chain having the reducing terminal residue) of the branched molecules, linear molecules, and branched molecules with isoamylase-resistant linkages[12,13]. The profile resembled that of the parent reduced and tritiated amylose, but was shifted to the side of small molecules due to removal of side-chains. The proportion of reduced and tritiated amylose with d.p. >710 decreased and that of molecules with 710–200 increased. The latter molecules were mainly C chains, but a portion of them might carry isoamylase–resistant linkages. Thus, the d.p. range of C chains of the branched molecules was 710–200, but the presence of C chains with d.p. >710 was suggested by the gel-permeation chromatograms of the large sub-fraction ($\overline{d.p.}_n$ 2720) and its isoamylolysate[12].

The branched molecules of maize amylose comprise short side-chains with $\overline{d.p.}_n$ 18 (ref. 13), as described for rice amylose[14], and probably also long side-chains[13]. The nature of the long side-chains was clarified by the labelling method. The isoamylolysate of the reduced amylose was reduced with [^{3}H]borohydride, where only the original side-chains were labelled. The long side-chains were precipitated as complexes with 1-butanol because the gel-permeation chromatography on Toyopearl HW-65F and HW-60F could not separate the short side-chains from a radioactive impurity in the [^{3}H]borohydride. The $d.p._n$ distribution of long side-chains (Fig. 3) was fairly wide (100–5000). Extremely long chains (d.p. ~ 5000) may be branched, since some branch linkages of the branched molecules are resistant to isoamylase, and this aspect is under investigation. The peak-top d.p. of the long side-chains was 200. The present and

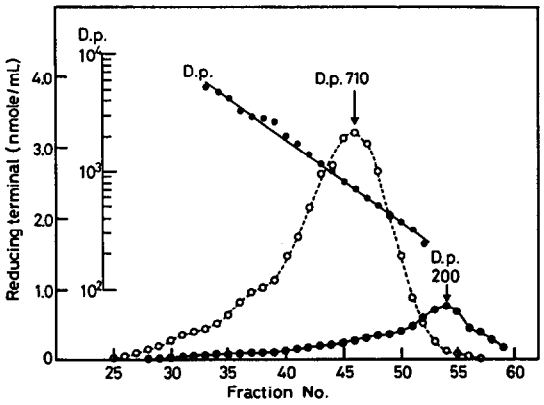


Fig. 3. Gel-permeation chromatogram and d.p. values of the long labelled side-chains (●) from the branched molecule in maize amylose on Toyopearl HW-65F and HW-60F. For reference, the chromatogram (○) and d.p. of the reduced tritiated amylose of maize (Fig. 1) are included.

previous[13] results indicate that the branched molecules of maize amylose contain side-chains having various sizes (d.p. >6, ref. 13) and support the model[13] for the structure of the maize branched molecules.

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