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MULTI-BRANCHED NATURE OF AMYLOSE AND THE ACTION OF DE-BRANCHING ENZYMES

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ABSTRACT

Convenient and sensitive methods are described for analyses of the reducing and non-reducing residues of amylose. The multi-branched nature of amylose samples from several plant sources was revealed quantitatively by these methods. *Pseudomonas* isoamylase partially (30%) split the branch linkage of potato amylose. The concurrent action of *Aerobacter* pullulanase and sweet-potato beta-amylase hydrolysed the amylose completely.

INTRODUCTION

The presence of anomalous linkages or branches in amylose [a long, linear (1→4)-α-D-glucan] was first suggested in 1952 by Peat *et al.*[1]. They observed that purified amylose from potato starch was, at most, 70% hydrolysed by pure beta-amylase. The main barrier to beta-amylase action on amylose is the minor degree of branching by (1→6)-α linkages as in amylopectin[2], since the cooperative action of beta-amylase with yeast isoamylase[3] or bacterial pullulanase[4,5], which acts specifically on such linkages, completely or partly overcomes the barrier. The main purpose of our study was to estimate the degree of branching of amylose quantitatively by determining the reducing and non-reducing residues.

The reducing residues or number-average molecular weight of amylose may be determined by photometry[6–8], periodate oxidation[9,10], polarimetry[11], osmometry[12,13], radiometry[14,15], and enzymic analysis[16,17]. However, for routine use, these methods are inconvenient, of low sensitivity, and/or require expensive facilities. We have now devised a method for routine analysis by modifying Park–Johnson's colorimetric procedure for glucose[18] and have determined the average degree of polymerisation ($\overline{d.p.}$) of amylose samples from several sources. The non-reducing residues were determined by modifying the rapid Smith-degradation method[19] so as to be suitable for amylose. The results indicate that some amylose samples have 9–20 chains.

MATERIALS AND METHODS

Materials. — Potato starch (Kenebec), which was prepared by the usual procedure using cold water, was a gift from Dr. S. Yoshioka (Hokkaido Agricultural Experimental Station). Commercial potato, Kuzu (*Pueraria hirusuta* Matsum), and wheat starches were the products of Hokuren (Hokkaido), Hirohachido-shoten (Kagoshima), and Sanwa Denpun Kogyo Co. (Nara), respectively. Tapioca starch was an imported, commercial product.

All amylose samples were isolated from starch by the method of Lansky *et al.*[20], but a mixture of 1-butanol and 3-methyl-1-butanol was used as the complexing agent instead of Pentasol, and the fractionation was performed under a nitrogen atmosphere. The amylose was purified by recrystallisation four times from hot, 10% aqueous 1-butanol by cooling.

Amylose of low molecular weight was prepared by debranching potato amylopectin with *Pseudomonas* isoamylase and isolating the larger unit-chain component by filtration through Bio-Gel P-30. The $\overline{d.p.}$ values per reducing and non-reducing residue were 40 and 41, respectively, and it was hydrolysed completely with beta-amylase. The reducing residue was determined by the methods of Somogyi[21] and Nelson[22], but the heating period was extended to 30 min. Under these conditions, maltosaccharides give the same reducing value as glucose[8].

Amylodextrin-NT (resembling Naegeli amylodextrin) was prepared by a rapid process of steeping potato starch in M HCl–70% ethanol at 65° for 48 h, as described elsewhere[23]. The $\overline{d.p.}$ values per reducing and non-reducing end of the specimen were 21 and 11, respectively.

Beta-amylase was prepared[24] from sweet potato, and recrystallised from an aqueous ammonium sulphate solution for safe storage. Isoamylase, crystallised from the culture broth of a *Pseudomonas* sp., was a gift from Professor T. Harada (Osaka University). Pullulanase (*Aerobacter aerogenes*, crystalline) was a product of Hayashibara Biochemical Laboratories Inc., Okayama. Other enzymes were obtained from Boehringer-Mannheim-Yamanouchi, Tokyo.

Procedures for determination of reducing residues. — To 1 ml of a specimen solution (described below) containing amylose equivalent to 1–5 μg of glucose was added 0.5 ml of sodium carbonate–sodium hydrogencarbonate buffer containing potassium cyanide (4.8 g of Na_2CO_3, 9.2 g of $NaHCO_3$, and 0.65 g of KCN/litre of water), and the mixture was heated for exactly 15 min in a vigorously boiling water-bath. After cooling for 10 min in running tap-water, 2.5 ml of ferric ammonium sulphate solution (3 g/litre of 50mM H_2SO_4) was added to the mixture under effective ventilation and the mixture was kept for 20 min at room temperature. The absorbance at 715 nm (any wavelength in the range of 650–730 nm is suitable) of the resulting blue solution was determined (Shimadzu UV 210A spectrophotometer). A reference solution, which contained all of the components of the specimen solution except amylose, and a standard solution containing 5 μg of D-glucose per ml of the reference solution were treated as above. The reducing residues and $\overline{d.p.}$ values were calculated

from the absorbance of the specimen and standard solutions and from the carbohydrate content as glucose, which was determined with anthrone–H_2SO_4[25].

The amylose solution was prepared by the following procedure. To dry amylose (up to 50 mg) were added, successively, 0.2 ml of 99% ethanol and 0.5 ml of water, and then 0.5 ml of 2M sodium hydroxide was added to the suspension with shaking. Amylose was dissolved completely by gradual dilution with 0.5–1.0 ml of water with shaking. The solution was neutralised to pH 6.5–7.0 with M hydrochloric acid and made up to 5 ml with water. The neutralised solution was kept in a warm water-bath (~45°) to avoid retrogradation, while other specimens were being treated.

Smith degradation for determination of non-reducing residues. — Amylose (5–8 mg/ml) was dissolved in sodium hydroxide, and the solution was neutralised with hydrochloric acid as described above. Smith degradation was effected by the procedure described previously[19], but the periodate oxidation was effected at 30° and the resulting glycerol (produced from the non-reducing residues) was determined fluorimetrically by the coupled reactions of glycerol 3-phosphate dehydrogenase and glycerol kinase. The Smith-degraded specimen (50 μl) was mixed with 150 μl of 0.2M glycine–M hydrazine hydrate containing 2mM $MgCl_2$ (pH 9.8), 2 μl of 100mM ATP (pH 7.0), 10 μl of 20mM NAD, and 3 μl of glycerol 3-phosphate dehydrogenase (600 U/ml) in a microcell, and the reaction was started by the addition of 1 μl of glycerol kinase (85 U/ml) to the microcell. The microcell was placed in an attached cell-holder which was covered, except for the slits (3 × 15 mm), with a black-plastic film. The total volume was 216 μl. The relative fluorescence intensity due to the formation of NADH was read at excitation and emission wavelengths of 340 and 456 nm, respectively (JASCO Model FP-4 spectrofluorometer). NADH formed was calculated from the relative fluorescence intensities of the specimen and a standard maltotriose (0.3 mg/ml oxidised with periodate at 30° for 3 h and treated under the same conditions as for the specimen) or authentic glycerol solution (containing all the components due to Smith degradation).

Hydrolysis with isoamylase. — To 10 ml of potato amylose (prepared from commercial starch) solution (10 mg/ml), prepared as described above and adjusted to pH 3.5, were added 0.5 ml of M acetate buffer (pH 3.5) and 11 U of isoamylase (6 μl), and the mixture was incubated at 45°. At intervals, aliquots (1 ml) were immersed in a boiling water-bath for 1.5 min, to terminate the reaction, and then cooled to room temperature, and the pH was adjusted to 6.5–7.0 with M sodium hydroxide. The volume was made up to 5 ml with water, and the $\overline{d.p.}$ of the hydrolysate was determined.

Hydrolysis with pullulanase. — To 0.5 ml of a solution containing 5 mg of amylose (prepared from commercial potato-starch) and 20mM acetate buffer (pH 5.5) was added 20 U of pullulanase (50 μl), and the mixture was incubated at 30°. The hydrolysis was terminated by immersing the mixture in a boiling water-bath for 1.5 min, and the coagulated protein was removed by centrifugation. The pullulanase was sensitive to temperature (35° was suggested as a stable temperature by the supplier)

and was inactivated easily by heating. The $\overline{d.p.}$ of the resulting amylose was determined after the pH of the supernatant solution was adjusted to 6.5–7.0.

Concurrent hydrolysis with pullulanase and beta-amylase. — The amylose was hydrolysed under the conditions described above, but beta-amylase (50 U/mg of substrate) was added to the reaction mixture.

Successive hydrolysis with isoamylase and pullulanase. — The amylose was debranched first with isoamylase (1.1 U/mg of amylose) at 45° for 2.5 h followed by the treatment with pullulanase (4 U/mg of amylose) at 30° for 24 h. After the isoamylolysis, the reaction mixture was heated in a boiling water-bath for 1.5 min and the pH was adjusted to 5.5–5.6 with sodium hydroxide.

Other analytical methods. — Iodine affinity was determined by amperometric titration[26].

The limit of beta-amylolysis was determined by hydrolysing the specimen (3 mg) at 30° in 50mM acetate buffer (pH 4.8) with crystalline beta-amylase[24] (150 U). The hydrolysis reached an upper-limit value after 30-min incubation and it was maintained for 6 h. Maltose produced was determined by Somogyi's method[21] using Nelson's reagent[22].

RESULTS AND DISCUSSION

The reducing residues of amylose may be determined sensitively by reactions forming Prussian Blue, as described by Nussenbaum and Hassid[7], but their method uses gum ghatti which is not usually available locally. The Park–Johnson procedure for the determination of glucose[18] is sensitive and convenient, but is not suitable for the determination of the molecular weight of amylose, as it was noticed that the method gave higher reducing values with increasing molecular weight of (1→4)-α-D-glucans. Therefore, the method was modified to give the same reducing value from glucose to amylose having $\overline{d.p.}$ 40, as shown in Table I. The main modification was the lowering of the pH of the reaction of the carbohydrate and ferricyanide. The buffer was changed from sodium carbonate to a mixture of sodium carbonate and sodium hydrogencarbonate. The concentration of the buffer was increased to two-fold, because the reaction was sensitive to pH. The modified method followed Beer's law up to a reducing value equivalent to 5 μg of glucose, and the molar absorption

TABLE I

RELATIVE, MOLAR REDUCING-VALUES OF (1→4)-α-D-GLUCANS

Method	*Glucose*	*Maltose*	*Maltotriose*	*Amylodextrin-NT ($\overline{d.p.}$ 21)*	*Short amylose ($\overline{d.p.}$ 40)*
Park–Johnson	1.00	1.27	1.51	2.14	1.96
Modified	1.00	1.01	1.00	0.97	0.98

TABLE II

PROPERTIES OF AMYLOSE

Source	$\overline{D.p.}$*/red. residue*	$\overline{D.p.}$*/non-red. residue*	*Chain number*	*Iodine affinity (g/100 g)*	*Beta-amylolysis limit (%)*
Potato	6340	520	12.2	19.6	68
Potato[a] (Kenebec)	4850	510	9.5	20.1	88
Tapioca	3390	170	20.0	19.0	64
Kuzu	1590	180	9.1	19.9	76

[a]Prepared from starch prepared in a laboratory.

TABLE III

TIME COURSE OF SMITH DEGRADATION OF MALTOTRIOSE[a]

Oxidation time (h)	1	3	5	7	16.5	24
Glycerol (mol/mol)	0.90	0.99	0.98	0.98	0.98	0.99

[a]Maltotriose was oxidised with periodate (3 mg/ml, 30mM sodium periodate) at 30°, followed by reduction with sodium borohydride and acid hydrolysis; glycerol formed was assayed by using enzymes[19].

(calculated for glucose) was $\sim 2.3 \times 10^7$ $cm^{-1}.mol^{-1}$. Sodium lauryl sulfate, a stabiliser of Prussian Blue, was omitted, because it formed an insoluble complex with amylose; without the detergent, the resulting blue solution was stable for ~30 min. The method is simple and may be useful for the determination of molecular weights of other polysaccharides. The $\overline{d.p.}$ values for amyloses from various sources, determined by the method, are listed in Table II. Potato amyloses prepared from two different origins gave the highest d.p. values (6340 and 4850), which are a little higher than those given by Greenwood[27]. The $\overline{d.p.}$ values of tapioca and kuzu amyloses were 3390 and 1840, respectively.

Determination of the non-reducing residues of amylose was performed by rapid Smith-degradation, but the periodate oxidation involved was performed at 30° because, at 50°, the formation of glycerol increased continuously as shown in Fig. 1, presumably due to slight degradation during the oxidation. This situation is different from that for amylopectin, which forms a constant amount of glycerol on rapid Smith-degradation[19] including periodate oxidation at 50°. Maltotriose yielded the theoretical amount of glycerol during the oxidation at 30° for 3–24 h in the Smith degradation (Table III). Amylose similarly gave a constant level of glycerol after 5 h of oxidation at 30° and after 24 h at 16°, as shown in Fig. 1. The maintenance of a constant level of glycerol for a reasonable period in the Smith degradation is an

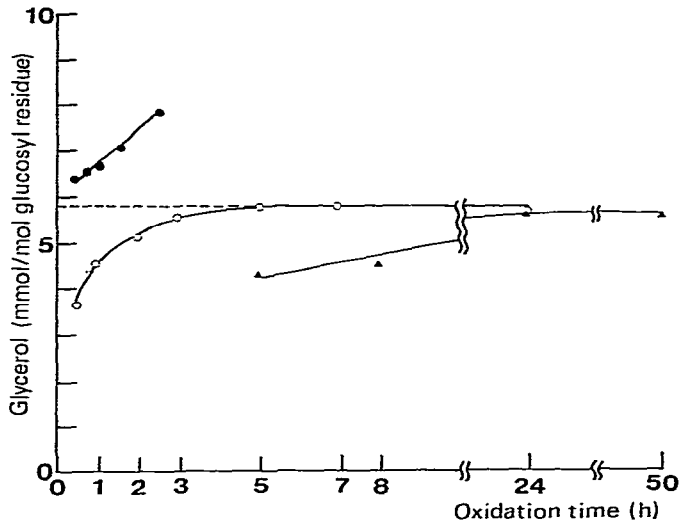

Fig. 1. Time courses of glycerol formation on Smith degradation. Periodate oxidation was performed at 50° (●), 30° (○), and 16° (▲).

advantage in quantitative analysis over simple periodate oxidation in which the liberation of formic acid keeps increasing slowly beyond complete and regular oxidation, even under well-controlled conditions. The over-oxidation interferes least with the formation of glycerol in Smith degradation of amylose, and the conditions described here are suitable for the determination of non-reducing residues of amylose. The unit-chain lengths of amyloses of various origins, determined by the Smith degradation–fluorimetric assay, are listed in Table II. Potato amylose gave high $\overline{\text{d.p.}}$ values (510 and 520) that are about 3-fold those of tapioca and kuzu amylose.

The foregoing data indicate that these amylose samples are composed of ~9–20 chains (Table II). It is improbable that the branched structure is due to the small proportion of accompanying amylopectin, as the amylose samples were recrystallised 4 times from 1-butanol and showed the high iodine-affinity of 19–20 g/100 g. It is conceivable that these amylose samples contain, in part, unbranched molecules, so that the branched molecules should have more branch linkages than 8–19, but such highly branched amylose molecules have not been described. Potter and Hassid[12] suggested that there are 0–3.3 branches per mole for amylose of various origins, by determining the molecular weight of acetylated amylose by osmometry and the non-reducing residues by titration of formic acid after periodate oxidation. The $\overline{\text{d.p.}}$ values for potato and tapioca amyloses were found to be 930 and 1300, respectively. These low values could be due to degradation in the fractionation of amylose under aerobic conditions and acetylation. In addition, some error might be inevitable in their determination of the non-reducing residues of amylose by periodate oxidation and titration.

The molecular structure of amylose of an individual plant source depends on

the conditions of the growth of the plant[28-30], storage after harvest, fractionation, and purification. Therefore, further studies are necessary to characterise the molecular structure of amylose of a particular origin, but the results presented here reveal the multibranched structure of whole amylose that is prepared by complete dispersion of starch granules. The high molecular weight and long unit-chain appear to be characteristic of potato amylose, since the amyloses obtained from two sources have a similar structure.

Potato amylose ($\overline{d.p.}$ 6340) was treated with pullulanase and isoamylase in order to characterise the branch linkages in the amylose and to see whether these linkages are cleaved completely by the enzymes. These enzymes degraded the amylose ($\overline{d.p.}$ 6340, chain number 12.2) rapidly at the beginning, and the resulting $\overline{d.p.}$ approached the constant values on prolonged incubation with large amounts of the enzymes, as shown in Figs. 2 and 3. However, the extents of debranching were far from complete under the conditions. Therefore, these debranching enzymes could not be used for the determination of the branch linkages of amylose. Isoamylase hydrolysed 30% of the branch linkages in amylose after 2.5-h incubation and produced a smaller molecule having $\overline{d.p.}$ 1470. Concomitantly, the limit of beta-amylolysis was increased from 68 to 82%. The incomplete debranching is not due to inactivation of the enzyme, since potato amylopectin (10 mg/ml, the same volume as the reaction mixture) added to the reaction mixture after 2.5-h incubation was debranched fully by extending the incubation to 5 h. Why the isoamylase is incapable of hydrolysing all of the branch linkages of the amylose is unknown, but the presence of maltosyl branches in the amylose, as suggested below, could be one reason. Pullulanase cleaved 43% of the branch linkages after 24-h incubation and lowered the $\overline{d.p.}$ of the molecule to 1090, and the limit of the beta-amylolysis was increased to 85%. The successive actions of isoamylase and pullulanase hydrolysed 54% of the branch linkages of the amylose and yielded molecules having $\overline{d.p.}$ 890. However, the amylose was completely hydrolysed into maltose after 24-h incubation with the concurrent action of pullulanase and beta-amylase, as reported by Banks and Greenwood[4], suggesting that all of the branch linkages in the amylose are (1→6)-α. The incomplete debranching with pullulanase alone appears to be mainly due to the retrogradation of amylose during the incubation, because the enzyme was fully active after 24-h incubation and the addition of beta-amylase (50 U/mg of substrate) after 24 h to the reaction mixture, without inactivation of pullulanase, gave only a slightly higher beta-amylolysis limit (88%) than by adding beta-amylase after inactivation of pullulanase. In fact, the reaction mixture became very faintly turbid during the incubation with pullulanase, but that with isoamylase remained crystal clear. That the isoamylolysis product was further debranched with pullulanase may suggest the presence of a few maltosyl stubs in the amylose, since *Pseudomonas* isoamylase hardly debranches maltosyl stubs in beta-limit dextrin of amylopectin[31,32], and *Aerobacter* pullulanase removes the maltosyl branches[33]. It is of interest that pullulanase hydrolyses the amylose more than isoamylase, since pullulanase is incapable of hydrolysing glycogen completely, in contrast to isoamylase[31]. Isoamylase hydrolysed only 30% of the branch

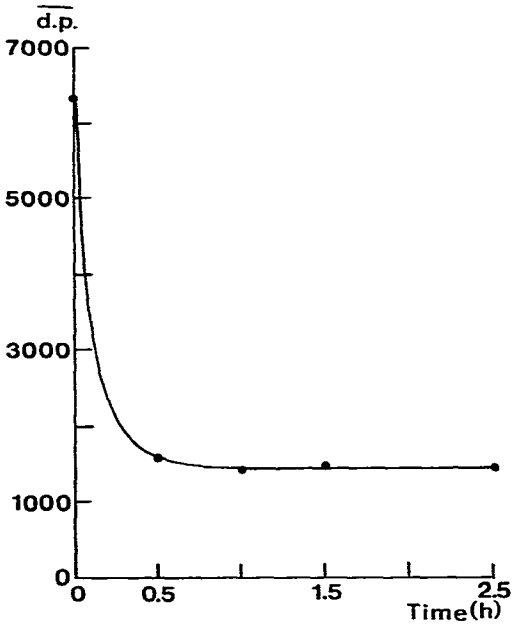

Fig. 2. Debranching of potato amylose (d.p. 6340, chain number 12.2; prepared from commercial starch) with *Pseudomonas* isoamylase. For conditions, see text. Debranching at the end of the incubation was 30%.

$$\text{Debranching (\%)} = \frac{(\overline{\text{d.p.}}\text{ of amylose}/\overline{\text{d.p.}}\text{ of debranched amylose}) - 1}{(\text{chain number of amylose}) - 1} \times 100.$$

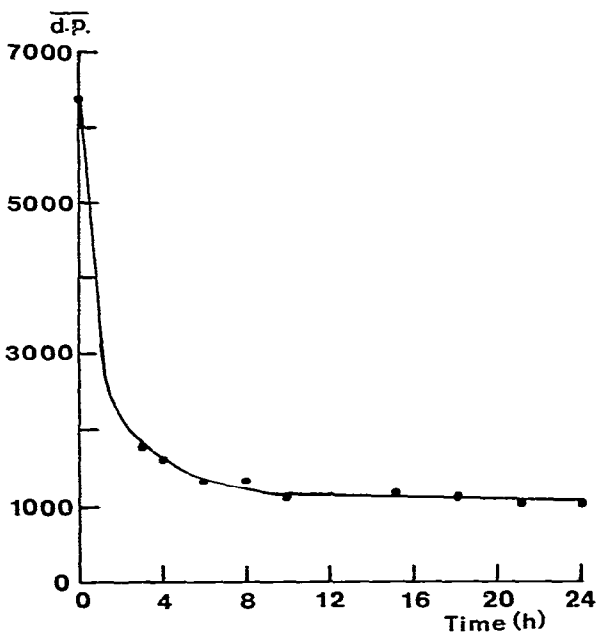

Fig. 3. Debranching of potato amylose ($\overline{\text{d.p.}}$ 6340, chain number 12.2; prepared from commercial starch) with *Aerobacter* pullulanase. For conditions, see text. Debranching at the end of the incubation was 43%.

linkages in the amylose, indicating that most of the branch linkages are not due to contamination by amylopectin, because the enzyme debranches amylopectin completely[31,32,34–36]. Further studies of the structure of amylose and the action specificities of the debranching enzymes are in progress.

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