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The Structure of Amyloses

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Determination of the degree of branching in amylose structure was made by noting the rate of sugar production during hydrolysis, both with amylo-glucosidase and with β-amylase. It has been shown that both enzymes hydrolyze only terminal units of starch structures, the former producing glucose and the latter, maltose. Accordingly, the rate at which sugar is produced in both cases depends upon the number of terminal groups present in a given volume of solution. Equimolar solutions of several amylose samples were hydrolyzed and the rates of sugar production compared with that determined for the linear substrate, corn crystalline amylose. The rates for corn A-fraction (butanol precipitable fraction) as well as for an A-fraction from an acid-hydrolyzed corn starch were substantially the same as for corn crystalline amylose, indicating that these amyloses are composed almost entirely of non-branched molecules. However, the rates for a potato A-fraction were very nearly double, and the rates for a tapioca A-fraction approximately triple that for a linear substrate. From these results it may be concluded that there is an average of one to two branches per molecule in the potato amylose and two to three branches per molecule in the tapioca amylose samples.

Although some starches such as corn contain an amylose fraction which very likely contains non-branched molecules,[1,2] it has been suspected that other starches contain amylose molecules which are of greater molecular weight and are slightly branched.[2] Methylation and periodate oxidation procedures used to advantage to determine branching in amylopectin involve experimental errors which are relatively large, however, in proportion to the degree of branching thought to exist in the larger of the amylose molecules. Thus, for example, Meyer and co-workers[1] obtained 0.32% tetramethylglucose from a methylated, relatively short, linear corn starch fraction with a DP_n of 300, but if this procedure were applied to tapioca amylose with a DP_n of approximately 1000,[3] then only 0.1% tetramethylglucose would be obtained if the molecules were linear and 0.2% if they contained a branch. These differences appear to be close to the experimental error of earlier methods.

Hess and Krajnc[4] obtained 0.47% tetramethylglucose from methylated potato amylose (by electrosedimentation) which gave a calculated chain length of 238. The DP_n is much larger, as we have confirmed, and Hess and Steurer[5] concluded that the amylose was slightly branched. Very recently, with improved techniques, Bourne, Fantes and Peat[6] found a chain length of 191 and Barker, Bourne and Wilkinson[7] a chain length of 204 by methylation for potato amylose (by thymol precipitation). Nevertheless, both Hassid and McCready[8] from methylation data and Potter and Hassid[9] from periodate oxidation measurements concluded that potato amylose (by Pentasol precipitation) with a DP_n of 930 was non-branched.

Kerr, Cleveland and Katzbeck[10] have shown that amylo-glucosidase from *Aspergillus niger* hydrolyzes amyloses to glucose by approaching the substrate only from terminals of the molecules. Equimolar solutions of several of the shorter corn amylose samples with a range of DP_n values from 135 to 480 gave the same rate of glucose production. Since this enzyme operates only by a terminalwise attack, even a very small degree of branching would result in a comparatively large and easily measured increase in the rate of sugar production. Furthermore, from studies with amylopectin,[10] it would be anticipated that as the amylo-glucosidase approached branched points, the rate, which is normally that of a first order reaction for strictly linear molecules, would be materially retarded. Therefore from measurements of the rate of glucose liberation one may determine the average number of branches per molecule and gain some idea from the decline in rate, whether the branches are, on the average, relatively long or short.

From previous work on the hydrolysis of amylose to maltose with β-amylase,[11-13] which shows an analogous type of hydrolysis, it would appear that hydrolysis rate studies with β-amylase may also be used to determine branching in the higher amyloses and thus to compare with the results found with amylo-glucosidase.

Experimental Section

Source of Materials.—The A-fractions were prepared by autoclaving dilute starch pastes at 17 p.s.i. steam pressure at *p*H 6.0 for 30 minutes, precipitating with Pentasol and recrystallizing the precipitate twice from aqueous butanol. The corn A-fraction (No. 27) has been extensively studied.[3,10,14] The tapioca A-fraction,[3] the potato A-fraction[14] from Idaho potato starch and corn crystalline amylose[10,13] samples have been studied heretofore. The A-fraction from the acid treated corn starch is the complementary sample to the B-fraction used in studies on amylopectin.[14]

The amylo-glucosidase was prepared as described in earlier work[10] by treating an *Aspergillus niger* (N.R.R.L. #330-1) culture filtrate with HCl at *p*H 2.25, storage at 5° followed by neutralization and filtration. The crystalline β-amylase was kindly supplied by Dr. A. K. Balls and was a 4× recrystallized product from sweet potatoes.

Enzyme Hydrolyses.—A carbohydrate concentration of 5.15×10^{-5} *M* was used in all cases. Molarity was computed from number average molecular weights ($DP_n \times$

(1) K. H. Meyer, M. Wertheim and P. Bernfeld, *Helv. Chim. Acta*, **23**, 865 (1940).

(2) R. W. Kerr, *Paper Trade J.*, (TAPPI) 115, 30 (1942), see also, R. W. Kerr, in "Chemistry and Industry of Starch," 2nd Ed., Academic Press, New York, N. Y., 1944, pp. 145, 333–334.

(3) F. C. Cleveland and R. W. Kerr, THIS JOURNAL, **71**, 16 (1949).

(4) K. Hess and B. Krajnc, *Ber.*, **73**, 796 (1940).

(5) K. Hess and E. Steurer, *ibid.*, **73**, 1076 (1940).

(6) E. S. Bourne, K. H. Fantes and S. Peat, *J. Chem. Soc.*, 1109 (1949).

(7) S. A. Barker, E. S. Bourne and I. A. Wilkinson, *ibid.*, 3027 (1950).

(8) W. Z. Hassid and R. M. McCready, THIS JOURNAL, **65**, 1157 (1943).

(9) A. L. Potter and W. Z. Hassid, *ibid.*, **70**, 3774 (1948).

(10) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *ibid.*, **73**, 3916 (1951).

(11) F. C. Cleveland and R. W. Kerr, *Cereal Chem.*, **28**, 133 (1948).

(12) R. W. Kerr, *Nature*, **164**, 757 (1949).

(13) R. W. Kerr and F. C. Cleveland, THIS JOURNAL, **73**, 2421 (1951).

(14) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *ibid.*, **73**, 111 (1951).

162), determined by osmometry; molarity = grams per liter/$DP_n \times 162$. One gram of corn A-fraction, or an equivalent weight of other fractions, was stirred at room temperature with 5 ml. of 2 N KOH until solution appeared to be complete. Then 15 ml. of water was added and the solution was stirred for 30 minutes. The solution was diluted and adjusted to the desired pH with HCl, immediately brought up to volume and placed in a water-bath at 45°. At zero time, an amount of enzyme solution was added which made the final volume 250 ml. A pH of 5.0 was used for amylo-glucosidase and a pH of 5.9 for the β-amylase. The exception to this procedure was for tapioca A-fraction which, because of the exceptionally high viscosity, required 2.5 times the amount of alkali and water to effect solution, although the final volume was also 250 ml. The longer hydrolyses were protected with toluene and the pH values were checked periodically.

At intervals aliquots were removed and reducing substance determined by alkaline ferricyanide oxidation. The results were calculated either as glucose or maltose. It has appeared from previous work that the only sugars produced from amylose by these enzymes are glucose by amylo-glucosidase and maltose by β-amylase.[10,13] A correction was applied for the slight reducing value of the unreacted substrate.

Osmotic Pressures.—These were determined on the triacetates of the amyloses in chloroform solution at 30° in a Fuoss-Meade cell by procedures outlined previously.[3,14] Acetates were prepared from amylose dispersed in formamide using acetic anhydride with pyridine as catalyst. Exceptions were that tapioca and potato A-fractions were dispersed in aqueous pyridine. However, identical DP_n values were obtained from potato A-fraction acetates prepared by the two methods. The osmotic pressure values for acetates of the several amyloses used in this study are shown in Fig. 1. The calculated DP_n values are shown in Table IV, together with the yield and iodine affinity of the amyloses.

Periodate Oxidation.—In order to show further that our linear standard, corn crystalline amylose, is composed substantially of unbranched molecules, oxidation with sodium metaperiodate and titration of formic acid produced was performed by a modification of procedures used by Potter and Hassid.[9] One-half gram samples were moistened with 3 ml. of petroleum ether in 125-ml. flasks and suspended in 10 ml. of 3% sodium chloride at 3°. At zero time, 10 ml. of 0.31 M recrystallized sodium metaperiodate was added and the flasks were shaken on a Fisher Gyrosolver at 3° in the dark. A glass marble was added to facilitate agitation. At the end of stated times the entire contents of each flask was titrated with 0.01 N barium hydroxide after first having eliminated the unused periodate with 1 ml. of redistilled ethylene glycol. A correction was applied for the slight titratable acidity of the amylose and for the blank composed of the periodate, sodium chloride and petroleum ether plus the final addition of ethylene glycol, all in the amounts used in the oxidation procedure. Average, corrected values were: 3.18, 3.49, 3.48, 3.58, 3.67 and 4.20 ml. of 0.01 N barium hydroxide, respectively, for oxidation times of 8, 16, 20, 24, 28 and 40 hr.

Titration was performed electrometrically. It was found that when 7 ml. of 0.01 N formic acid was titrated electrometrically with barium hydroxide in a volume of approximately 40 to 50 ml. at 25°, the mid-point in the titration curve was pH 7.1. Thus as claimed by several investigators[15] the use of methyl red as employed by Potter and Hassid[9] gives titration values which are too low. However, teh use of phenolphthalein in formic acid titrations[15] may give values which are too high. Accordingly, in these periodate oxidation studies, titration was made electrometrically to a pH value of 7.1.

A value of 3.60 ± 0.10 ml. of 0.01 N formic acid was estimated to have been produced from 0.5 g. of the amylose during the primary phase of the reaction. This corresponds to one non-reducing terminal per 255 ± 15 glucose units. The DP_n determined by osmometry was 235. Therefore, the results of periodate oxidation indicate that the corn crystalline amylose is composed substantially of non-branched molecules.

(15) See for example, Jeanes and Wilham, THIS JOURNAL, **72**, 2655 (1950); Meyer and Rathgeb, *Helv. Chim. Acta*, **32**, 1102 (1949).

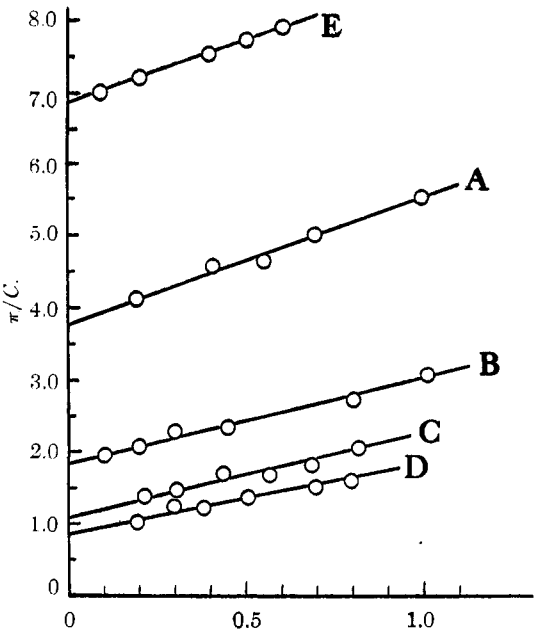

Fig. 1.—Osmotic pressures of amylose acetates in chloroform: A, corn crystalline amylose; B, corn A-fraction; C, potato A-fraction; D, tapioca A-fraction; E, A-fraction from 90-F corn starch.

Discussion of Results

The hydrolyses of equimolar (5.15×10^{-5} M) solutions of several amylose samples by amylo-glucosidase are compared in Table I. Although the three corn amylose samples, with DP_n values between 135 and 480, were hydrolyzed to produce glucose at approximately the same rate, the potato amylose sample gave almost double this amount of glucose per unit time over the early stages of hydrolysis and the tapioca amylose sample almost triple. A high rate of glucose production was maintained in the hydrolysis of potato amylose for a relatively long period whereas in the hydrolysis of tapioca amylose the rate declined and soon fell off to a value below that for the potato amylose.

Since it has been shown that corn crystalline amylose is linear, we may conclude first, that the butanol recrystallized corn A-fraction is very nearly linear also. We may conclude furthermore from this experiment that there is an average of approxi-

TABLE I

HYDROLYSIS OF 5.15×10^{-5} M SOLUTIONS OF AMYLOSES WITH AMYLO-GLUCOSIDASE CALCULATED AS MG. GLUCOSE PRODUCED PER 100 ML.

Time, hr.	Corn, 90-F A-fraction	Corn crystalline amylose	Corn A-fraction	Potato A-fraction	Tapioca A fraction
3	8.1	6.1	7.3	12.3	19.1
4.5	14.3	10.3	12.3	19.1	25.3
21	46.9	43.0	46.7	80.8	84.0
45	80.1	85.0	81.8	135.8	138.4
69	Retrograded		117.9	182.5	176.2
120		157.4	159.6	268.0	225.4
144		Retrograded	211.4	302.0	253.7
168			223.8	336.7	278.0
239			274.3	428.0	347.8

TABLE II

HYDROLYSIS OF 5.15×10^{-5} M SOLUTIONS OF AMYLOSES WITH CRYSTALLINE β-AMYLASE

Time, min.	Corn, 90-F, A		Corn crystalline		Corn A		Potato A		Tapioca A	
	Amylose hydrolyzed, %	Maltose, mg./100 ml.	Amylose hydrolyzed, %	Maltose, mg./100 ml.	Amylose hydrolyzed, %	Maltose, mg./100 ml.	Amylose hydrolyzed, %	Maltose, mg./100 ml.	Amylose hydrolyzed, %	Maltose, mg./100 ml.
5	21.1	24.8	10.3	20.8	6.4	26.8	9.7	72.0	9.0	82.4
10	31.9	37.6	17.5	35.2	8.7	35.5	15.3	113.6	12.1	111.2
15	39.4	46.4	21.9	44.0	10.2	42.9	18.6	137.6	13.0	120.0
20	42.3	50.0	25.8	52.0	11.1	47.0	21.5	160.0	13.9	128.0
30	48.8	57.6	30.2	60.8	12.4	52.1	27.6	186.4	15.5	142.4

mately two non-reducing terminals in the potato amylose preparation and that the branches are relatively long whereas there is an average of nearly three non-reducing terminals per molecule in the tapioca amylose sample and the branches are relatively shorter.

Table II shows the hydrolysis of equimolar (5.15×10^{-5} M) solutions of these same amylose samples by crystalline β-amylase, giving the mg. of maltose per 100 ml. produced per unit of time. The rates at which maltose was produced from the three corn amylose samples are very nearly the same and again it would appear that all of the corn amylose samples are composed almost entirely of linear molecules. However, maltose production from the potato amylose proceeded at a rate more than double that for the corn amylose samples, indeed almost three times as fast initially, and from the tapioca amylose sample, somewhat more than triple the rate for corn amylose. Again a high rate for the potato amylose hydrolysis was sustained for a relatively long time whereas the rate for tapioca, initially higher, rapidly declined to a value less than that for the potato amylose; from this it may also be inferred that the branches in the potato amylose are longer than those in tapioca.

Some difficulties worthy of note were experienced in performing these enzymic end-group analyses. The relatively short molecules of the 90-F, A-fraction showed a tendency to retrograde even at the high dilutions used and at the high pH levels maintained in these experiments. This was particularly noticeable in the hydrolysis with amyloglucosidase, the solution being visibly retrograded after 45 hours. Corn crystalline amylose did not show visible signs of aggregation until after about 120 hours. It should be borne in mind that in enzymic hydrolyses, the state of solution of the amylose will affect the results obtained. Our conclusions are based on the assumption that the amylose samples were molecularly dispersed during the early phases of hydrolysis, at least, so that the rates of sugar production during this interval were dependent on molecular structure. In the hydrolysis of solutions of lower solids content, the crystalline β-amylase was surprising labile, particularly with other conditions such as pH 5.9 and 45°, imposed in order to minimize retrogradation. This lability very likely accounts for the regular and pronounced decline in monomolecular reaction constants which occurred with time even in the hydrolysis of corn crystalline amylose. Although the hydrolysis of the 90-F, A-fraction and corn crystalline amylose virtually ceased at about 45 minutes, the enzyme apparently had more protection in the potato and tapioca hydrolyses wherein the carbohydrate concentrations on a weight per volume basis were of a much higher order. Here, enzyme activity was apparent even after 5 or 6 hours.

Because of the last mentioned difficulty, the hydrolyses of corn crystalline amylose, corn A- and potato A-fractions were repeated with the following variations. The solutions were maintained at a pH level of 5.25 by inclusion of sufficient sodium acetate buffer to make the solution 0.1 M with respect to the salt. Instead of crystalline β-amylase, 0.33 ml. of a liquid β-amylase preparation, made from barley,[16] was added per 100 ml. of hydrolysis solution (see Table III).

TABLE III

HYDROLYSIS OF STARCH FRACTIONS WITH BARLEY β-AMYLASE

Amyloses, 5.15×10^{-5} M concentration; potato amylopectin, 0.2500 g. per 100 ml.; hydrolysis products shown as mg. maltose per 100 ml.

Hydrolysis time, min.	Corn crystalline amylose	Corn A-fraction	Potato A-fraction	Potato amylopectin
5	23.7	22.1	48.2	41.4
10	43.0	39.4	80.8	71.9
15	59.7	52.9	113.3	91.1
20	71.9	65.9	138.1	107.1
30	92.0	82.0	178.3	120.2
45	109.1	105.3	224.2	127.2
90	146.2	149.3	315.5	133.8
180	...	203.2	395.2	135.5
300	...	244.9	462.9	138.6

Comparing the results of the several enzymic hydrolyses, we may conclude that there is an average of approximately 2 to 3 non-reducing terminals per molecule in the potato amylose and approximately 3 to 4 in the tapioca samples. One may reason, furthermore, that inasmuch as some of the shorter amylose molecules, particularly in the case of potato[17] appear to be non-branched, some of the larger potato amylose molecules may have as many as 4, and some of the tapioca amylose molecules as many as 5 or 6 branches.

Our enzymic end-group methods do not possess one of the limitations of other end-group methods. This limitation is that, from the analytical results alone, one is unable to distinguish between a sample of amylose in which the molecules are slightly branched and a sample that is a mixture of non-balanced chains and highly branched amylopectin impurities. If we assume that in the 5.15×10^{-5}

(16) R. W. Kerr, O. R. Trubell and G. M. Severson, *Cereal Chem.*, **19**, 64 (1942).

(17) Potato crystalline amylose, prepared by hot water leaching of the starch and crystallization of the extracted solids with butanol, was found to have a β-amylase conversion limit of 97% and an $[\eta]$ of 1.12.

M solution of the potato A-fraction (see Table III) there was slightly less than 5.15×10^{-5} mole per liter ($0.7083 - x$ gram per 100 ml.) of non-branched molecules of about DP_n 850 and approximately 5.15×10^{-5} molar equivalent per liter (or 0.0183 g. per 100 ml., which equals x) of potato amylopectin with a commonly reported "chain length" of 22, then this amylose sample containing less than 3% amylopectin impurity would show apparently 2 non-reducing terminals per molecule by both the periodate and methylation end group methods. In the very earliest stages of the enzymic hydrolysis, it would be anticipated that the rate of maltose production from this mixture also would approach double the value for a non-branched amylose of DP_n 850. However, certainly by the time the first sample was taken (at 5 minutes) the amount of maltose produced would not be double the value for the non-branched amylose. Bearing in mind that amylopectins are at best only about 60% hydrolyzed at completion of the reaction with β-amylase, then the maximum yield of maltose from 0.0183 g. of amylopectin would be 0.0116 g. or somewhat less than one-fourth of the maltose actually found. With increasing reaction time the discrepancy between calculated and found values for maltose would become larger.

At a reaction time of 90 minutes, if we assume that half of the maltose found was due to an unbranched fraction with DP_n 850, then of the original 0.7083 g./100 ml. of substrate

$$\frac{0.3155}{2} \times \frac{1}{1.05} \times \frac{1}{0.6} = 0.250 \text{ g.}$$

would have been amylopectin, or we are led to the very unlikely conclusion that the original potato A-fraction contained some 35% amylopectin impurity.

Table III shows the amounts of maltose produced at various reaction times from potato amylopectin at a concentration of 0.250 g./100 ml., pH 5.25 and 45°, using the same concentration of barley β-amylase employed with the amyloses. This is approximately the concentration of amylopectin which should have been present in the potato A-fraction sample to obtain the amount of maltose found at 90 minutes if potato amylose molecules are non-branched and of the average size indicated. As would be expected from the larger number of end-groups per unit weight, and as demonstrated by Hopkins and Jha[18] the initial rate of maltose production from a given weight concentration of amylopectin is many times greater than the rate from amylose under the same experimental conditions. Therefore, because of the high rate at which maltose is produced during the early stages of amylopectin hydrolysis followed by a decline in rate to zero as branched points are approached, the presence of highly branched material in an amylose sample would lead to a distorted maltose rate curve for the mixture so that it would be quite dissimilar in shape to the rate curve for a non-branched sample or one that was composed of molecules with relatively few long branches.

From consideration of the kinetics of enzymic reactions used in the end-group analyses, we conclude that both potato and tapioca amyloses contain a large proportion of slightly branched molecules rather than that these samples contain a small amount of highly branched amylopectin either in stable union with non-branched molecules or as a minor impurity.

Because of the very high limits of hydrolysis to maltose frequently reported for these slightly branched amyloses, it may be concluded that their structure is a V or a Y, or possibly a star-like shape, with relatively a small number of glucose units in the tail which terminates in the reducing end-group.

The corn amylose (A-fraction) used in this study was a butanol recrystallized product representing only about 20% by weight of the starch. Although this fraction is shown to consist largely of non-branched chains, there is, quite possibly, another 10% or more of the starch, precipitable with Pentasol and other amylose complexing agents, which is slightly branched.[19-23] Also, it should be pointed out that the potato A-fraction used in this study was prepared from Idaho potato starch and was twice recrystallized from aqueous butanol. More recently, a butanol, double recrystallized A-fraction was prepared from Maine potato starch in 20.3% yield with a DP_n of 970, which showed an average of only 1.3 non-reducing terminals per molecule by the β-amylase end-group method. This suggests that some potato starches, depending on source, contain a larger proportion than others of non-branched molecules and this may possibly account, in part at least, for discrepancies between several reports concerning the composition of this starch and the structure of its amylose fraction.[6-9,23,24]

TABLE IV

DATA FOR A-FRACTIONS AND FOR CORN CRYSTALLINE AMYLOSE

A-Fraction or amylose	Yield from starch, %	Iodine affinity	Osmotic pressure, DP_n
Corn	21.0	19.2	480
Corn, 90-F	12.0	16.3	135
Corn crystalline amylose	5.0	20.1	235
Potato (Idaho)	20.0	18.7	850
Tapioca (Dominican R.)	15.4	18.9	1050

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(18) R. H. Hopkins and B. K. Jha, *Biochem. J.*, **46**, 319 (1950).

(19) R. W. Kerr and O. R. Trubell, *Paper Trade J.*, **117**, No. 15, 25 (1943).

(20) W. N. Haworth, S. Peat and P. E. Sargott, *Nature*, **157**, 19 (1946).

(21) J. E. Hodge, E. Montgomery and G. E. Hilbert, *Cereal Chem.* **25**, 19 (1948).

(22) S. Lansky, M. Kooi and T. J. Schoch, THIS JOURNAL, **71**, 4066 (1949).

(23) A. L. Potter and W. Z. Hassid, *ibid.*, **73**, 593 (1951).

(24) S. Peat, W. J. Whelan and S. J. Pirt, *Nature*, **164**, 490 (1949).