[CONTRIBUTION FROM THE GEORGE M. MOFFETT LABORATORIES, CORN PRODUCTS REFINING COMPANY]

The Action of Crystalline β-Amylase on Corn Crystalline Amylose

BY RALPH W. KERR AND FRANK C. CLEVELAND

The object of this investigation was to study the action of a crystalline β-amylase on a highly purified amylose in order to decide which of two possible mechanisms proposed, "single-chain" or "multiple-chain" action, is the more probable one. The hydrolysis was interrupted at approximately the mid-point. The high polymer fraction of the hydrolysate was separated by alcoholic fractionation and crystallized as platelets from aqueous butanol. Osmotic pressure measurements, ferricyanide oxidation and intrinsic viscosity determinations showed it to be the same as the original amylose in average chain length and in chain length distribution. The low polymer fraction, estimated as maltose by ferricyanide oxidation, was analyzed by paper chromatography and found to consist only of maltose with possibly trace amounts of maltotriose.

Quantitative determination of the hydrolysate as maltose and residual amylose molecules having a chain length identical to the original substrate, strongly favors the theory that β-amylase operates by a "single-chain" mechanism. These results disprove the theory that at any ratio of enzyme to substrate, all linear chains are attacked simultaneously.

In a previous report[1] it was shown that the high polymer residue recovered from the hydrolysate at different times during the hydrolysis of corn amylose (corn A-fraction, the butanol-precipitable fraction of corn starch) with β-amylase, possessed physical and chemical properties very nearly the same as those of the original amylose. The results suggested the theory that when the enzyme attacked an amylose molecule and split off maltose groups, it hydrolyzed this molecule completely before making an attack on another molecule.

Recently, Hopkins and Jelinek[2] and Bourne and Whelan[3] have questioned this "single-chain" theory; they favor the view that all amylose chains are attacked simultaneously by the β-amylase. From earlier studies, Hopkins, Jelinek and Harrison[4] concluded that longer chains of amylose are hydrolyzed at a more rapid rate than shorter ones and that this explained, on the basis of the multi-chain hydrolysis theory, why the hydrolysis reaction exhibits an abnormal reduction in rate with time, and explained their observation that residues recovered from hydrolysates are more difficult to hydrolyze with β-amylase than the original sample. They also found that the wave length for peak light absorption by the iodine complex shifted toward the red as hydrolysis progressed, in support of the multi-chain hydrolysis theory, whereas Swanson[5] found the wave length of peak absorption to remain constant for corn A-fraction, in support of the single chain hydrolysis theory. In the more recent work referred to above, Hopkins and Jelinek[2] found that an acid-hydrolyzed potato amylose sample of *DP* 20 and the parent potato amylose showed about the same maximal velocities when hydrolyzed by β-amylase and this result was taken to support the view that the enzyme will attack all amylose molecules simultaneously and indiscriminately.

In one of our reports,[1] a comparison of the initial rate constants for corn crystalline amylose of DP_n 225 and for corn A-fraction of DP_n 455 favored the assumption that the shorter chains were hydrolyzed at a measurably greater rate when molar concentrations were taken into consideration although it was pointed out at that time, however, that the apparent lesser rate for the corn A-fraction could be due to the presence of some slightly branched molecules which may possibly exist in the whole amylose fraction. It was pointed out[6] furthermore that if these amylose preparations do contain some branched molecules, then as hydrolysis progressed there would be an accumulation of branched residues which would be hydrolyzed with great difficulty, if at all. Thus, it could very well be that some of the uncertainty which exists regarding the theory of the action of β-amylase may be due to the use of amylose samples which do not consist entirely of unbranched chains. Possibly also, the enzyme preparations used in some of the past work have not been of the highest purity obtainable. Accordingly, our hydrolysis studies have been repeated using corn crystalline amylose, prepared by hot water leaching of starch granules and butanol precipitation of the extract, followed by two additional recrystallizations of the amylose from aqueous butanol. This procedure was found to give amylose preparations of the highest degree of linearity as measured by β-amylase conversion limits[7] and this conclusion has been supported by iodometric analyses.[8,9]

The enzyme used was four times recrystallized β-amylase, prepared by Dr. A. K. Balls from sweet potatoes.[10]

Experimental

Hydrolysis of Amylose.—Corn crystalline amylose, twice recrystallized from aqueous butanol, was prepared by the method of Kerr and Severson.[7] Characteristics of the product are shown in Table II.

Fifteen grams of the amylose, containing 7.8% volatile material, was dissolved in 75 ml. of 2 *N* potassium hydroxide solution at 25°. Within 2 hours the digest was diluted with 2 liters of water, adjusted to *p*H 6.0 with dilute hydrochloric acid and brought to a volume of 3750 ml. at 47°. Immediately, a very small amount, about 0.05 ml. of a suspension of β-amylase crystals in saturated $(NH_4)_2SO_4$ solution, prepared by Dr. Balls, was added and the temperature maintained at 47° with vigorous stirring. Aliquots were removed from time to time and tested for *p*H value and maltose equivalent. The *p*H value was found

(1) F. C. Cleveland and R. W. Kerr, Am. Assoc. of Cereal Chemists, Abstracts of Papers for the 32nd Annual Meeting at Kansas City, Mo., May, 1947, p. 24. See also *Cereal Chem.*, **25**, 133 (1948).

(2) R. H. Hopkins and B. Jelinek, *Nature*, **164**, 955 (1949).

(3) E. J. Bourne and W. J. Whelan, *ibid.*, **166**, 258 (1950).

(4) R. H. Hopkins, B. Jelinek and L. E. Harrison, *Biochem. J.*, **43**, 32 (1948).

(5) M. A. Swanson, *J. Biol. Chem.*, **172**, 805, 825 (1948).

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

(7) R. W. Kerr and G. M. Severson, THIS JOURNAL, **65**, 195 (1943). See also, R. W. Kerr, in "Chemistry and Industry of Starch," Academic Press, New York, N. Y., 1944, p. 145.

(8) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

(9) K. G. Krishnaswamy and A. Sreenivasan, *J. Biol. Chem.*, **176**, 1253 (1948).

(10) A. K. Balls, R. R. Thompson and M. K. Walden, *ibid.*, **173**, 9 (1948).

not to change from 6.0 during the period of the experiment. Maltose was determined by ferricyanide oxidation, essentially as outlined by Gore and Steele[11] and as used in our earlier work.[1] Recrystallized maltose, assayed for dry substance maltose content by the well known iodine oxidation method of Kline and Acree, was used as a standard.

Deducting volatiles from the weight of substrate used and deducting the dry substance removed in the aliquots for testing purposes, actually 13.68 g. of amylose remained in the conversion liquors.

At a reaction time of 15 minutes, an estimated 20.4% of the amylose had been hydrolyzed to maltose; at 45 minutes, 43.3%. The remaining liquors were heated to boiling. Analysis of a sample cooled to 47° showed that 47.3% of the amylose had been hydrolyzed to maltose. When the conversion liquor had been cooled to about 90°, 750 ml. of butanol and 3700 ml. of methanol were added slowly with stirring. On standing overnight at room temperature a non-gummy precipitate formed, which could readily be centrifuged and resuspended in a very fine state in a mixture of 150 ml. of water, 30 ml. of butanol and 150 ml. of methanol. The *p*H of the centrifugate was 6.3. The washed product was recentrifuged and completely dissolved in 600 ml. of boiling water. This was accomplished without any retrogradation by forming a slurry of the product with a part of the water, which had been saturated with butanol, and adding this slurry with stirring to the balance of the water which was kept vigorously boiling. Then 100 ml. of butanol was added and the solution cooled slowly to room temperature. After standing for 3 days the liquors containing a crystal growth of platelets was refrigerated at 3° for 24 hours. The crystals were centrifuged, resuspended in 250 ml. of water saturated with butanol and recentrifuged. The product was washed 5 times with 300-ml. portions of methanol, each wash extending over a period of 24 hours. The washed crystals were freed of methanol in a vacuum desiccator at room temperature. Yield of crystals, dry basis, was 7.038 g., or 51.4% of the original amylose, as shown in Table I.

Osmotic Pressure Measurements.—Acetates were prepared by the formamide dispersion method and osmotic pressures of the acetates were determined in chloroform essentially as described in our previous studies.[1,12,13] Yields of triacetates, after two acetylations were approximately 95%.

Chromatographic Analyses.—The low polymer fraction of the hydrolysate, consisting of the first water–butanol–methanol centrifugate and the washings from the first high polymer precipitate, were concentrated under reduced pressure at 30° to a volume of one liter. Droplets containing 0.068, 0.136 and 0.272 mg. of dissolved solids were applied to strips of Schleicher and Schuell paper (No. 507), 7.5 mm. wide and 584 mm. long at a distance of 100 mm. from one end. These droplets, applied from a micropipet, spread out into a band either side of the origin totalling 25 mm. After drying, the ends were immersed in a solvent mixture consisting of butanol (6 parts), pyridine (4 parts) and water (3 parts), and suspended in a closed chamber at room temperature for 24 hours. At this time the solvent boundary was noted and the strips removed and air-dried. After spraying the strips with a solution consisting of one gram of 3,5-dinitrosalicylic acid in 100 ml. of *N* potassium hydroxide, air-drying again and heating in an oven at 110° for 15 min., the positions of the red-brown colored bands on the paper strips were determined. From these, the per cent. migration (R_F values) of the solute in the bands was calculated in reference to the solvent.

Only one band developed with the low polymer fraction. This was approximately the width of the original droplet and had an average R_F value of 0.235. No material which develops color with dinitrosalicylate was left at the origin. The intensity of color in the band varied with the amount of solute applied.

The R_F values were similarly determined for 0.05 and 0.1 mg. of glucose, maltose (using amounts also up to 0.25 mg.), amyloheptaose and amylodextrin. These were 0.333, 0.239, 0.041 and 0, respectively. The amylodextrin, an acid-hydrolyzed, linear potato fraction, having a *DP* of 25 by reducing methods, failed to move from the position of its original band at the origin. Both the amylodextrin and amyloheptaose bands were clearly discernible using amounts as low as 0.05 to 1.0 mg.

The low polymer fraction of the hydrolysate was then compared against maltose, using massive applications of 0.7 mg. dry solids, by further concentration of the hydrolysate to 100 ml., after an ion-exchange treatment to remove the potassium chloride present. Again only one band developed at approximately the position for maltose. The remainder of the paper showed only the yellow color of the dinitrosalicylate, both at the origin as well as throughout the intermediate portions. R_F values, run in duplicate, were hydrolysate, 0.263 and 0.266; maltose, 0.263 and 0.265.

From the R_F values as well as the intensities of color in the bands, it may be concluded that the low polymer fraction consisted of maltose, with possibly trace amounts of maltotriose; no sugars of greater chain length could be detected.

The amyloheptaose and amylodextrin samples were kindly supplied by Dr. Dexter French.

The methods for the determination of iodine affinities, ferricyanide numbers and intrinsic viscosities have been outlined.[14]

Discussion of Results

Table I summarizes the distribution in yields when the crystalline amylose was treated with crystalline β-amylase, the hydrolysis interrupted when 47.3% by weight of the amylose had been converted to maltose and the residual high polymer fraction in the hydrolysate isolated by a butanol crystallization technique.

TABLE I

DISTRIBUTION IN YIELDS FROM THE HYDROLYSIS OF AMYLOSE WITH β-AMYLASE

Material	Amount in grams	Yield as % of original amylose
Original amylose	13.680	
Maltose		47.3
Butanol recrystallized residue	7.038	51.4
Butanol–water mother liquor	0.1304	0.95
Butanol–water wash	0.0515	0.37

The maltose reported was determined by reduction methods. That the reducing material was actually maltose was confirmed by chromatographic analysis of the hydrolysate after precipitation of the high polymer fraction. Except for possible trace amounts of maltotriose, chain lengths intermediate to maltose and amylose were absent.

Out of a possible 52.7% of the original amylose not converted to maltose, 51.4% was recovered and isolated as a butanol crystallizable product in

TABLE II

PROPERTIES OF ORIGINAL AMYLOSE AND RESIDUE RECOVERED AFTER HYDROLYSIS WITH β-AMYLASE

	Original amylose	Recovered amylose residue
DP_n	235	235
Ferricyanide no.	3.43	3.53
Intrinsic viscosity in *N* KOH at 35°	0.46	0.48
Iodine affinity	20.1[a]	20.4[a]

[a] Iodine affinity values, calculated by extrapolation to zero free iodine concentration are slightly lower than iodine absorption percentages, calculated from the point of intersection of tangents to the iodine titration curve.

(11) H. C. Gore and H. K. Steele, *Ind. Eng. Chem., Anal. Ed.*, **7**, 324 (1935).

(12) R. W. Kerr and F. C. Cleveland, THIS JOURNAL, **71**, 3455 (1949).

(13) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, "The Molecular Magnitude of Amylopectin," *ibid.*, **73**, 111 (1951).

(14) R. W. Kerr in "Chemistry and Industry of Starch," 2nd ed., Academic Press, New York, N. Y., 1950, pp. 674–681.

the form of platelets, quite similar to the original amylose. Other characteristics of the recovered amylose residue are compared against those of the original amylose in Table II.

Both the osmotic pressure results and the ferricyanide numbers indicate very little difference, if any, between the number average molecular weights of the original corn crystalline amylose and the residual amylose recovered when nearly half of the amylose sample had been hydrolyzed to maltose. From the osmotic pressure data shown in Table III, and from the extrapolation of these data given in Fig. 1, it will be seen that the osmotic pressure–concentration relationship of the recovered amylose acetate is substantially identical to that of the original amylose acetate and accordingly a DP_n value of 235 was calculated for both samples. A very slight though measurable increase in viscosity in favor of the recovered amylose may indicate a minor change in chain length distribution. However, these differences are so small that for all practical purposes one may say that the recovered amylose is identical to the original sample both in average chain length and in chain length distribution.

TABLE III

OSMOTIC PRESSURES IN CHLOROFORM FOR ACETATES OF CORN CRYSTALLINE AMYLOSE AND THE RESIDUE AFTER β-AMYLOLYSIS

Concentration, C, in g. per 100 ml.	Pressure, P, in cm. chloroform	π, g. per sq. cm.	π/C
Original amylose (calcd. DP_n 235)			
0.0	(by extrapolation)		3.75
.1970	0.55	0.809	4.107
.4068	1.26	1.852	4.553
.5575	1.75	2.573	4.614
.6934	2.36	3.469	5.003
.9915	3.73	5.483	5.530
Residual amylose (calcd. DP_n 235)			
0.0	(by extrapolation)		3.75
.2110	0.58	0.853	4.041
.4110	1.27	1.867	4.540
.5522	1.78	2.617	4.739
.7074	2.45	3.602	5.094
.9849	3.76	5.527	5.612

The iodine affinity values indicate, particularly in view of the relatively low DP values, a high degree of linearity in the molecules making up the samples.

Table I shows that of 13.680 g. of original amylose 98.7% could be accounted for after the hydrolysis by maltose production or as recovered amylose, leaving only 1.3% (or 178 mg.) which could possibly be amylose chains partially hydrolyzed to some intermediate length. From this unaccounted 1.3%, moreover, there must be subtracted errors in measurements, unknown mechanical losses in the several isolation procedures and the loss due to solubility of the amylose–butanol complex in aqueous butanol. The latter is appreciable when solubility is approached from supersaturation and the result varies with precipitation conditions, such as time, temperature and agitation. Under the conditions we have used to isolate

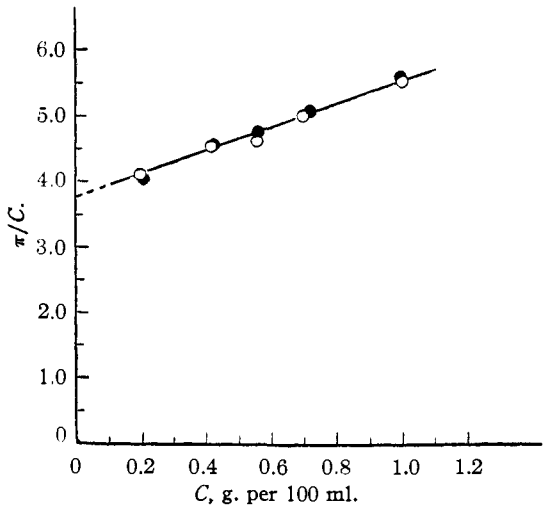

Fig. 1.—Osmotic pressures in chloroform, acetates of corn crystalline amylose and of residue from β-amylolysis: O, original amylose acetate; ●, residual amylose acetate.

the residue, solubility of the corn crystalline amylose–butanol complex is of the order of 10 mg. per 100 ml. and this accounts for a total of about 0.7% loss, leaving less than 1% to be attributed to mechanical losses and errors in weighing or measuring volumes.

Therefore, it may be concluded that, as one uses amylose preparations which approach perfection in respect to linearity of the constituent molecules, for hydrolysis by β-amylase, it becomes increasingly easier to demonstrate the absence of products intermediate to amylose and maltose during the hydrolysis period, except possibly during the last stages when the number of remaining amylose molecules approaches the number of available enzyme molecules. These results favor the view that, after making contact, an enzyme molecule completely hydrolyzes an amylose molecule before attacking another; they are incompatible with the theory that all amylose molecules are simultaneously and progressively shortened in length as hydrolysis proceeds.

It is of considerable interest to note in this connection that in a study of the hydrolysis of the seven membered chain, amyloheptaose, by β-amylase, French and co-workers[15] very recently found no products in the hydrolysates other than maltose, maltotriose and unconverted amyloheptaose. Even after 55% of the sample had been hydrolyzed, no amylopentaose could be found in the hydrolysis liquors. Since the triose is an end product (in considerable yield in this case[16]) and could not be further hydrolyzed by the enzyme and amylopentaose is the only possible intermediate product, these workers concluded that β-amylase hydrolyzes each molecular chain before attacking the next.

Under unfavorable conditions of temperature or pH value, such as 70° or pH 10, which normally

(15) D. French, M. L. Levine, J. H. Pazur and E. Norberg, THIS JOURNAL, 72, 1746 (1950).

(16) In the hydrolysis of chain lengths of DP_n 235, however, assuming an even distribution between odd and even numbered chain lengths, the molar ratio of the triose to maltose on hydrolysis in this case should be of the order of only (1:235).

lead to inactivation of the enzyme, French and co-workers[17] found measurable quantities of amylopentaose. It is possible that some enzyme molecules became inactivated sufficiently so that, because of their changed physical state, they were unable to form a complex with the entire substrate molecule.

French and co-workers explain the single chain hydrolysis theory by assuming that intermediate products of hydrolysis do not diffuse from the locality of their production with sufficient rapidity to escape further attack by the enzyme, whereas we have been led to speculate that complex formation between the enzyme and substrate involves a considerably longer portion of the substrate chain than the two terminal glucose units, possibly the entire length of some linear amylose molecules, and that virtually complete hydrolysis ensues at the instant of dissociation of the complex.

(17) D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 1866 (1950).

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[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

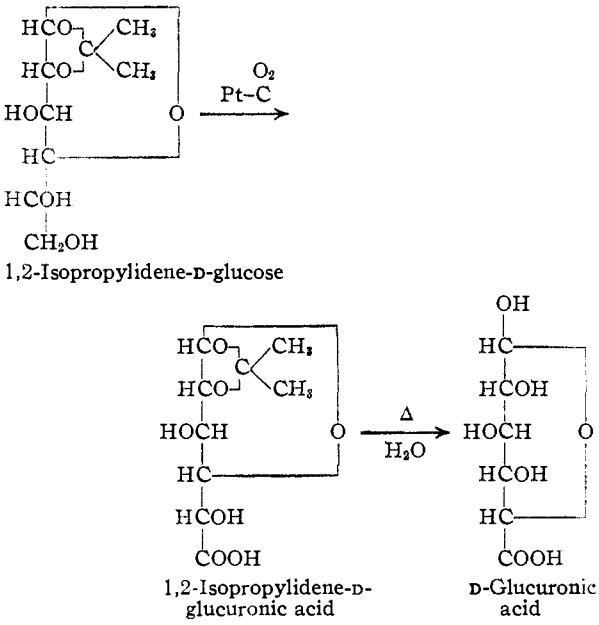
A Practical Synthesis of D-Glucuronic Acid through the Catalytic Oxidation of 1,2-Isopropylidene-D-glucose[2]

BY C. L. MEHLTRETTER, B. H. ALEXANDER, R. L. MELLIES AND C. E. RIST

A practical synthesis of D-glucuronic acid has been developed in which 1,2-isopropylidene-D-glucose is catalytically oxidized to 1,2-isopropylidene-D-glucuronic acid in a yield of 50–60% of theory using platinum–carbon catalyst and air. By simple hydrolysis 1,2-isopropylidene-D-glucuronic acid is nearly quantitatively converted to D-glucuronic acid. A key step in the synthesis is the separation of 1,2-isopropylidene-D-glucuronic acid from contaminating by-products in the oxidation mixture as the insoluble calcium salt. The barium and sodium salts of 1,2-isopropylidene-D-glucuronic acid also have been prepared. To facilitate this synthesis an improved procedure has been devised for the preparation of 1,2-isopropylidene-D-glucose in aqueous solution in 93% yield. A small quantity of by-product isolated was shown to be isopropylidene-bis-(diisopropylidene-D-glucose). Its structure, however, has not yet been completely established.

The oxidation of carbohydrate substances to sugar acids with oxygen in the presence of noble metal catalysts has been shown to be preferential for aldehyde and primary alcohol groups. Busch[3] quantitatively converted D-glucose to D-gluconic acid with air in the presence of palladium–calcium sulfate catalyst. With the more active platinum–activated carbon catalyst Heyns[4] was able to oxidize L-sorbose to 2-keto-L-gulonic acid under neutral or slightly alkaline conditions. Similarly Trenner[5] converted 2,3-isopropylidene-L-sorbose in aqueous solution to 2,3-isopropylidene-2,5-anhydro-L-gulosaccharic acid. More recently[6] D-glucose has been catalytically oxidized to D-glucosaccharic acid in 54% yield by use of platinized–activated carbon and air, a result contradictory to the findings of Heyns and Heinemann.[7]

This procedure has now been successfully used as the basis of a synthesis of D-glucuronic acid from 1,2-isopropylidene-D-glucose in an over-all yield of at least 43%. Optimum conditions have been found for the catalytic oxidation of 1,2-isopropylidene-D-glucose to 1,2-isopropylidene-D-glucuronic acid. The latter substance was best isolated as the calcium salt from which a mixture of D-glucuronic acid and D-glucuronolactone was obtained in 85% yield by acid hydrolysis. The barium and sodium salts of 1,2-isopropylidene-D-glucuronic acid have also been prepared. The sequence of reactions may be illustrated as



The results of several experiments are given in Table I to show the effect of temperature, air velocity and rate of stirring on the reaction time and on the yield of 1,2-isopropylidene-D-glucuronic acid.

The temperature of the reaction appeared to be optimum at about 50°. Lowering the temperature to 28° decreased the rate of oxidation below the limit of practicability. On the other hand, a temperature of 100°, although it hastened the reaction, caused enough decomposition to reduce

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) This paper was presented before the Division of Sugar Chemistry and Technology at the 118th Meeting of the American Chemical Society, Chicago, Illinois, September 3–8, 1950.

(3) M. Busch, German Patent 702,729 (1941).

(4) K. Heyns, *Ann.*, **558**, 177 (1947); O. Dalmer and K. Heyns, U. S. Patent 2,190,377 (1940).

(5) N. R. Trenner, U. S. Patent 2,428,438 (1947).

(6) C. L. Mehltretter, C. E. Rist and B. H. Alexander, U. S. Patent 2,472,168 (1949).

(7) K. Heyns and R. Heinemann, *Ann.*, **558**, 187 (1947).