[FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY NEW YORK]

Further Studies of the Action of Pancreatic Amylase: Hydrolysis of Waxy Maize Starch[1a]

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Recent advances in the chemistry of starches have made available better characterized substrates for the study of the action of amylases. Such studies are promising, especially if they are carried out with purified amylases and under conditions designed to favor the action of the enzyme and to protect it from inactivation. The work reported here deals with the hydrolysis of waxy maize starch by highly purified maltase-free pancreatic amylase.[2] Unlike ordinary starches, the waxy varieties contain no linear components[3] and, therefore, offer opportunities to study the action of amylases on branched whole starches.

Experimental

Two samples of waxy maize starch[4] were defatted by exhaustive treatment with methanol[5] and washed repeatedly with distilled and with redistilled water to remove substances which might influence the activity of the amylase. After defatting, there was less than 0.04%[6] fat in the starch. The starches gave no evidence of the presence of linear components either by potentiometric titration[7] or by precipitation procedures.[8] They gave a reddish violet color with iodine. Good dispersions could be obtained in water with concentrations up to one per cent. Results of studies of amylase action with the two samples of starch agreed very closely.

The linear fraction from corn starch used in comparisons with waxy maize starch was kindly supplied by Dr. T. J. Schoch. It was hydrolyzed completely to fermentable sugar by beta amylase and corresponded to 94% "amylose"[9] by potentiometric titration.[7,9,10] The corn starch used in the comparisons was defatted.[11]

The starch was hydrolyzed at 40° with highly purified maltase-free pancreatic amylase.[1,2] The methods used and the experimental procedures have been described elsewhere.[2]

Portions of some of the hydrolysis mixtures were examined for maltose, and for glucose by selective fermentation with yeasts. Maltose was determined with washed bakers yeast by a modification of the method of Somogyi.[12,13,14] Glucose was determined with a special strain of yeast, no. 2019,[15] which ferments glucose but not maltose at *p*H 4.7 to 4.9.[16] Dextrins were determined from the reducing values by difference.[12,13,14] When very low concentrations of reducing sugars were present, a manometric technique[17,18] was used. In this way, it was possible to detect less than 1 mg. of glucose in solutions containing more than 100 mg. carbohydrate. All procedures were standardized with known mixtures of glucose and maltose.

Average degrees of polymerization have been calculated for the reducing dextrins from their weights and reducing values. Although iodometric methods are stoichiometric for reducing sugars of low molecular weights, the application of the method to dextrins formed in the earlier stages of the hydrolysis of starch may introduce certain errors. Therefore, the values for the average degrees of polymerization of the dextrins cannot be taken as absolute values. However, this method seems to be the most satisfactory of those at present available by which to obtain an indication of the breakdown of the substrate.

Results

Rates of Hydrolysis.—The data summarized in Table I show that waxy maize starch is hydrolyzed more slowly than unfractionated corn starch and much more slowly than the linear

TABLE I

A COMPARISON OF THE ACTION OF PURIFIED MALTASE-FREE PANCREATIC AMYLASE ON WAXY MAIZE STARCH AND ON OTHER SUBSTRATES[a]

	Relative concentrations of amylase					
	1			8		
	Reducing values as per cent. theoretical maltose					
Reaction Time, hr.	Waxy maize starch	Corn starch	Linear substrate	Waxy maize starch	Corn starch	Linear substrate
2.5	36	40	60	62	65	84
5	50	56	80	64	66	85
10	55	61	81	65	68	86
20	60	66	86	65	70	85
30	62	68	87	68	70	85
45	63	69	87	69	72	88
60	63		87	70	74	89
90	65	70	87	71	74	90
120	65	71	87	73	75	91
180	67	71	87	75	79	93
300	69	72	90	79	82	94

[a] Defatted waxy maize starch, defatted corn starch or linear fraction from corn starch; 0.5%; 0.01 *M* phosphate; 0.02 *M* sodium chloride; *p*H 7.2. Amylase preparation 1.0 or 8.0 mg. per 1000 mg. carbohydrate. Hydrolyses at 40°.

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(2) (a) Sherman, Caldwell and Adams, *J. Biol. Chem.*, **88**, 295 (1930); (b) Alfin and Caldwell, THIS JOURNAL, **70**, 2534 (1948).

(3) Schopmeyer, Felton and Ford, *Ind. Eng. Chem.*, **35**, 1168 (1943).

(4) Courtesy of the National Starch Products Co., Inc., and of the Northern Regional Laboratories.

(5) Schoch, THIS JOURNAL, **64**, 2954 (1942).

(6) A. O. A. C., "Official and Tentative Methods of Analysis," 5th Ed., Washington, 1940,

(7) Bates, French and Rundle, THIS JOURNAL, **65**, 142 (1943).

(8) Horan, Dissertation, Columbia University, 1944.

(9) Kerr and Severson, THIS JOURNAL, **65**, 193 (1943).

(10) Schoch, "Advances in Carbohydrate Chemistry," Vol. I, 1945, p. 260.

(11) Daly and Caldwell, unpublished.

(12) Somogyi, *J. Biol. Chem.*, **119**, 741 (1937).

(13) Somogyi and Stark, *ibid.*, **142**, 579 (1942).

(14) Meyer and Bernfeld, *Helv. Chim. Acta*, **24**, 359 E (1941).

(15) Kindness of The Fleischmann Laboratories.

(16) Schultz, Fisher, Atkin and Frey, *Ind. and Eng. Chem., Anal. Ed.*, **15**, 496 (1943).

(17) Dixon, "Manometric Methods," The Macmillan Co., New York, N. Y., 1943.

(18) Umbreit, Burris and Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minnesota, 1946.

fraction from corn starch. These comparisons are based upon the total reducing values of the hydrolyzates obtained when the same concentrations of pancreatic amylase reacted with the different substrates under strictly comparable conditions.

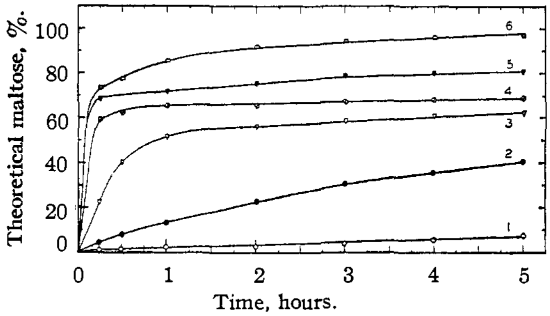

Fig. 1.—Influence of concentration of pancreatic amylase upon the extent of the hydrolysis of waxy maize starch, amylase preparation per 1000 mg. of starch: Curve 1, 0.001 mg.; 2, 0.01 mg.; 3, 0.1 mg.; 4, 1.0 mg.; 5, 10 mg.; 6, 100 mg.; hydrolyses at 40°; optimal conditions (2).

Extent of Hydrolysis.—The data given in Fig. 1 show that the extent of the hydrolysis of waxy maize starch by pancreatic amylase depends within wide limits upon the concentration of the amylase. Similar results have been reported for the action of purified pancreatic amylase on potato starch,[2] corn starch,[11] and on the linear fraction from corn starch.[2] With this branched substrate, also, the hydrolysis time-curves with pancreatic amylase show a change from a rapid to a slow phase of the reaction and tend to flatten at higher values as the concentration of amylase is increased. The extents of hydrolysis attained with different concentrations of amylase do not tend to reach a common limit such as is observed with beta amylase from malted barley,[19,20] and as has been reported for this amylase.[21,22] It should be pointed out that pancreatic amylase is not inactivated to any appreciable extent under the conditions of these experiments.[2] Therefore, the slowing down of these reactions cannot be attributed to any appreciable inactivation of the amylase.

Differentiation of Reducing Products.—The data given in Table II show that the branched chain substrate, waxy maize starch, like other starches[11,23] and their linear components[23] is hydrolyzed relatively rapidly by pancreatic amylase to products of relatively low average molecular weights and of relatively high reducing values. Thus, when approximately 20% of the glucosidic linkages of the substrate had been broken, reducing dextrins with an average degree of polymerization of 6.8 glucose units accounted for 62% of the total reducing value and for 87% by weight of the total products. These results strengthen the conclusion that pancreatic amylase causes the random hydrolysis of the branched chain components of starches as well as of their linear components.[11,21]

TABLE II

PRODUCTS FORMED FROM WAXY MAIZE STARCH BY PURIFIED MALTASE-FREE PANCREATIC AMYLASE

Amylase concentration mg./g. starch,[a] mg.	Reducing values as per cent. theoretical maltose			Glucose[b] as per cent theoretical glucose	Dextrins[b]	
	Total, %	Reducing dextrins,[b] %	Maltose, %		Total products by weight, %	Average degrees of polymerization,[c] $\overline{DP}$
0.01	41.5	25.6	9.7	3.1	87	6.8
0.10	63.3	27.0	23.3	6.5	70	5.2
1.00	69.5	24.3	29.3	8.0	63	5.2
10.0	80.9	19.5	34.3	13.6	52	5.3
100	97.5	16.4	46.1	17.5	36	4.4

[a] Waxy maize starch, 0.5%; 0.01 M, phosphate; 0.02 M, chloride; pH 7.2; hydrolyses, five hours at 40°. [b] Glucose, by selective fermentation with a special strain of yeast; maltose, by selective fermentation with washed bakers yeast; dextrins, by difference (refs. 12, 13, 14, 15). [c] Average degrees of polymerization of dextrins were calculated from their reducing values as follows:

$$\overline{DP} = \frac{\text{weight of dextrins (mg.)}}{\text{reducing value of dextrins as maltose equivalents (mg.)}} \times 2$$

Upon more extensive hydrolysis of waxy maize starch, the molecular weights of the reducing dextrins decrease more slowly. Dextrins with average degrees of polymerization of 4.4 glucose units accounted for 36% by weight of the products when the hydrolysis corresponded to 98% theoretical maltose. These residual dextrins are of somewhat higher average molecular weights than those reported for equivalent stages in the hydrolysis of unfractionated potato starch[23] or of the linear fraction from corn starch.[23] These differences are probably due in part to a higher concentration of 1,6-α-D-glucosidic linkages in the residual dextrins from waxy maize starch. It is also possible that the hydrolysis of a branched chain substrate such as waxy maize starch may yield relatively large concentrations of low molecular weight products such as maltotriose, that contain only 1,4-α-D-glucosidic linkages, and that these low molecular weight products are hydrolyzed very slowly if at all by pancreatic amylase. It may be of interest to note that Montgomery and co-workers[24] found 6-(α-D-glucopyranosyl)-D-glucose among the products after the action of a number of enzymes upon waxy maize starch.

The data summarized in Table II show that both maltose and glucose are formed from waxy maize starch by maltase-free pancreatic amylase. Moreover, measurements made at earlier stages of

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(19) van Klinkenberg, *Z. physiol. Chem.*, **212**, 173 (1932).

(20) Hopkins, Murray and Lockwood, *Biochem. J.*, **40**, 507 (1946).

(21) Willstätter, Waldschmidt-Leitz and Hesse, *Z. physiol. Chem.*, **126**, 143 (1923).

(22) Blom, Bok and Braae, *ibid.*, **250**, 104 (1937).

(23) Alfin and Caldwell, THIS JOURNAL, **71**, 128 (1949).

(24) Montgomery, Weakley and Hilbert, *ibid.*, **69**, 2249 (1947).

the hydrolysis of waxy maize starch than those reported in Table II showed that maltose was present in significant concentrations from the very early stages of the hydrolysis of this substrate, at stages which corresponded to approximately 2 to 5% of theoretical maltose. On the other hand, reaction mixtures at these very early stages of hydrolysis gave no evidence of the presence of glucose even when the concentrated hydrolyzates were examined by the sensitive manometric technique. Concentrated dialyzates of these early reaction mixtures, also, failed to show traces of glucose. At later stages of the reaction, whenever both sugars appear in the hydrolyzates of waxy maize starch, the concentrations of glucose are much lower than those of maltose.

The data given in Table II are also of interest because they show that the relative concentrations of the products were different in comparable hydrolyzates which had reached stages of very slow rates of change in the same time (five hours) under the influence of different concentrations of maltase-free pancreatic amylase. These data confirm and extend previous observations[2] that the slowing down of the hydrolysis of starch by pancreatic amylase under conditions which prevent its inactivation[2] is due largely to the replacement of the original substrate by products which the amylase hydrolyzes slowly, for which it has low affinities.

While the results obtained in the earlier stages of the hydrolysis suggest that pancreatic amylase causes a random hydrolysis of waxy maize starch, the accumulation of low molecular weight dextrins in the later stages of the hydrolysis indicates that the action of the amylase is not perfectly random.

Summary and Conclusions

A study has been made of the hydrolysis of the branched chain substrate, waxy maize starch, by highly purified maltase-free pancreatic amylase.

It has been found that the extent of the hydrolysis of waxy maize starch like that of other starches and of their linear components depends within wide limits upon the concentration of pancreatic amylase.

Under comparable conditions and judged by the total reducing values of the reaction mixtures, waxy maize starch is hydrolyzed more slowly by pancreatic amylase than unfractionated corn starch and much more slowly than the linear fraction from corn starch.

Maltose is present in significant concentrations from the very early stages of the hydrolysis of waxy maize starch by purified pancreatic amylase.

Glucose, also, is liberated in the hydrolysis of waxy maize starch by purified maltase-free pancreatic amylase but this sugar is set free in smaller concentrations than maltose and does not appear in the very early stages of the hydrolysis.

Waxy maize starch is hydrolyzed rapidly by pancreatic amylase to products of relatively low average molecular weights and of relatively high reducing values.

The attack of pancreatic amylase on waxy maize starch appears to be random in the early stages of the hydrolysis but the accumulation of low molecular weight products in the later stages of the hydrolysis indicates that the action of the amylase is not perfectly random.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

The Dienone-Phenol Rearrangement. II. Rearrangement of 1-Keto-4-methyl-4-phenyl-1,4-dihydronaphthalene

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If the dienone–phenol rearrangement proceeds by a mechanism[1,2] similar to that for the conversion of pinacols to pinacolones, the same migratory aptitudes of groups should be observed in both rearrangements. It is well established[3,4] that a phenyl group migrates preferentially with respect to a methyl group in the pinacol rearrangement, and now the same migratory aptitude has been found in the dienone–phenol rearrangement of 1-keto-4-methyl-4-phenyl-1,4-dihydronaphthalene (VI) to 4-methyl-3-phenyl-1-naphthol (VIII).

Synthesis of the dienone VI involved the preparation of γ,γ-diphenylvaleric acid[5] and the cyclization of this acid to 4-methyl-4-phenyl-1-tetralone (IV).[6c] Bromination of this ketone gave a

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(1) Arnold, Buckley and Richter, THIS JOURNAL, **69**, 2322 (1947).

(2) Huang-Minlon, *ibid.*, **70**, 611 (1948).

(3) Kon, *Annual Reports*, **30**, 182 (1933).

(4) Thorner and Zincke, *Ber.*, **13**, 644 (1880).

(5) This acid has been obtained as a side product in the preparation of phenacylacetone from "Lävulinsäurechlorides" by Helberger (*Ann.*, **522**, 270 (1936)). It has been prepared also by Eykman (*Chemisch Weekblad*, **4**, 727 (1907)) by treating $\Delta\beta$-angelica lactone with benzene and aluminum chloride. We have found that under normal conditions for the condensation of lactones with benzene[6a] a 46% yield of phenacyl acetone can be obtained from $\Delta\beta$-angelica lactone. This cleavage between the carbonyl carbon and the oxygen is similar to that observed by Boese[6b] in the reaction of diketene with benzene and aluminum chloride.

(6a) Beyer, *Ber.*, **70**, 1101, 1482 (1937).

(6b) Boese, *Ind. Eng. Chem.*, **32**, 16 (1940).

(6c) Preliminary experiments under varied conditions showed that it was not feasible to prepare this ketone by a direct Friedel–Crafts reaction between γ-methyl-γ-phenylbutyrolactone and benzene, as originally proposed (1).