[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Action of Taka Amylase[1]

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The action of taka amylase, an α-amylase produced by the mold, *Aspergillus oryzae*, has been investigated with a number of well characterized substrates. Both highly purified but uncrystallized preparations of taka amylase and crystalline taka amylase were employed. The results showed that these two types of amylase solutions could be used interchangeably. In both cases, the amylase solutions were free from all detectable traces of maltase activity. The uncrystallized preparations had also been found by selective inactivation measurements to be free from any significant traces of contaminating dextrinase or other glucosidase activities. Comparisons show that the linear fraction from corn starch is hydrolyzed more rapidly and more extensively by taka amylase than several branched substrates. Given sufficient time for the hydrolyses and sufficiently high concentrations of enzyme, taka amylase hydrolyzes the linear substrate completely to maltose and glucose. No evidence of other products was obtained in the final hydrolyzates when these were examined by several methods including selective fermentation and chromatographic techniques. These results show that taka amylase hydrolyzes trisaccharides and higher sugars composed of 1,4-α-D-glucosidic chains. Taka amylase does not hydrolyze maltose. Branched chain substrates including the branched fraction from corn starch, waxy maize starch, glycogen and β-amylase limit dextrins are hydrolyzed less readily and less extensively under the same conditions and by equivalent concentrations of taka amylase than the linear fraction from corn starch. Moreover, the rate and the extent of the hydrolyses of the branched substrates decrease as the proportion of 1,6-α-D-glucosidic linkages in the substrate increases. A bacterial dextran composed of D-glucose united mainly by 1,6-α-D-glucosidic linkages but with 1,4-α-D-glucosidic linkages at the points of branching was not hydrolyzed by taka amylase. These results confirm and extend previous evidence that taka amylase does not hydrolyze 1,6-α-D-glucosidic linkages nor short chain saccharides that contain 1,6-α-D-glucosidic linkages. Taka amylase hydrolyzes both the alpha and the beta Schardinger dextrins completely to maltose and glucose. The Michaelis–Menten constants were determined for the action of taka amylase with the different substrates. The action of taka amylase is discussed.

Introduction

The work reported here deals with the action of taka amylase, the α-amylase produced by the mold, *Aspergillus oryzae*, on a number of well characterized substrates.

Experimental

Amylase.—The major portion of the work was carried out with highly purified preparations of taka amylase[3,4] that had been freed from traces of maltase[4] and had been shown, by selective inactivation measurements,[5] to be free from detectable traces of contaminating dextrinase or other glucosidase activities.[4] The main points of the work with the different substrates were repeated with crystalline taka amylase prepared essentially by the method of Fischer and de Montmollin.[6] The results obtained with the two types of amylase solutions and any given substrate were found to agree very well. This finding is not surprising as solutions of the highly purified maltase-free but uncrystallized preparations had the same saccharogenic activities[7] and other properties as solutions of the crystalline amylase. In both cases, the amylase produced 2400 times its weight of maltose equivalents in 30 minutes at 40° from 1% Lintner soluble potato starch adjusted to 0.01 *M* acetate and *p*H 5.0.[7] Like the crystalline amylase,[6] the uncrystallized amylase preparations were found to be approximately 90% one protein by electrophoresis and by sedimentation measurements.[4]

Both the crystalline taka amylase and the highly purified but not crystalline preparations were free from traces of maltase activity. Thus, no appreciable increase in reducing value was observed with either type of amylase solution when 1% maltose was incubated at 40° for 24 hours with 100 times the concentration of taka amylase required to cause 20% hydrolysis of 1% Lintner soluble potato starch in 30 minutes at 40° under the same conditions, 0.01 *M* acetate, *p*H 5.0.[7]

Substrates.—The substrates investigated included: a linear fraction from corn starch,[8] a branched fraction from corn starch,[8] unfractionated potato starch, waxy maize starch,[9–12] glycogen,[13] maltose,[14,15] a bacterial dextran,[16] β-amylase limit dextrins,[17,18] alpha and beta Schardinger dextrins.[19]

For use, the substrates were dissolved in 0.1 *M* potassium hydroxide, neutralized with hydrochloric acid and adjusted to a final concentration of 1% substrate, 0.02 *M* acetate, 0.1 *M* potassium chloride, 0.02 *M* calcium chloride[4] and *p*H 5.0.[7] The hydrolyses were carried out at 40°. When reactions were continued for more than 4 hours, toluene was added as a preservative. Toluene does not influence the activity of taka amylase under the conditions used. With the exception of the Schardinger dextrins, the

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(2) The data reported here are taken in part from a dissertation submitted by Virginia M. Hanrahan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry under the Faculty of Pure Science of Columbia University.

(3) M. L. Caldwell, R. M. Chester, A. H. Doebbeling and G. W. Volz, *J. Biol. Chem.*, **161**, 361 (1945).

(4) Virginia M. Hanrahan, Dissertation, Columbia University, New York, 1950.

(5) R. B. Alfin and M. L. Caldwell, THIS JOURNAL, **70**, 2534 (1948).

(6) Ed. H. Fischer and R. de Montmollin, *Helv. Chim. Acta*, **34**, 1987 (1951).

(7) M. L. Caldwell and S. E. Doebbeling, THIS JOURNAL, **59**, 1835 (1937).

(8) The authors wish to thank Dr. T. J. Schoch who kindly furnished the linear and the branched fractions from corn starch.

(9) The waxy maize starch was kindly furnished by the National Starch Products Co., Inc. It was defatted by treatment with methanol[10] and washed repeatedly with water. It gave no evidence of the presence of linear components either by potentiometric titration[11] or by precipitation procedures.[12]

(10) T. J. Schoch, THIS JOURNAL, **64**, 2954 (1942).

(11) F. L. Bates, D. French and R. E. Rundle, *ibid.*, **65**, 142 (1943).

(12) T. J. Schoch, "Advances in Carbohydrate Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1945, p. 247.

(13) The authors wish to thank Dr. James McBride for the highly purified oyster glycogen.

(14) The maltose was prepared by action of β-amylase on starch and recrystallized: $[\alpha]^{25}D$ 131.25°; reducing value 98.5 by iodometric method.[15]

(15) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(16) The authors wish to thank Dr. Allene Jeanes for the dextran. It is described as Dextran A or C, Table II, A. Jeanes, C. A. Wilham and J. C. Miers, *J. Biol. Chem.*, **176**, 603 (1948).

(17) The β-amylase limit dextrins were prepared by the exhaustive action of β-amylase on a portion of the waxy maize starch described above.[9] β-Amylase reacted at *p*H 4.5 and 0.01 *M* acetate[18] upon 2% waxy maize starch. The hydrolyzate reached a limit of hydrolysis at 49% theoretical maltose. This limit was not changed by prolonging the time of hydrolysis nor by the addition of more β-amylase. The hydrolyzate was boiled to inactivate the β-amylase, dialyzed until free from maltose, adjusted to a known concentration of dextrins, 0.02 *M* acetate, *p*H 5.0 and used as substrate for taka amylase.

(18) M. L. Caldwell and S. E. Doebbeling, *J. Biol. Chem.*, **110**, 739 (1935).

(19) The authors wish to thank Dr. Evelyn Tilden who kindly supplied the Schardinger dextrins. Both the alpha and the beta Schardinger dextrins were recrystallized several times.

total reducing values of the hydrolyzates were determined by an iodometric method[15] which gives a stoichiometric measure of the glucosidic linkages broken in the substrate. The total reducing values were calculated to their equivalents of maltose and are reported as such, although they are known to represent the reducing values of a number of products including reducing dextrins and other reducing sugars.[20] The reducing values of hydrolyzates of the Schardinger dextrins were determined by the copper method of Somogyi.[21] The unit concentration of the amylase refers to the concentration required to cause the formation of maltose equivalents corresponding to 20% theoretical maltose in 30 minutes at 40° from 1% Lintner soluble potato starch adjusted to 0.01 *M* acetate and *p*H 5.0.[7] Glucose and maltose were determined in the hydrolyzates by selective fermentation[22-24] and by chromatographic techniques.[25-27] Glucose was determined also by a copper acetate method.[28]

Results and Discussion

Hydrolysis of Linear Substrate, Reducing Values. The data given in Table I are typical of the results obtained when highly purified[4] or crystalline[6] maltase-free taka amylase reacted with the linear fraction from corn starch.[8] Increasing concentrations of taka amylase increased the rate and the extent of the hydrolysis of the substrate until a limit was reached, at the equivalent of 109% of the theoretical maltose. This percentage hydrolysis was obtained in 24 hours with the highest concentration of amylase used. As will be shown later, the only reducing products in these final hydrolyzates were glucose and maltose and in proportions which accounted for the total reducing values found. These results show that taka amylase is capable of hydrolyzing the 1,4-α-D-glucosidic linkages of straight chain low molecular weight dextrins and of higher sugars. It does not hydrolyze maltose.

TABLE I

INFLUENCE OF CONCENTRATION OF TAKA AMYLASE UPON RATE AND EXTENT OF HYDROLYSIS OF A LINEAR FRACTION FROM CORN STARCH

Hydrolysis,[a] min.	Relative concentrations[b] of taka amylase: 1 [c]	1 [d]	2.5 [c]	5 [c]	10 [c]	25 [c]	25 [d]	100 [c]
	Theoretical maltose,[e] %							
5	4.2	6.0	10.3	17.7	31.7	54.1	60.7	72.5
10	8.3	10.3	18.6	33.5	54.3	70.1	..	77.2
15	12.5	15.2	28.8	46.2	65.0	75.1	76.0	79.0
20	16.3	19.8	36.1	53.3	68.3	..	76.5	80.1
30	24.7	29.8	48.4	63.1	74.5	75.7	77.5	81.5
45	33.5	34.7	58.5	68.3	74.8	77.0	78.0	85.0
60	41.8	42.3	65.7	70.4	78.0	80.4	78.5	87.6
90	54.2	54.4	73.2	74.8	78.0	82.3	80.0	89.3
120	58.2	61.8	73.6	77.1	78.5	83.9	83.0	93.7
180	67.0	67.8	76.7	81.3	81.6	85.3	87.8	100
240	71.7	71.8	78.7	84.1	83.4	..	89.5	..
300	74.4	75.1	80.8	..	84.1	90.9	89.6	105
1440	79.5	81.2	84.1	89.2	96.2	102	102	109

[a] Linear fraction from corn starch: 1%; 0.1 *M* KCl; 0.02 *M* $CaCl_2$; 0.02 *M* acetate; *p*H 5.0; 40°. [b] Unit concentration, 0.089 mg. amylase preparation or 0.095 crystalline amylase per g. substrate. [c] Highly purified but not crystallized maltase-free taka amylase. [d] Crystalline maltase-free taka amylase. [e] Reducing values by iodometric method.[15]

Hydrolysis of Branched Chain Substrates, Reducing Values.—The data given in Tables II and III summarize the results obtained when maltase-free taka amylase reacted with the branched chain substrates, waxy maize starch[9] and glycogen.[13] Again, as with the linear substrate, increasing concentrations of taka amylase increased the rate and the extent of the hydrolysis of waxy maize starch and of glycogen and no evidence was obtained in either case of the formation of high molecular weight resistant dextrins such as are formed when β-amylases react with branched chain substrates.[29-32]

Comparison of the Rates and Extents of the Hydrolyses of Different Substrates.—The data given in Tables I, II, III and in Figs. 1, 2 and 3 show that the linear fraction from corn starch is hydrolyzed more rapidly and more extensively than the branched chain substrates when these are treated under the same conditions with the same concentrations of taka amylase. The data also

TABLE II

INFLUENCE OF CONCENTRATION OF TAKA AMYLASE UPON RATE AND EXTENT OF HYDROLYSIS OF WAXY MAIZE STARCH

Hydrolysis,[a] min.	Relative concentrations[b] of taka amylase: 1 [c]	1 [d]	2.5 [c]	5.0 [c]	10 [c]	25 [c]	25 [d]	100 [c]
	Theoretical maltose,[e] %							
2	..	..	..	..	11.4	26.3	23.7	51.2
4	..	..	..	..	20.7	39.2	41.2	58.2
5	3.5	2.8	7.8	15.4	..	..	..	..
6	..	..	..	..	..	..	47.6	57.4
8	..	..	..	..	..	52.5	52.1	61.2
10	6.1	..	15.4	27.4	42.3	54.6	54.7	62.6
15	9.2	10.1	21.3	35.0	48.3	57.5	56.3	64.3
20	12.4	12.7	27.2	40.2	51.4	..	59.6	..
25	15.4	15.2	32.0	44.7	..	..	58.0	..
30	18.2	18.9	36.1	47.1	56.1	59.0	60.9	67.0
45	24.7	25.9	42.5	51.2	59.5	62.4	59.9	69.1
60	30.6	31.0	47.5	54.7	60.3	64.5	63.9	72.8
90	40.5	39.8	51.8	60.9	61.1	65.8	66.5	74.3
120	43.5	43.2	54.6	61.1	62.6	..	65.2	77.5
180	50.0	50.0	59.6	63.0	..	72.3	69.5	85.0
240	52.2	53.3	58.2	63.7	65.8	70.9	72.8	88.6
300	54.0	55.9	60.4	64.1	..	78.6	79.8	87.1
330	..	..	..	..	69.4	..	..	..
1440	62.1	62.9	65.8	69.4	73.6	82.3	86.2	91.0
2880	..	..	..	..	..	..	..	91.1[f]

[a] Waxy maize starch: 1%; 0.1 *M* KCl; 0.02 *M* $CaCl_2$; 0.02 *M* acetate; *p*H 5.0, 40°. [b] Unit concentration, 0.089 mg. amylase preparation or 0.095 mg. crystalline amylase per g. substrate. [c] Highly purified but not crystallized maltase-free taka amylase. [d] Crystalline maltase-free taka amylase. [e] Reducing values by iodometric method.[15] [f] Two hundred times unit concentration of crystalline maltase-free taka amylase.

(20) G. W. Volz and M. L. Caldwell, *J. Biol. Chem.*, **171**, 667 (1947).
(21) M. Somogyi, *ibid.*, **160**, 61 (1945).
(22) M. Somogyi, *ibid.*, **119**, 741 (1937).
(23) I. E. Stark and M. Somogyi, *ibid.*, **142**, 579 (1942).
(24) A. S. Schultz, R. A. Fisher, L. Atkin and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 496 (1943).
(25) D. French, D. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 5150 (1950).
(26) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).
(27) R. J. Block, *ibid.*, **22**, 1327 (1950).
(28) L. L. Phillips and M. L. Caldwell, *ibid.*, **23**, 1172 (1951).
(29) E. Ohlsson, *Z. physiol. Chem.*, **189**, 17 (1930).
(30) G. A. van Klinkenberg, *ibid.*, **209**, 253 (1932).
(31) R. W. Kerr and F. C. Cleveland, THIS JOURNAL, **71**, 3455 (1949).
(32) K. H. Meyer, "Advances in Colloid Science," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1942, p. 143.

TABLE III

INFLUENCE OF CONCENTRATION OF TAKA AMYLASE UPON RATE AND EXTENT OF HYDROLYSIS OF GLYCOGEN

Time of hydrolysis,[a] min.	Relative concentrations[b] of taka amylase 1	2.5	5	10	25	100
	Theoretical maltose,[c] %					
10	4.6	9.5	13.3	19.8	28.4	39.6
20	7.6	13.6	18.1	25.6	33.4	41.4
30	9.9	17.3	22.4	28.6	35.8	44.7
45	13.0	..	25.0	31.4	38.0	48.1
60	14.3	23.3	28.4	34.8	40.0	50.8
90	19.2	26.8	30.9	36.4	43.0	52.8
120	21.5	28.3	32.8	36.4	43.7	54.9
180	27.1	31.7	..	40.1	47.1	59.3
240	28.8	35.1	39.8	43.9	51.0	64.2
300	31.7	37.9	40.5	46.0	55.1	65.8
1440	40.6	46.2	50.6	54.1	65.0	74.7

[a] Oyster glycogen: 1%; 0.1 *M* KCl; 0.02 *M* $CaCl_2$; 0.02 *M* acetate; *p*H 5.0, 40°. [b] Unit concentration, 0.089 mg. amylase per gram glycogen. Highly purified but not crystallized maltase-free taka amylase. [c] Reducing values by iodometric method.[15]

show that the branched chain fraction from corn starch[8] and the waxy maize starch[9] were hydrolyzed at very nearly the same rate and to very nearly the same extent under the same conditions while the glycogen[13] was hydrolyzed much more slowly and less extensively than the other branched substrates. The similarity in the rate and extent of the hydrolysis of the branched substrate from corn starch and of waxy maize starch is not surprising because these two substrates probably are very similar in their architecture.[33] The slower and less extensive hydrolysis of the glycogen may be due to its more extensive branching.[34] This may interfere with the union of taka amylase with the glycogen, and, therefore, may lower the affinity of the amylase for this substrate. As would be expected, the hydrolysis curves for unfractionated potato starch fall between those for the linear and the branched fractions from corn starch and somewhat nearer the curves for the branched substrate, Figs. 1, 2 and 3.

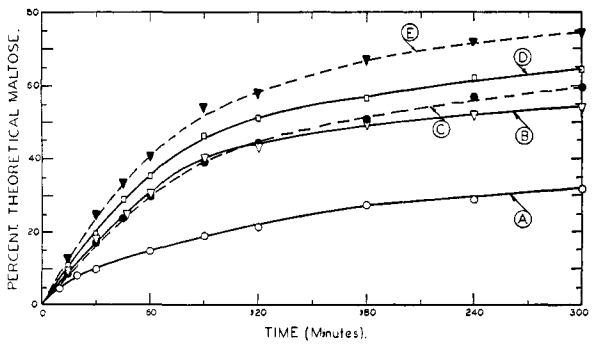

Fig. 1.—A comparison of the rate and extent of the hydrolysis of several substrates with the same concentration of taka amylase. Amylase: unit concentration 0.089 mg. per gram substrate; substrates: A, glycogen; B, waxy maize starch; C, branched fraction from corn starch; D, unfractionated potato starch, E, linear fraction from corn starch; in all cases, 1% substrate, 0.02 *M* acetate, 0.10 *M* KCl, 0.02 *M* $CaCl_2$, *p*H 5.0, 40°.[7]

(33) H. H. Schopmeyer, G. E. Felton and C. L. Ford, *Ind. Eng. Chem.*, **35**, 1168 (1943).

(34) K. H. Meyer, "Advances in Enzymology," Vol. 3, Interscience Publishers, Inc., New York, N. Y., 1943, p. 109.

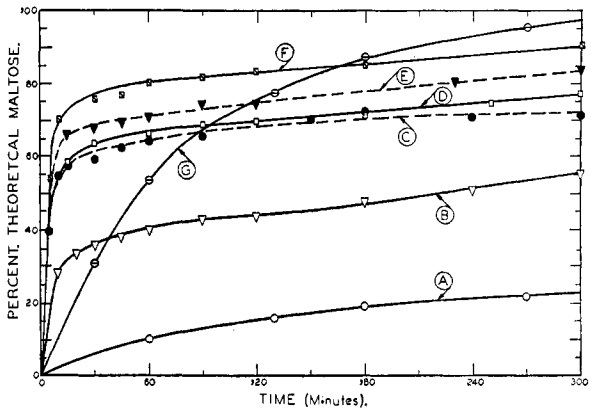

Fig. 2.—A comparison of the rate and extent of the hydrolysis of several substrates by taka amylase. Conditions the same as for Fig. 1 except for a 25-fold increase in the concentration of taka amylase. Substrates: A, alpha Schardinger dextrin; B, glycogen; C, waxy maize starch; D, branched fraction from corn starch; E, unfractionated potato starch; F, linear fraction from corn starch; G, beta Schardinger dextrin.

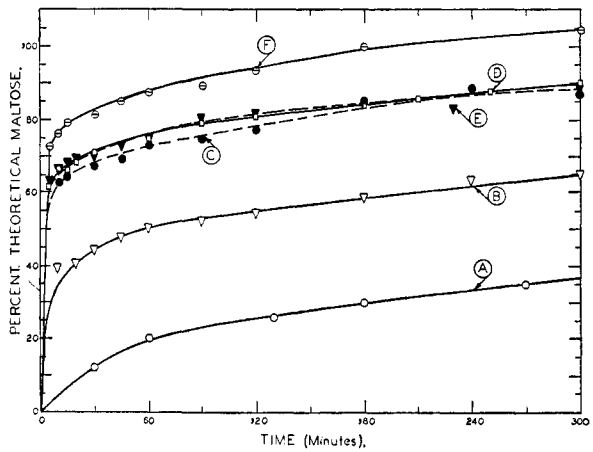

Fig. 3.—A comparison of the rate and extent of the hydrolysis of several substrates by taka amylase. Conditions the same as in Fig. 1 except for a 100-fold increase in the concentration of taka amylase; A, alpha Schardinger dextrin; B, glycogen; C, waxy maize starch; D, branched chain fraction from corn starch; E, unfractionated potato starch; F, linear fraction from corn starch.

Hydrolysis of β-Amylase Limit Dextrins.—A study was made of the action of highly purified maltase-free taka amylase on β-amylase limit dextrins[17] formed by the exhaustive action of β-amylase on defatted waxy maize starch.[9] Comparisons showed that β-amylase limit dextrins were hydrolyzed much more slowly than the other branched substrates investigated. In one comparison, a 0.3% concentration of β-amylase limit dextrins was hydrolyzed less completely in 24 hours at a relative taka amylase concentration of 10 than a 0.3% concentration of glycogen in 24 hours at a relative taka amylase concentration of 1. The slower hydrolysis of the β-amylase limit dextrins than of the original waxy maize starch by

taka amylase suggests that an increase in the proportion of 1,6-α-D-glucosidic linkages in its substrate has an unfavorable influence upon the action of taka amylase. On the other hand, the slower hydrolysis of the β-amylase limit dextrins than of glycogen by taka amylase suggests that the presence of free non-reducing glucosidic chains or outer branches in its substrate aids the action of taka amylase. Thus, the removal of the outer branches from waxy maize starch by β-amylase[32,35-38] formed a substrate that was less readily hydrolyzed by taka amylase than glycogen in spite of the fact that the highly branched glycogen[34] presumably has a higher proportion of 1,6 α-D-glucosidic linkages than the β-amylase limit dextrins from waxy maize starch.[39]

Hydrolysis of Alpha and Beta Schardinger Dextrins.—The data given in Table IV and Fig. 4 show that highly purified maltase-free or crystalline maltase-free taka amylase causes extensive hydrolysis of both the alpha and the beta Schardinger dextrins.[19] These data also make it evident that the beta Schardinger dextrin, the seven 1,4-α-D glucose unit cyclic compound,[40] is hydrolyzed much more rapidly, about 5 times as fast and also much more extensively by equivalent concentrations of taka amylase than the alpha Schardinger dextrin, the six 1,4-α-D-glucose unit cyclic compound.[40] Thus, a relative concentration of taka amylase of 25 brought about more rapid and more extensive hydrolysis of the beta Schardinger dextrin in 24 hours than was attained with the alpha Schardinger dextrin with a relative concentration of taka amylase of 100.

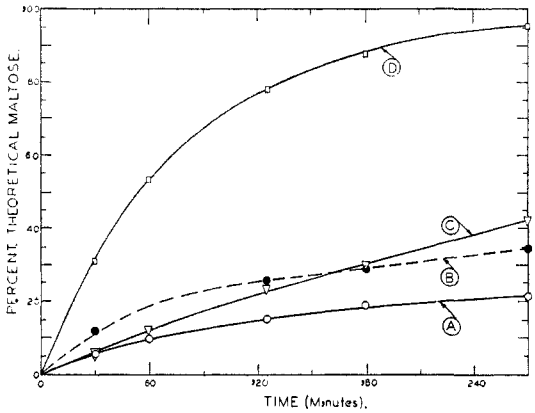

Fig. 4.—A comparison of the rate and extent of the hydrolysis of alpha and beta Schardinger dextrins by taka amylase: A, alpha Schardinger dextrin, relative concentration of taka amylase, 25; B, alpha Schardinger dextrin, relative concentration of taka amylase, 100; C, beta Schardinger dextrin, relative concentration of taka amylase, 5; D, beta Schardinger dextrin, relative concentration of taka amylase, 25. Substrates 1%, 0.02 *M* acetate, 0.10 *M* KCl, 0.02 *M* $CaCl_2$, *p*H 5.0.[7]

TABLE IV

INFLUENCE OF CONCENTRATION OF TAKA AMYLASE UPON RATE AND EXTENT OF HYDROLYSIS OF ALPHA AND BETA SCHARDINGER DEXTRINS

Time of hydrolysis,[a] min.	Alpha Schardinger dextrin, Relative concentrations[b] of taka amylase: 25[c]	100[d]	Beta Schardinger dextrin, Relative concentrations[b] of taka amylase: 5[c]	25[c]	100[d]
	Theoretical maltose,[e] %				
30	..	12.1	12.4	5.9	30.8	30.8
60	10.1	19.1	20.1	12.5	53.5	49.5
130	15.3	25.7	27.3	23.5	77.9	78.5
180	18.9	29.9	29.0	30.7	87.8	86.2
270	21.4	35.3	36.5	41.9	95.7	95.7
1440	35.0	83.4	81.1	84.6		106
2880			127[f]			115[g]

[a] Alpha or beta Schardinger dextrin: 1%; 0.1 *M* KCl; 0.02 *M* $CaCl_2$; 0.02 *M* acetate; *p*H 5.0, 40°. [b] Unit concentration; 0.089 mg. amylase preparation or 0.095 mg. crystalline amylase per g. dextrin. [c] Highly purified but not crystallized maltase-free taka amylase. [d] Crystalline maltase-free taka amylase. [e] Reducing values by copper method of Somogyi.[21] [f] Five hundred times unit concentration of crystalline maltase-free taka amylase. [g] Two hundred times unit concentration of crystalline maltase-free taka amylase.

A comparison of the data given in Tables I, II, III and IV and in Figs. 2 and 3, shows that the initial rates of hydrolysis by taka amylase were much slower for both Schardinger dextrins than for any of the other substrates in the comparison. On the other hand, the data also show that although the alpha Schardinger dextrin had the slowest initial rate of hydrolysis by taka amylase of any of the substrates compared, it was hydrolyzed more completely in 24 hours at a relative concentration of taka amylase of 100 than was glycogen and approached the value attained under the same conditions by the hydrolyzate of waxy maize starch.

In addition, a comparison of the data given in Tables I, II, III and IV and in Fig. 2 for a relative concentration of taka amylase of 25 shows that the beta Schardinger dextrin, after a slow initial rate of reaction, eventually was hydrolyzed more extensively by this relative concentration of taka amylase of 25 than any of the other substrates studied.

The slow initial rate of the hydrolysis of the Schardinger dextrins probably is due to a slow rate of breaking of the cyclic compounds. After this rupture, the 7 glucose unit chains of their fragments are hydrolyzed more rapidly and more extensively by equivalent concentrations of taka amylase than the 6 glucose unit chains or their fragments. The results with the alpha and beta Schardinger dextrins give additional direct evidence that taka amylase does not require free glucosidic chains or ends for its action even though these may be an aid as indicated by the results already discussed for beta amylase limit dextrins.

In passing, it should be emphasized that the alpha Schardinger dextrinase and the beta Schardinger

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

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

(37) S. Hestrin, *ibid.*, **179**, 943 (1949).

(38) G. T. Cori and J. Larner, *ibid.*, **188**, 17 (1951).

(39) The following summary was suggested by Dr. T. J. Schoch. Assuming a branch length of 25–30 glucose units for waxy maize starch, and also assuming a 50% yield of the β-amylase limit dextrins,[32] then the β-amylase limit dextrins would possess one branch point per 12–15 glucose units. Glycogen, however, is believed to have an average branch length of 9–11 glucose units, or one branch point per 10 glucose units. Hence, the glycogen contains a slightly higher proportion of branch points than the β-amylase limit dextrins from waxy maize starch.

(40) D. French and R. E. Rundle, THIS JOURNAL, **64**, 1651 (1942).

dextrinase activities appear to be properties of taka amylase itself and not to be due to the action of traces of contaminating enzymes. Thus, in addition to evidence already presented concerning the homogeneity of the amylase,[4] no difference was observed in the ratios of the amylase activity (saccharogenic, starch as substrate) to the alpha Schardinger dextrinase activity or to the beta Schardinger dextrinase activity when a solution of the purified maltase-free taka amylase containing 11 mg. of amylase preparation per ml., 0.02 *M* acetate, 0.02 *M* calcium chloride and *p*H 5.8 was held at 60° for 30 minutes or when it was adjusted to *p*H 3.4 and held at 5 to 10° for 3 weeks. In the latter case, 50% inactivation of the amylase activity was accompanied by 48% inactivation of the beta Schardinger dextrinase activity and by 45% inactivation of the alpha Schardinger dextrinase activity.

Failure to Cause the Hydrolysis of Dextran.—The bacterial dextran[16] conforms to the following description. It is a polymer of α-D-glucopyranose. The molecule has a branched structure in which the predominant glucosidic linkage is 1,6 while 1,4-linkages occur at points of branching.[41] Hydrolysis of the dextran was attempted with highly purified maltase-free taka amylase but no increase in the reducing value of the reaction mixture was detected over a 48-hour period at 40° and at a relative concentration of taka amylase of 25. That the amylase was still active was evident from the fact that a portion of the 48-hour dextran reaction mixture caused extensive hydrolysis of a starch substrate to which it was added.

The results with this dextran confirm and extend the evidence obtained with other branched substrates that taka amylase does not hydrolyze 1,6-α-glucosidic linkages. In addition, the failure of taka amylase to hydrolyze either maltose or this dextran leads to the conclusion that taka amylase does not hydrolyze single 1,4-α-D-glucose linkages whether these occur between two free glucose residues, as in maltose, or between two glucose residues that in turn are united to other glucose residues by 1,6-α-D-glucosidic linkages.

Additional Comparisons.—The data given in Fig. 5 show that the extent of the hydrolysis of any given substrate by taka amylase depends upon the duration of the reaction and upon the relative concentration of amylase to substrate. These data also emphasize the fact that taka amylase hydrolyzes the linear substrate much more readily than the branched substrates and that glycogen is hydrolyzed less readily than waxy maize starch, presumably because it is more highly branched and has a greater proportion of 1,6-α-D-glucosidic linkages.

The comparison given in Fig. 6 also emphasizes the influence of the extent of the branching of its substrates upon the action of taka amylase. The data for the linear fraction from corn starch give two typical S-shaped curves with definite points of inflection. These curves suggest that the hydrolysis of the substrate is taking place in at least two general stages in which the over-all rates of reaction are different and probably depend on the chain length of the substrate fragments. The data for waxy maize starch give similar curves with definite points of inflection but the data for the more highly branched glycogen give a curve in which no point of inflection is apparent. It is evident that the hydrolysis of a substrate becomes more complicated as its branching is increased probably because straight chain and branched chain fragments are hydrolyzed at different rates. Curves with definite points of inflection similar to those given by the linear substrate were obtained for both the alpha and the beta Schardinger dextrins. These substrates also yield only straight chain fragments upon hydrolysis.[40]

(41) A. Jeanes, N. C. Schieltz and C. A. Wilham, *J. Biol. Chem.*, **176**, 617 (1948).

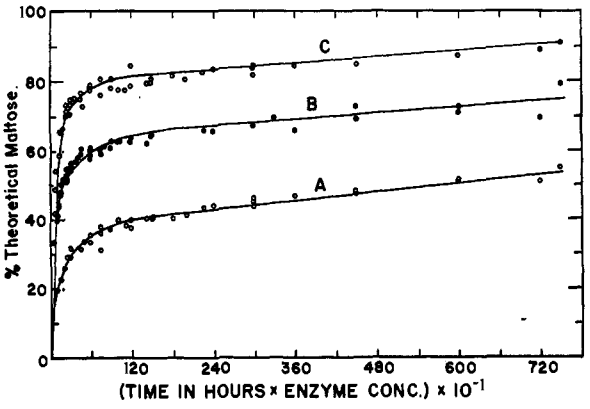

Fig. 5.—Comparison of the hydrolysis of different substrates by maltase-free taka amylase: A, glycogen; B, waxy maize starch; C, linear fraction from corn starch. Substrates, 1%. Relative concentrations of taka amylase for each substrate: 1, 2.5, 5, 10, 25 and 100; $E_1t_1 = E_2t_2$.

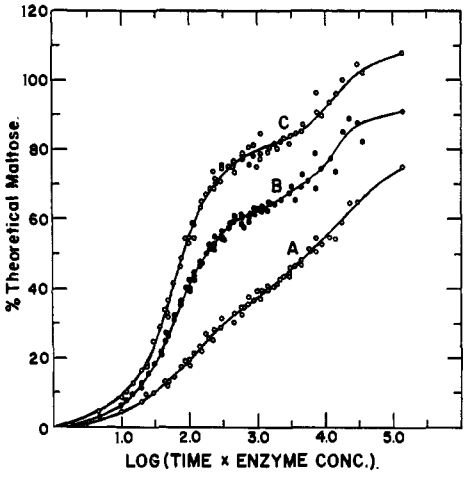

Fig. 6.—Comparison of the hydrolysis of different substrates by maltase-free taka amylase: A, glycogen; B, waxy maize starch; C, linear fraction from corn starch. Substrates, 1%. Relative concentrations of taka amylase for each substrate: 1, 2.5, 5, 10, 25, 100.

Products Formed.—The data given in Table V summarize information about the products that remained in the hydrolyzates of the different substrates after 24 or 48 hours hydrolysis with increasing concentrations of highly purified and crystallized maltase-free taka amylase. The average degrees of polymerization of the unfermented

TABLE V

PRODUCTS FORMED FROM DIFFERENT SUBSTRATES BY MALTASE-FREE TAKA AMYLASE

Substrate[a]	Relative concn. of taka amylase, units[b]	Total reducing value as % theoretical maltose,[c] %	Maltose as % theoretical maltose,[d] %	Glucose as % theoretical glucose,[e] %	Dextrins[f] as % theoretical maltose, %	Dextrins[f] as % total products by weight, %	Dextrins average degrees of polymerization,[g] D.P.
Linear fraction from corn starch	1.0	79.5	53.6		25.9	46.4	3.5
	2.5	84.1	59.6		24.5	40.4	3.5
	5.0	89.2		1.8			
	10.0	96.2	68.6	2.7	22.5	28.6	2.5
	25.0	104.8	(83.5)	11.2	Not detectable		
	100.0	109.0	87.2	11.5	None[h]	None[h]	
Unfractionated potato starch	1	67.1	21.1		46.0	78.9	3.5
	2.5	76.0	52.1		23.9	47.9	4.0
	5.0	77.6	54.0	1.3	21.1	44.5	4.0
	10.0	84.9	58.4	3.1	20.6	38.4	4.0
	25.0	91.0	55.5	9.0	18.4	35.0	4.0
Waxy maize starch	1.0	62.1	36.5		25.6	63.5	5.0
	5.0	69.4	48.3		21.1	51.7	5.0
	10.0	73.6	51.2	1.3	19.9	47.3	5.0
	25.0	82.8		3.6			
	100.0	91.0	65.5	4.5	16.9	29.8	3.5
	200.0[i]	91.1					
Branched fraction from corn starch	1.0	64.1	34.1		30.0	65.9	4.5
	2.5	66.8	44.6		22.2	55.4	5.0
	5.0	72.6	53.5		19.1	46.5	5.0
	10.0	78.3	55.3	2.7	17.9	41.7	4.5
	25.0	87.8	63.5	6.3	12.3	29.8	5.0
	100	91.1	63.7	9.5	9.3	26.5	5.5
Glycogen	1.0	40.6	25.3		15.3	74.7	10.0
	2.5	46.2	22.4		23.8	77.6	6.5
	5.0	50.4	30.6		19.8	69.4	7.0
	25.0	65.0	49.6	3.6	8.6	46.4	11.0
	100	74.7	57.0	4.1	9.9	38.8	8.0
α-Schardinger dextrin	25	35					
	100	83.4					
	500[i]	127.0			None[h]	None[h]	
β-Schardinger dextrin	25[i]	106.0					
	200[i]	115.0			None[h]	None[h]	

[a] Substrate: 1%; 0.02 *M* acetate; 0.02 *M* $CaCl_2$; 0.1 *M* KCl; *p*H 5.0; hydrolyses at 40° for 24 hours. [b] Unit concentration of highly purified maltase-free taka amylase, 0.089 mg. per g. substrate. [c] Determined by iodometric method.[15] [d] Determined from total reducing value and selective fermentation by difference.[15,22-24,28] [e] Determined by copper acetate method.[28] [f] Determined selective fermentation methods.[22-24] [g] *D.P.* = weight of dextrin (mg.)/reducing value as maltose (mg.) × 2. [h] Only glucose and maltose detectable by chromatographic techniques.[25-27] [i] Crystalline maltase-free taka amylase, hydrolyses of 48 hours. [j] Crystalline maltase-free taka amylase, hydrolyses of 24 hours.

products were calculated from their weights and reducing values. Such values are only approximations but are of interest for comparison.

Examination of the data given in Table V shows that glucose is formed in relatively small concentrations by maltase-free taka amylase from starch or its components or from glycogen. Moreover, glucose appears only during the later stages of the hydrolyses, when approximately 89% of the theoretical maltose had been formed from the linear substrate, 65% from glycogen and 74 to 78% from the other substrates.

For any given substrate, the production of glucose increases as the hydrolysis proceeds. The linear component from corn starch is hydrolyzed more extensively than the branched chain substrates and eventually produces the most glucose. On the other hand, comparisons of the hydrolyzates at equivalent stages in the hydrolyses of the different substrates, when the equivalent of the same per cent. of theoretical maltose had been formed, show that glucose is produced earlier and in slightly higher concentrations from the branched-chain substrates than from the linear substrate. Similar results have been obtained with pancreatic amylase.[42]

The data given in Table V show that glucose and maltose account for all of the reducing value of the 24-hour hydrolyzate of the linear substrate at a relative concentration of taka amylase of 100. The analytical data from the reducing values were confirmed by selective fermentation[22-24] and by chromatographic measurements[25-27] which failed to show any trace of hydrolysis products other than glucose and maltose.

The data given in Table V confirm and extend the evidence that the branched chain substrates are

(42) R. B. Alfin and M. L. Caldwell, THIS JOURNAL, **71**, 128 (1949).

hydrolyzed less extensively under the same conditions by maltase-free taka amylase than the linear substrate. Significant concentrations of low molecular weight dextrins were present in the later stages of the hydrolyses of the branched substrates. The concentration and the average molecular weights of these products were higher in the hydrolyzates of glycogen than in those of the other branched chain substrates. The products in these hydrolyzates are being investigated.

Michaelis-Menten Constants.—The action of α-amylases in causing the rapid break-down of their substrates to products of relatively low molecular weights[43,44] makes uncertain the applicability of calculations of Michaelis constants[45,46] to their action and introduces questions about the interpretation and significance of such constants when calculated for α-amylase action. In spite of these drawbacks, it seemed of interest to determine the Michaelis-Menten constants[45,46] for the comparable action of taka amylase upon the substrates studied here. The concentrations of maltose equivalents produced by the action of taka amylase upon each of several different concentrations of each substrate were plotted against time and the initial velocities calculated for each of the substrate concentrations. Values for the Michaelis-Menten constants then were determined by two of the graphical methods recommended by Lineweaver and Burk[46] and averaged for each substrate.

The substrate concentrations and consequently the Michaelis-Menten constants are expressed on the basis of per cent. concentration of substrate as is customary[47,48] with substrates for which accurate molecular weights are not known. The values for K_s are translated also into molar values using the best available data for the molecular weights of the substrates.[40,49-51] The data are summarized in Table VI.

TABLE VI

MICHAELIS-MENTEN CONSTANTS OF TAKA AMYLASE FOR SEVERAL SUBSTRATES

Substrate	Michaelis-Menten constants K_s, %	K_s M	Affinity constant $K_m = 1/K_s$, %	Molecular weights
Linear fraction from corn starch	0.035	2.7×10^{-6}	29	1.5×10^5 (49,50)
Potato starch	.041	.....	25	.....
Waxy maize starch	.048	[3.2×10^{-7}]	21	[1.5×10^6][a]
Branched fraction from corn starch	.050	3.3×10^{-7}	20	1.5×10^6 (49,50)
Glycogen	.42	1.3×10^{-7}	2.4	1.5×10^6 (51)
β-Schardinger dextrin	.57	5.0×10^{-8}	1.8	1134 (40)
α-Schardinger dextrin	.93	9.5×10^{-8}	1.1	978 (40)

[a] Molecular weight assumed from value for branched fraction from corn starch.

When calculated on the basis of percentage concentration of substrate which we prefer, the Michaelis-Menten constants for taka amylase given in Table VI are similar for the linear and branched substrates from corn starch, for unfractionated potato starch and for waxy maize starch. In this respect taka amylase resembles malt α-amylase[47] and pancreatic amylase[48] and differs from malt β-amylase[52,53] and from gluc amylase.[54] On the other hand, examination of the data shows that the Michaelis-Menten constant for taka amylase is somewhat lower with the linear fraction from corn starch than with the other substrates in the comparison. This finding suggests that taka amylase has the highest affinity for this linear substrate. The Michaelis-Menten constant for taka amylase with glycogen is approximately 10 times higher than those for the other branched substrates. Malt α-amylase[47] also is reported to have a low affinity for glycogen.

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(43) K. Myrbäck, "Advances in Carbohydrate Chemistry," Vol. III, Academic Press, Inc., New York, N. Y., 1948, p. 252.
(44) M. L. Caldwell and M. Adams, *ibid.*, Vol. V, 1950, p. 229.
(45) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).
(46) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(47) S. Schwimmer, *J. Biol. Chem.*, **186**, 181 (1950).
(48) P. Bernfeld and H. Studer-Pécha, *Helv. Chim. Acta*, **30**, 1904 (1947).
(49) T. Deszczynski, Dissertation, Columbia University, New York, N. Y., 1949.
(50) C. O. Beckmann and Q. Landis, THIS JOURNAL, **61**, 1495 (1939).
(51) R. Basil, *J. Chem. Soc.*, 1567 (1948).
(52) R. H. Hopkins, *Advances in Enzymology*, **6**, 389 (1946).
(53) K. H. Meyer and J. Press, *Helv. Chim. Acta*, **24**, 50 (1940).
(54) L. L. Phillips and M. L. Caldwell, THIS JOURNAL, **73**, 3563 (1951).