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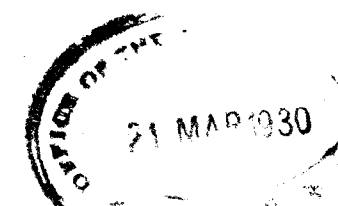
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[Vol. 13A, Part IV, pp. 31 to 41.]

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CONTENTS

I. STUDIES ON DEXTRINS. PART I. ACTION OF AMYLASE FROM CHOLAM (*SORGHUM VULGARE*) ON POTATO STARCH.

BY

Vinayak Narayan Patwardhan.

II. AMYLASE FROM RAGI (*ELEUSINE CORACANA*).

BY

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O. FORSTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.

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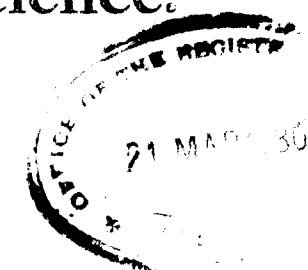
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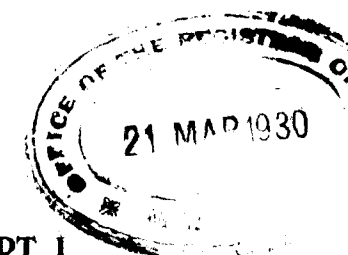
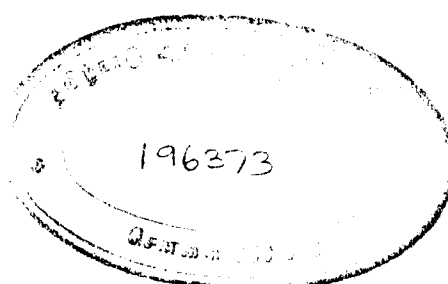
Vinayak Narayan Patwardhan.

II. AMYLASE FROM RAGI (*ELEUSINE CORACANA*).

BY

V. N. Patwardhan and Nuggihalli Narayana.

DR. M. O. FORSTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.



I.—STUDIES ON DEXTRINS. PART I.
Action of amylase from Cholan (*Sorghum vulgare*)
on potato starch.

By Vinayak Narayan Patwardhan.

The dextrins form an important group of carbohydrates occurring under various conditions and invariably formed as intermediate products during enzyme-hydrolysis of starch; they possess considerable interest from the academic and the nutritional point of view, but our knowledge of them is comparatively obscure. The only criteria at present available for establishing their identity and purity are reducing power, specific rotation and behaviour on enzyme-hydrolysis. It is therefore not possible to state from our present knowledge whether any of the dextrins so far prepared is a single substance or a mixture.

Musculus (*Compt. rend.*, 1860, 50, 785) was the first to show that sugar and dextrins were formed simultaneously during the hydrolysis of starch. O'Sullivan (*J.C.S.*, 1872-76), in his systematic studies on the breakdown of starch by diastase showed conclusively that maltose was the sugar formed in the hydrolysis and was always accompanied by dextrins. He stated that the formation of dextrin increased with rise of temperature, more dextrin than maltose being formed between 60° and 70°.

Brown and Heron (*J.C.S.*, 1879, 35, 596) stated that diastatic hydrolysis of starch takes place by stages, one molecule of maltose being set free at each step accompanied by a lower dextrin. Brown and Morris (*J.C.S.*, 1889, 55, 449) isolated an amylo-dextrin by the action of dilute mineral acids on ungelatinised starch in the cold, and showed that it differed from soluble starch. Lintner (*Ber.*, 1893, 26, 2533) observed that by the action of diastase on starch three dextrins were formed, besides maltose and isomaltose. He named them amylo-dextrin, $(C_{12}H_{20}O_{10})_{54}$, erythro-dextrin, $(C_{12}H_{20}O_{10})_{17} \cdot (C_{12}H_{22}O_{11})$ and achroo-dextrin, $(C_{12}H_{20}O_{10})_5 \cdot (C_{12}H_{22}O_{11})$ respectively. Amylo-dextrin did not reduce Fehling solution, but gave a blue colour with iodine: erythro-dextrin reduced Fehling solution and gave a brown colour with iodine, while achroo-dextrin reduced Fehling solution, but gave no colour with iodine. Ling and Baker (*Proc. C. S.*, 1896-1897, 3) separated two dextrins, maltodextrin- α and maltodextrin- β from the reaction mixture of starch and diastase at 70°. Brown and Millar (*J.C.S.*, 1899, 75, 315) obtained a 20 per cent. yield of stable dextrin and 80 per cent. of maltose by the action of diastase on starch at 60°.

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The dextrin had a reducing power (R), 5.5–5.9 (maltose, 100) and specific rotation, $[\alpha]_D$ 195–196°. When hydrolysed by diastase this dextrin yielded equal quantities of maltose and glucose, the hydrolysis being very slow. Zulkowski and Franz (*J.C.S., Abstr.*, 1896, i, 120) showed that dextrans were also formed by heating starch with glycerol at temperatures between 190° and 210°. The action of *Bacillus macerans* on starch (Schardinger, *J.C.S., Abstr.*, 1911, i, 181, from *Centr. Bakt.*, 1911, 29, ii, 188) produced bodies resembling dextrans; some were crystalline and others amorphous, but all were unfermentable by yeast.

Latterly Gatin-Gruzevska (*Compt. rend.*, 1908, 146, 540, and *ibid.*, 1911, 152, 785) claimed to have separated amylopectin and amylose from the granules of starch. Amylose was soluble in cold water, while amylopectin only gelatinised in hot water. Pringsheim and Eissler (*Ber.*, 1913, 46, 2959) isolated two kinds of dextrin by the action of Schardinger's bacillus on starch, naming them dextrin- α or tetra-amylose and dextrin- β or hexa-amylose; underlying this nomenclature was the idea that polyamyloses formed the structural basis of the starch molecule. They subjected the polyamyloses to the action of malt diastase, taka-diastase, emulsin, *Penicillium africanum* and yeast. Pringsheim and his collaborators devoted much attention in later years to the chemical constitution of polyamyloses, mention of which will be made in a later paper dealing with that particular aspect.

Ling and Nanji (*J.C.S.*, 1923, 123, 2666) determined the ratio of amylose to amylopectin in the molecule of starch and found it to be 1:2. They found that when alcohol-dried amylase from ungerminated barley was allowed to act on starch-paste at 50°, amylose was converted quantitatively to maltose, while amylopectin was left entirely unaffected. Undried barley amylase, on the other hand, was observed to attack amylopectin and give $\alpha\beta$ -hexa-amylose. Ling and Nanji (*J.C.S.*, 1925, 127, 629) also studied the stable dextrin isolated by Brown and Morris in 1885. The dextrin had the following constants:—R, 14; $[\alpha]_D$ 185.0° and molecular weight, 1923; it could be hydrolysed by malt diastase, emulsin and maltase to give mixtures of glucose, maltose and isomaltose.

Baker and Hulton (*J.C.S.*, 1929, 1655) observed that precipitated amylase from ungerminated oats hydrolysed potato starch to maltose only, but that when the reaction was stopped before completion, α -amylodextrin (R, 1.5 and $[\alpha]_D$ 184.1°) identical with that obtained by the action of amylase from ungerminated rye on potato starch (Baker and Hulton, *J.C.S.*, 1921, 119, 805) was the product. Amylase from germinated oats gave a dextrin with R, 9.3 and $[\alpha]_D$ 185.9°.

while germinated rye amylase gave a dextrin with the constants R, 10.8 and $[\alpha]_D$ 181.9°. These results of Baker and Hulton do not fit in with the theory first brought forward by Maquenne and Roux (*Compt. rend.*, 1905, 140, 1303) and later, elaborated by Ling and Nanji (*loc. cit.*), namely, that starch is not a homogeneous substance but is built up of two substances, polymerised amylose and amylopectin.

The work in this laboratory supports the views of Baker and Hulton. During the hydrolysis of potato starch by amylase from cholam (*Sorghum vulgare*) four different dextrans were isolated. To avoid confusion of nomenclature they have been tentatively named Dextrans-I, II, III and IV. Dextrin-I was isolated from the reaction mixture containing cholam malt amylase and potato starch when it just failed to give a purple colour with iodine. Dextrin-II represents the stable dextrin of Brown and Morris. It was obtained when the reaction between cholam malt amylase and potato starch had reached an equilibrium, which was after about 80 per cent. starch had been converted into maltose. Alcohol-dried amylase from ungerminated cholam saccharified starch to the extent of 30 per cent. in sixteen hours; nearly 66 per cent. of non-saccharine solids could be precipitated from the reaction mixture and were separated into two fractions, Dextrin-III and Dextrin-IV. The former was non-reducing and the latter only feebly reducing.

The crude dextrans were purified by redissolving and reprecipitating from water several times, using 95 per cent. alcohol as the precipitant, till reducing power and specific rotation were found to remain unchanged by subsequent treatment. They were then subjected to prolonged extraction with dry methyl alcohol and later, with 80 to 95 per cent. ethyl alcohol to ensure complete removal of sugar. The dextrans as finally obtained were white amorphous powders soluble in water. Dextrans-I and II dissolved in cold water giving clear solutions, while the other two dissolved only on boiling. The constants for the pure dry substances were as follows:—

Dextrin	I	II	III	IV
Reducing power (glucose 100) ...	3.0	5.0	0	less than 1
Specific rotation, degrees ...	174.0	155.9	170.6	184.2

The constants for Dextrin-IV suggested that it was probably identical with α -amylodextrin of Baker and Hulton.

The dextrans were submitted to hydrolysis by amylases from malted and ungerminated cholam. Dextrin-II was resistant to the action of malted cholam amylase at P_H 7.0, while at P_H 4.67 it was hydrolysed to the extent of about 20 per cent. The other dextrans

were rapidly hydrolysed by both the enzymes at the optimal temperature and hydrogen-ion concentration (P_H 4.67). The enzyme from ungerminated cholam attacked all four dextrans more effectively than that from malted cholam and carried the hydrolysis much further. In no case, however, did hydrolysis proceed to completion; under the most favourable circumstances the dextrans were hydrolysed as follows:—

Dextrin	I	II	III	IV
Hydrolysis per cent. ...	61.6	35.8	66.3	74.8

EXPERIMENTAL.

Preparation and hydrolysis of Dextrin-I.

Malted cholam powder (10 gms.) was extracted with cold water for two hours with frequent shaking, the filtered extract being diluted to 100 c.c. Potato starch (100 gms.) was made into paste with three litres of boiling water and after boiling for a few minutes cooled to 50°, at which temperature the paste and the enzyme were mixed. The reaction was allowed to proceed at the same temperature and stopped by boiling the mixture after it ceased to give a purple colour with iodine. The mixture was then concentrated on the water bath and the dextrin precipitated with 2½–3 volumes of 95 per cent. alcohol. The precipitate was purified in the manner described already.

Hydrolysis of Dextrin-I at 50°.—Flasks containing 50 c.c. of dextrin solution (2 per cent.), 20.85 c.c. of malted cholam enzyme (0.24 per cent.) with 19.15 c.c. of buffer (McIlvaine's) and 1 c.c. of toluene were kept in a thermostat. Sugar was determined at intervals.

TABLE I.

Time in hours	Mgms. maltose	
	P_H 4.67	P_H 7.05
1	23.8	...
2	31.2	17.3
4	40.0	...
24	59.6	32.7
48	65.0	37.5

Preparation and hydrolysis of Dextrin-II.

Starch paste and enzyme solution were prepared as before; the reaction proceeded at 45° with 5 c.c. of toluene for 54 hours. The dextrin was obtained and purified as stated above.

Hydrolysis of Dextrin-II at 37° and P_H 4.67.—Four flasks were arranged, each containing 25 c.c. dextrin solution (2 per cent.) and 15 c.c. of buffer solution with (1) 10 c.c. of malted cholam enzyme (0.2 per cent.), (2) 10 c.c. of ungerminated cholam enzyme (0.2 per cent.), (3) 10 c.c. of mixture of equal volumes of malted and ungerminated cholam enzymes and (4) 10 c.c. of water. The dextrin being very resistant to hydrolysis sugar was determined only after 48 and 90 hours. At the end of the latter period, the percentages of hydrolysis in the different cases were as follows:—(1), 20.8; (2), 35.8; (3), 29.8; (4), 0.

Preparation and hydrolysis of Dextrans-III and IV.

Alcohol-dried amylase (0.5 gm.) was shaken with cold water for half an hour and made up to 100 c.c. after filtration; 100 gms. of potato starch were made into a paste with three litres of water and the paste cooled to 50°. The solutions were mixed and the reaction allowed to proceed at 50° after adding 5 c.c. of toluene. In sixteen hours the paste was observed to be completely liquefied and the coloration with iodine was purple. The mixture was raised to the boiling-point and concentrated on the water-bath to 800 c.c. when 1300 c.c. of 95 per cent. alcohol was added, the precipitate being allowed to settle for 24 hours. The precipitate was observed to be in two layers, a stiff, horny mass below surmounted by a white, fluffy powder; these were separated and dehydrated. On further treatment and purification they were found to be two different dextrans, the horny mass on complete dehydration giving a white powder (Dextrin-III). The yields of crude substances were 35 and 33 gms. respectively; there was not much loss during the purification.

Dextrans-III and IV were only slightly soluble in cold water, but dissolved on boiling, and remained in solution on cooling.

Hydrolysis of Dextrin-III.—Three flasks each containing 50 c.c. of dextrin solution (2 per cent.) and 25 c.c. of buffer solution together with, in flasks 1 and 2, 25 c.c. malted cholam enzyme, and in the third flask 25 c.c. of ungerminated cholam enzyme, were kept in a thermostat at 30°, 0.5 c.c. toluene being added to each flask. Sugar was determined at intervals.

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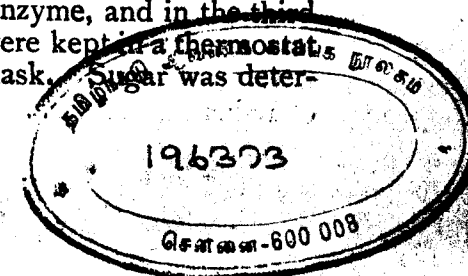


TABLE II.

Time in hours	Mgms. of maltose		
	Cholam Malt		Ungerminated cholam
	P _H	7.05	4.67
24		21.1	31.1
48		27.6	39.4
72		31.2	44.5
92		...	..
			70.3

Hydrolysis of Dextrin-IV.—Reaction mixtures were prepared as described in the previous trial except that only two flasks were arranged for malted and ungerminated enzymes at P_H 4.67. The reaction was conducted in a thermostat at 30°.

TABLE III.

Time in hours	Mgms. of maltose	
	Cholam malt	Ungerminated cholam
5	25.6	30.1
22	36.5	55.9
92	54.4	79.0

The composition of these dextrins is unknown, but attempts are being made to determine their nature.

SUMMARY.

1. Four dextrins were isolated from the products of hydrolysis of potato starch, and their reducing power and specific rotation determined; one dextrin appears to be identical with the α -amylodextrin of Baker and Hulton.

2. The dextrins were hydrolysed by the enzymes from malted and ungerminated cholam. The latter attacked the dextrins more readily and carried the hydrolysis farther than the former.

The author has great pleasure in gratefully acknowledging the useful advice and help rendered by Prof. Roland V. Norris during the course of the work.

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II.—AMYLASE FROM RAGI (*ELEUSINE CORACANA*).

By Vinayak Narayan Patwardhan and Nuggehalli Narayana.

The abnormal results obtained as a result of prolonged dialysis of the amylase from *Zea mais* (Patwardhan, *J. Indian Inst. Sci.*, 1929, 12A, 185) interested the author to extend the investigation to similar enzymes from other seeds. The present investigation deals with the enzyme from ragi.

That the activity of an amylase is diminished after dialysis and that the loss is more pronounced in case of an animal amylase than in one of vegetable origin is well known (Guyenot, *Compt. rend. Soc. Biol.*, 1907, 63, 768; Brunacci, *Chem. Absts.*, 1910, 4, 2324; Lisbonne, *Compt. rend. Soc. Biol.*, 1911, 70, 62; Biery, *Compt. rend. Soc. Biol.*, 1922, 87, 1111). The activity thus lost by an animal amylase can, however, be restored by the addition of neutral salts and certain amino-acids (Sherman and Walker, *J. Amer. Chem. Soc.*, 1923, 45, 1960; Haehn, *Biochem. Zeit.*, 1923, 143, 516). The behaviour of amylases from vegetable sources is not however, so well established. Contradictory evidence is forthcoming regarding loss of activity by barley malt amylase on dialysis, and the possible reactivation of vegetable amylases in general is still an open question (Eadie, *Biochem. J.*, 1926, 20, 1016). Like the maize amylase, the enzyme from ragi lost its activity on prolonged dialysis and was not reactivated on addition of neutral salts, amino-acids or buffer solutions. The cause of such behaviour might have been the same in both cases, but it was felt that an intensive study of the subject was necessary before any conclusions could be drawn.

The ragi enzyme had greater saccharifying power than cholam malt and maize malt enzymes, but less than that of barley malt amylase. The optimum temperature lay between 55° and 60°. It was less liable to destruction at higher temperatures, which were observed to cause rapid inactivation of the cholam enzyme. The optimum reaction was between P_H 4.86 and 5.07. There was no evidence to show that a thermostable amylase complement occurred with the enzyme.

EXPERIMENTAL.

Malting and preparation of the enzyme.—The methods were identical with those described in the earlier communications (Patwardhan, *loc. cit.*)

Hydrolysis of potato starch by malted ragi amylase.—(a) A flask containing 40 c.c. of 2 per cent. starch paste, 30 c.c. water and 2 c.c.

toluene was brought to 30° and 10 c. c. of enzyme solution (0.502 per cent.) added. Sugar was determined at intervals.

TABLE I A.

Time in mins.	Mgms. maltose in 10 c.c.	Colour with iodine
0	0	Deep blue
15	4.8	Deep purple
30	13.4	Purple
60	26.8	Brown
100	38.9	Brown
1430	84.7	Nil

The reaction reached the equilibrium point in 24 hours, when about 80 per cent. of the starch had been saccharified to maltose. Comparative figures for maltose production in 100 minutes from the start of the hydrolysis as observed for different enzymes are given below.

Mgms. maltose ...	AMYLASES			
	Barley	Ragi	Maize	Cholam
	76.8	38.9	29.0	18.4

(b) To 8 c.c. of starch paste (2 per cent.) and 6 c.c. of water at 30° 2 c.c. enzyme solution (0.502 per cent.) was added in a viscosimeter and the readings taken at intervals.

TABLE I B.

Change of Viscosity in terms of time of flow (t) in secs.

Time of reading in minutes	1	15	30	45	60	102
t	211.0	125.5	120.5	114.0	114.0	110.0

Effect of change of temperature.

Eight flasks containing 15 c.c. each of starch paste (2 per cent.) were brought to the desired temperature and 15 c.c. of 2 per cent. ragi malt extract added. Readings were taken after hydrolysis for 30 minutes in each case.

TABLE II.

Temperature Centigrade	Mgms. maltose in 10 c.c.	Mgms. ppt. (dry) in 10 c.c.
30	18.9	85.6
40	19.2	83.5
45	22.7	67.9
50	24.2	...
55	35.8	59.3
60	38.2	52.3
65	27.9	77.4
70	25.6	81.5

Effect of Change of hydrogen-ion concentration.

A series of flasks containing 15 c.c. of starch paste and 10 c.c. of McIlvaine's buffer solution were brought to 30° and 5 c.c. ragi malt extract (6 per cent.) added to each. Readings were taken after hydrolysis for 30 minutes.

TABLE III.

P _H	Mgms. maltose in 10 c.c.	Mgms. dry ppt. in 10 c.c.
7.0	19.5	175.3
6.6	27.4	190.5
6.15	28.5	175.0
5.96	28.5	173.0
5.8	29.4	171.4
5.6	29.4	163.8
5.26	31.7	164.8
5.07	32.3	159.4
4.86	32.3	155.2
4.67	29.9	163.0
4.50	29.0	160.5
4.34	29.0	164.4

Effect of dialysis on ragi malt enzyme.

Tubes prepared from 6 per cent. collodion were used for dialysis. All dialyses were carried out in a cold chamber between 0° and 3° with toluenated water in presence of toluene.

(a) 0.502 per cent. enzyme when dialysed lost its activity as shown below:—

No. of days	...	0	2	5	9	9
c.c. KMnO ₄ for 10 c.c.	4.70	3.50	3.05	1.05	1.10 (Salt	added to the last one)

(b) Ragi malt extract (22.5 per cent.) lost its activity practically completely in sixteen days. Extract in a glass tube kept in the same dialyser as control had lost only a little of its activity even after 28 days.

No. of days	...	...	...	...	0	3	16	17	28
c.c. KMnO_4 required for 10 c.c. (corrected)	...	9.30	5.30	0.05	0.05	...			
Control— KMnO_4 required for 10 c.c. (corrected)	9.30	...	...	...	8.00				

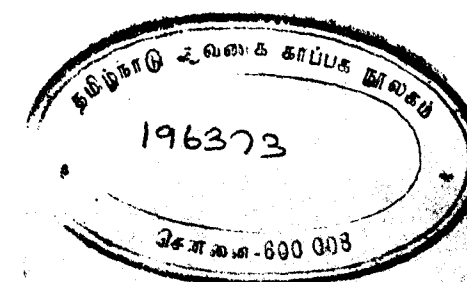
The activity could not be regenerated by addition of salt, buffer solutions of known P_H or asparagine. The details of the trials were the same as those described in the paper on maize amylase.

(c) Experiment to test the regenerating action of boiled enzyme.

Three flasks, A, B and C each containing 12.5 c.c. of dialysed enzyme and 12.5 c.c. of 2 per cent. starch paste were treated as follows:—To A, 7.5 c.c. of boiled, undialysed enzyme was added, to B, 7.5 c.c. of undialysed, unboiled enzyme, and to C, 7.5 c.c. of distilled water. The volumes of permanganate required after 1 hour at 30° were, A, 0.05; B, 4.85; and C, 0.40 c.c. respectively. The results show clearly that the boiled and dialysed enzymes were inactive, singly and together.

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