the volume made up to 10 ml. After standing overnight the maltose produced was determined by the alkaline ferricyanide method[10]; observed, 57.5% maltose; calcd. for two moles of maltose per mole of amyloheptaonate, 56.7%. A similar digest was subjected to electrophoretic analysis and the amylotrionate produced in the enzymolysis identified by its characteristic electrophoretic mobility.

Amylotriose.—A 4-g. sample of amyloheptaose was treated with an excess of β-amylase solution and allowed to stand overnight. The products of hydrolysis were worked up by fractional precipitation with 95% ethanol, retaining and reprecipitating the least soluble fraction. After four precipitations there was obtained 1.0 g. (58%) of an amorphous saccharide with the properties: $[\alpha]_D$ +158.0 (*c*, 1 in H_2O); mol. wt. by hypoiodite[11] 501.9, by alkaline ferricyanide[10] 477; calcd. for amylotriose[12]: $[\alpha]_D$ +158.1,[10] mol. wt. 504.

Rate of Action of β-Amylase on Amyloheptaose.—Parallel digests of amyloheptaose and soluble starch were set up as follows: 0.25 g. of substrate was treated with an amount of enzyme solution corresponding to 0.25 mg. of the dry preparation, diluted to 25 ml. and incubated in a water-bath at 40°. From time to time samples were withdrawn and the increase in reducing value determined. In the initial phase of the digest (0–30% maltose formed) the rate of hydrolysis was constant and the same with both starch and amyloheptaose (7 mg. maltose/min. under these conditions). After complete hydrolysis of the amyloheptaose sample, the increase in reducing power corresponded to the formation of 62.5% maltose; calcd. for two moles of maltose, 57.5%.

(10) Levine, Foster and Hixon, THIS JOURNAL, **64**, 2331 (1942).

(11) Kline and Acree, *Ind. Eng. Chem., Anal. Ed.*, **2**, 413 (1930).

(12) Sugihara and Wolfrom, THIS JOURNAL, **71**, 3357 (1949), report $[\alpha]^{23}_D$ +160°.

Summary

1. Soy bean β-amylase hydrolyzes amyloheptaose or amyloheptaonate to give two moles of maltose and one mole of amylotriose or amylotrionate, respectively.

2. The preparation and analytical values for amorphous amylotriose are reported.

3. Amylotriose and amylotrionate are stable to the action of β-amylase.

4. β-Amylase produces maltose from amyloheptaose and starch at essentially the same rate.

5. Amylopentaose does not accumulate in significant amount during the enzyme digests.

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[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY AGRICULTURAL EXPERIMENT STATION, AND THE DEPARTMENT OF PHYSICS, PURDUE UNIVERSITY]

Behavior of Low Molecular Weight Amylose with Complexing Agents[1]

BY WILLIAM DVONCH, H. J. YEARIAN[2] AND ROY L. WHISTLER

A number of fractionating agents have been discovered which selectively precipitate amylose from a starch–water dispersion. Schoch initially discovered the action of *n*-butyl alcohol[3] and later[4] the similar action of other monohydroxy alcohols and of oleic acid.[5] The number of compounds with a selective precipitation action for amylose was further extended by Whistler and Hilbert[6] to include those containing nitro, ester, ketone, mercapto groups and cyclic nitrogen as in pyridine. Most recently, Haworth, *et al.*,[7] have recommended thymol.

All these complexing agents give approximately the same yield of amylose from the same source, about 22–29% from corn starch, and the purity as determined by iodine titration does not vary significantly. This uniformity of behavior among a group of compounds of such widely different nature is rather surprising. The present work was undertaken to see if this similarity exists with amylose of low molecular weight. The agents chosen for investigation were *n*-butyl alcohol, 2-nitropropane, *n*-amyl acetate, *n*-amyl methyl ketone and nitrobenzene[8] as being representative of the various types. Amylose was degraded by acid hydrolysis until only a portion of it would precipitate in the presence of each agent after two to four days of slow cooling in the manner in which amylose is usually isolated. Unlike the behavior of high molecular weight amylose, precipitation was not complete at this point, but continued for several weeks. These precipitates were also collected. The fractions so obtained were analyzed for molecular weight by the spectrophotometric method of Swanson.[9] Iodine sorption[10] and X-ray diffraction patterns were also obtained.

Experimental

Hydrolysis and Fractionation Procedure.—Approximately 240 g. of once-recrystallized butanol–amylose paste prepared by butanol precipitation from potato or

(1) Journal Paper No. 367 of the Purdue University Agricultural Experiment Station.

(2) Department of Physics, Purdue University.

(3) T. J. Schoch, THIS JOURNAL, **64**, 2957 (1942).

(4) T. J. Schoch, "The Fractionation of Starch" in *Advances in Carbohydrate Chemistry*, Academic Press, Inc., New York, N. Y., 1945, Vol. 1, p. 247.

(5) T. J. Schoch, THIS JOURNAL, **66**, 1232 (1944).

(6) R. L. Whistler and G. E. Hilbert, *ibid.*, **67**, 1161 (1945).

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

(8) Nitrobenzene is recommended as a fractionating agent in unpublished work of Whistler, Johnson and Hilbert. As the amount of a complexing agent needed is roughly determined by its solubility in water, nitrobenzene is useful in large-scale fractionations as only limited quantities are required in contrast to the large amounts required for other agents. With nitrobenzene the yields of amylose are dependent on *p*H. A maximum occurs in the range *p*H 7.5 to 9.0.

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

(10) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943); E. J. Wilson, T. J. Schoch and C. S. Hudson, *ibid.*, 1380.

TABLE I

OBSERVED SPACING AND ORDER OF INTENSITY OF DIFFRACTION RINGS OF AMYLOSE[a] COMPLEXES

Complexing agent	Spacing, Å.	Order of intensity
	11.8	VS (very strong)
	7.5	F (faint)
	6.7	VF
	5.9	M (moderate)
Nitrobenzene	5.3	S
	4.6	VF
	4.2	F
	3.9	F
	3.4	VF
	2.6	VF
	9.5	F
n-Amyl acetate	7.2	F
	4.5	S
	13.1	M
	10.2	F
	9.3	F
n-Amyl methyl ketone	7.2	F
	5.9	VF
	5.1	M
	4.6	S

[a] Once-recrystallized potato amylose.

corn starch was dispersed in 6 liters of boiling water and heated for one and one-half hours to remove the butanol. The solution was poured through cheesecloth into a warmed 9-liter serum bottle and made up to 5,900 ml. (1% solution) at about 60°. The solution was placed in a large water-bath maintained at 98° and stirred by means of a glass centrifugal-type stirrer operating through a short air condenser. After temperature constancy had been attained, 15.00 ml. of 2.00 *N* sulfuric acid was added to make the acid concentration about 0.005 *N*. At the end of the hydrolysis period of six to nine hours, the solution was neutralized with sodium hydroxide and supercentrifuged. Ten-ml. samples were withdrawn for analysis for dry substance,[11] and 50.00-ml. samples for determination of reducing substance.[12]

The solution was reheated to 90° and sufficient complexing agent was added to saturate the solution. With nitrobenzene, 25 ml. of a saturated sodium bicarbonate solution was added to bring the *p*H to 7.5–9.0. After the solution was cooled for two to four days, the precipitate was collected by supercentrifugation, freed from water and complexing agent by four passages through ethanol in the Waring Blendor and dried over calcium chloride *in vacuo*. Yields were based on this weight of sample as the 2–3% moisture content does not appreciably affect the results. After drying, the fraction was ground to pass a 60-mesh screen and equilibrated to room conditions.

The centrifugate from the initial precipitation was allowed to stand at room temperature and the precipitate which formed was collected at a suitable time. During this period, no evidence of microbial growth was noted.

Analytical Methods.—For the determination of the degree of polymerization (DP) by spectrophotometric absorption,[9] a 17-mg. sample was added to 50 ml. of boiling water, boiled until solution was complete and diluted to 100 ml. A 10-ml. aliquot of this was taken to prepare a solution 5 mg. per cent. in amylose, 10 mg. per cent. in iodine, 20 mg. per cent. in potassium iodide and 0.01 *N* in sulfuric acid for analysis.

All X-ray patterns were obtained by exposing for two hours a 1-mm. thick portion of the fresh moist precipitate to filtered copper radiation at 35 K. V. P., 23 ma. and a specimen-to-film distance of 5.0 cm. As they were necessary for comparison, the diffraction patterns of nitrobenzene–, *n*-amyl acetate– and *n*-amyl methyl ketone–amylose complexes were obtained and the spacing and the relative order of intensity of the rings are shown in Table I.

Results and Discussion

The precipitates from *n*-butyl alcohol and 2-nitropropane are gelatinous and appear microscopically as irregular fragments with some clusters of fine needles present in the *n*-butyl alcohol precipitate. In contrast, the precipitates from nitrobenzene, *n*-amyl acetate and *n*-amyl methyl ketone are granular and appear as uniform spherical particles. Particles similar to these from the second group of agents were obtained when a hydrolyzed amylose solution was allowed to retrograde under the conditions similar to those for complex formation. X-Ray examination of the initial precipitates from the second group of complexing agents showed that they all gave a "B" pattern identical with the retrograded material. On the other hand, the initial *n*-butyl alcohol and 2-nitropropane precipitates gave their typical amylose-complex patterns. While the second *n*-butyl alcohol precipitate gave the typical complex pattern, the second 2-nitropropane precipitate gave a pattern whose

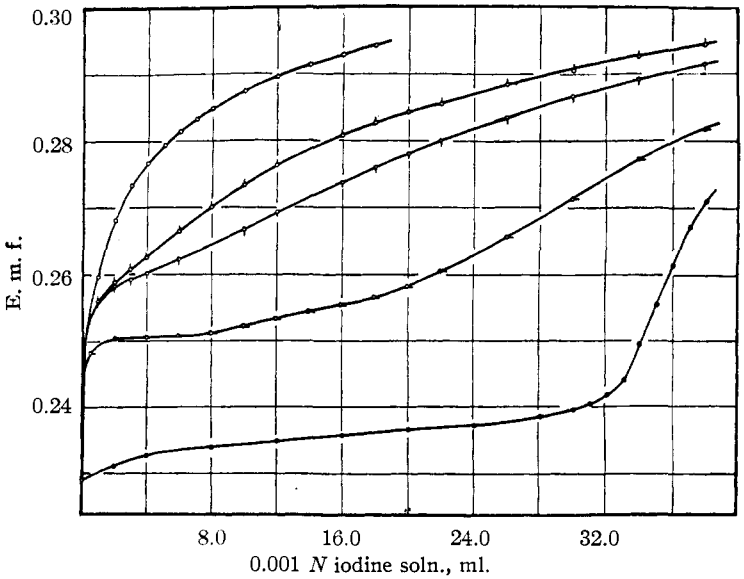

Fig. 1.—Titration curves of amylose fractions of different DP: ○, 18; ○, 20; ○-, 46; ●, undegraded potato amylose; O, blank.

(11) J. E. Cleland and W. P. Fetzer, *Ind. Eng. Chem., Anal. Ed.*, **13**, 858 (1941).

(12) P. A. Shaffer and A. F. Hartmann, *J. Biol. Chem.*, **45**, 365 (1921).

TABLE II

DP OF AMYLOSE FRACTIONS[a]

Hydrolysis, hr.	Red.[b] val., %	Complexing agent	First fraction Time, d.	First fraction Yield, %	First fraction DP	Second fraction Time, d.	Second fraction Yield, %	Second fraction DP
6.5	...	*n*-Butyl alc.	2	25	56	18	2	23
5.5	...	*n*-Butyl alc.	2[c]	8	35	37[c]	5	27
9.0	7.0	2-Nitropropane	2	20	40	39	4	27
7.0[d]	4.9	None	2	25	40	38	16	29
7.0[d]	4.9	Nitrobenzene	2	16	40	46	14	35
7.0[e]	5.0	*n*-Amyl acet.	4	47	40	40	13	29
7.0[e]	5.0	*n*-Amyl methyl ketone	4	48	40	40	12	27

[a] All fractions obtained from potato amylose except as noted. [b] Reducing substance calculated as dextrose divided by dry substance. [c] Obtained from corn amylose. [d,e] Hydrolyzate was divided into two parts and separately precipitated.

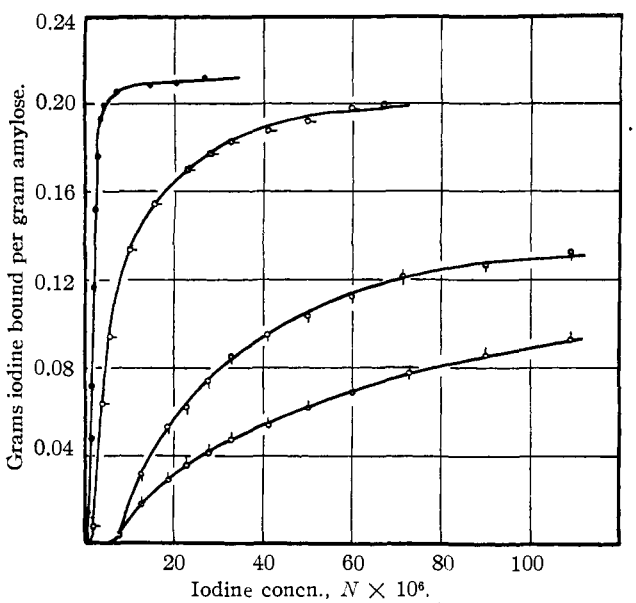

Fig. 2.—Amount of iodine bound by amylose fractions of different DP: ○, 18; ○, 20; ○-, 46; ●, undegraded potato amylose.

4 rings (*d*, Å.; 16.3, VF; 5.3, VF; 4.5, S; 3.7, VVF) correspond in position to a "B" pattern.

The values for the average DP of the precipitates are shown in Table II. It is evident that each of the complexing agents tested should have some limit to the amylose molecular weight it is able to precipitate. Under the experimental conditions used here, the limit for *n*-butyl alcohol lies below a DP of about 20. For 2-nitropropane, the limit is in the range of DP 30–40. Nitrobenzene, *n*-amyl acetate and *n*-amyl methyl ketone have this limit above a DP of 40.

In their paper on the potentiometric iodine titration of amylose, Bates, *et al.*,[10] report that a very short chain amylose required a very much larger iodine activity for complex formation than potato amylose. This is borne out by the data in Fig. 1 which shows the iodine titration curves of amylose fractions of different DP. It is seen that the e. m. f. of complex formation varies inversely with DP and the curve approximates an amylopectin curve with decreasing DP.

Plotting grams of iodine bound *vs.* iodine concentration, as in Fig. 2, in order to attempt to differentiate between complex formation characteristic for amylose and surface adsorption characteristic for amylopectin as done by Bates, *et al.*,[10] it is evident that the two processes merge and it becomes impossible to separate them at a DP of less than about 50.

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Summary

When amylose is acid-hydrolyzed to a DP of about 20–40, it can no longer form insoluble complexes with nitrobenzene, *n*-amyl acetate and *n*-amyl methyl ketone. However, it is still capable of forming an insoluble complex with *n*-butyl alcohol and to some extent with 2-nitropropane.

The e. m. f. of iodine complex formation varies inversely with the DP of amylose and with a DP of less than about 50 it is not possible to separate surface adsorption from complex formation.

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