microns, which is near Simerl and Browning's[10] optimum wave length values for the estimation of this blue color. The photocolorimeter was adjusted so that the blank which had a light yellow color, due to the iodine, gave a reading of 0.

The readings on the logarithmic scale of the colorimeter are directly proportional to the concentration, if Beer's law holds. In Fig. 1 direct scale readings are plotted against percentage concentrations of mixtures of amylose and amylopectin. The total starch concentration, whether a pure sample or a mixture, is kept constant at 1.0 mg. per 100 cc. of solution. As evident from Fig. 1, artificial mixtures of amylose and amylopectin (20, 40, 50, 60 and 80% amylose) fall on a straight line connecting pure amylopectin and pure amylose.

Hydrolysis of Starch Fractions with β-Amylase.—A criterion for purity of the starch fractions was based on 100% hydrolysis with β-amylase to maltose. The degree of hydrolysis of unfractionated potato starch by this enzyme is from 60 to 64%. Complete hydrolysis of amylose (also Samec and Mayer's amyloamylose) with β-amylase coincided with a reading of 310 on the photocolorimeter. Enzymatically synthesized potato starch hydrolyzed to the extent of 98% and its color intensity corresponded to a reading of 305.

The hydrolyses were carried out with β-amylase prepared from ungerminated barley, according to Hanes and Cattle.[11] The correlation between the degree of hydrolysis with the enzyme and the intensity of the blue color with iodine of starch and its different fractions is summarized in Table II.

The authors express their appreciation to Dr. C. S. Hanes, Cambridge University, for his helpful suggestions on the separation of the two starch fractions.

Summary

1. A simple method for separation of potato starch into two fractions, amylose and amylopectin, is described.

2. The amylose is almost insoluble in water, completely hydrolyzed with β-amylase to maltose and gives a brilliant blue color when treated with iodine.

3. The amyloamylose fraction of starch prepared by Samec and Mayer's method possessed properties similar to amylose and is therefore considered to be identical with the latter.

4. Enzymatically synthesized potato starch also resembles the amylose fraction of starch in all its properties.

5. A quantitative method based on the color reaction with iodine, for estimation of the relative proportion of amylose and amylopectin in mixtures, or in unfractionated potato starch is described.

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(10) L. E. Simerl and B. L. Browning, *Ind. Eng. Chem., Anal. Ed.*, **11**, 125 (1939).

(11) C. S. Hanes and M. Cattle, *Proc. Roy. Soc.* (London), **B125**, 387 (1938).

[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

The Molecular Constitution of Amylose and Amylopectin of Potato Starch

BY W. Z. HASSID AND R. M. MCCREADY[1]

The molecular structure of the two starch components, amylose and amylopectin, known to possess different physical properties, was first studied by Hirst, Plant and Wilkinson.[2] These authors concluded that both fractions are made up of chains consisting of 24 to 30 glucopyranose units and that their molecular constitution is essentially the same. They attributed the differences in physical properties of the two starch components to the difference in state of hydration and degree of aggregation, or interlocking of the chains to form large colloidal micelles. Recently Meyer and collaborators[3] presented evidence, based on methylation experiments, showing that the two starch fractions possess a different molecular structure: the amylose, consisting of long unbranched chains of glucopyranose units, and the amylopectin of branched chains of about 27 units. Haworth, Heath and Peat,[4] however, appear to doubt the accuracy of their experimental results. They state that Meyer and collaborators "have attempted a very rough end-group estimation of an incompletely methylated amylose from potato starch . . . ."

The question as to whether starch is a uniformly structural compound, or whether it exists as a mixture of two chemical entities, therefore, requires further study. In view of the importance of this question, the present writers undertook a reinvestigation of the problem.

Amylose and amylopectin were prepared by the

(1) Present address, Western Regional Research Laboratory, Albany, California.

(2) E. L. Hirst, M. M. T. Plant and M. D. Wilkinson, *J. Chem. Soc.*, 2375 (1932).

(3) K. H. Meyer, M. Wertheim and P. Bernfeld, *Helv. Chim. Acta*, **23** 865 (1940); **24**, 378 (1941).

(4) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 55 (1942).

method previously described.[5] The degree of hydrolysis with β-amylase and the color intensity with iodine were used as criteria for purity of the two fractions. The amylose fraction was completely hydrolyzed with β-amylase to maltose and had a color intensity with iodine corresponding to a reading of 310 on the Klett–Summerson photocolorimeter.[5] Its solubility in water was about 0.1% and its specific rotation in 1 *N* sodium hydroxide was $[\alpha]_D$ + 155°. The amylopectin was hydrolyzed with β-amylase to maltose to the extent of 54% and its color intensity with iodine corresponded to 52 on the photocolorimeter. It formed a viscous opalescent solution in water and had a specific rotation in 1 *N* sodium hydroxide $[\alpha]_D$ +161°. The methylated amylose was insoluble in hot and also cold water. The methylated amylopectin was insoluble in hot water but soluble in cold. The approximate molecular weight of the methylated amylose determined by Staudinger's viscosity method was 50,000 and of the methylated amylopectin, 92,000. The methylated amylose (OCH_3, 44.8%) on hydrolysis yielded 0.32% tetramethylglucose, a value corresponding to a chain length of about 300 to 400 glucose units. On hydrolysis of the methylated amylopectin 4.67% of tetramethylglucose was obtained. This amount corresponds to a repeating chain length of 25 glucose units. These data clearly show that potato starch consists of two distinct fractions, each having a different molecular constitution. The amylose is made up of long chains of about 300 to 400 glucose units corresponding to a molecular weight of approximately 80,000. Since this molecular weight is of the same order of magnitude as that obtained by the viscosity method, there is good reason to believe that these chains represent whole molecules of amylose in which no branching occurs. The amylopectin is made up of chains of 25 glucose units and has a branched structure. The amylose content of potato starch is about 20%. On the basis that this fraction contains an insignificant amount of end group (0.32% tetramethylglucose), the theoretical repeating chain length of natural starch may be calculated to be 30 glucose units. This value agrees within experimental error with the directly determined repeating chain lengths of natural starches (24 to 30 glucose units).

Hirst and collaborators[2] obtained the same chain lengths for amylose and amylopectin, but apparently they dealt with a mixture, not with pure components. These authors used the method of Ling and Nanji[6] for the separation of the two fractions. We in this Laboratory found that no adequate separation of the two fractions could be accomplished by this method. The extent of β-amylase hydrolysis to maltose of a sample of amylose, prepared by the Ling and Nanji method, was 70% and the color with iodine corresponded to a reading of 112 on the photocolorimeter. These values correspond to a mixture of the two components, in which the amylose exists only in slightly higher proportion than is found in natural starch.

The difference in behavior of β-amylase toward amylose and amylopectin seems to bear a direct relation to their structures. In the case of amylopectin, with a repeating chain length of 25 glucose units and a β-amylase hydrolysis limit of 54%, the enzyme attacks these chains at the non-reducing ends, splitting off successive terminal maltose fragments, until it encounters some modification in structure. Since it is established that side linkages occur in starch on the sixth carbon atom of some of the glucose units in the chains,[7] it can be postulated that these linkages are responsible for stopping the hydrolysis at those points. An hydrolysis limit of 54% would correspond to a rupture of the chain at approximately the thirteenth to fifteenth glucose unit. Since amylose (also synthetic starch) is made up of long chains without branching, and therefore no side linkages, the hydrolysis of the β-amylase continues until the whole molecule is degraded to maltose. The idea that β-amylase attacks the starch chains at the non-reducing ends, splitting off maltose until the enzyme is stopped by a modification in structure, originated with Hanes.[8] Figure 1 represents diagrammatically the manner in which β-amylase attacks the two starch fractions.

Experimental

Properties of Amylose.—The amylose was prepared as described in a previous paper.[5] It dissolved with difficulty in hot water (about 0.1%) and rapidly retrograded from solution. It was soluble in 3% sodium hydroxide but retrograded again when the solution was made neutral. Its specific rotation (*c*, 1) in 1 *N* sodium hydroxide was $[\alpha]_D$ +155°. It produced an intensely blue color when

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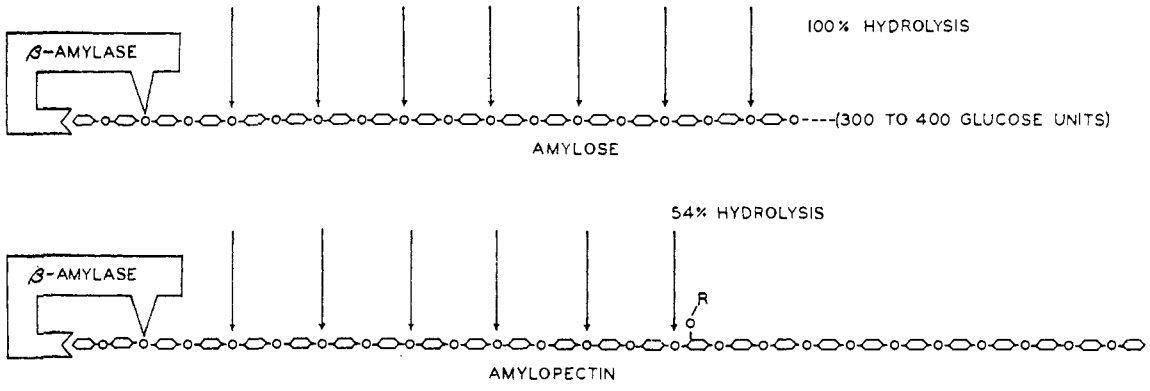

Fig. 1.—Scheme illustrating the mode of action of β-amylase in relation to the structure of amylose and amylopectin.

treated with iodine and corresponded to a reading of 310 on the Klett-Summerson photocolorimeter.[5] It was completely hydrolyzed with β-amylase to maltose.

Preparation of Amylose by the Ling and Nanji Method.—A 5% starch paste was frozen and left overnight at 15°. The frozen mass was then extracted several times at 50°, the extract concentrated under reduced pressure and the amylose precipitated with alcohol. Upon hydrolysis of this product with β-amylase 70% was converted to maltose. Its coloration with iodine was blue and the reading on the photocolorimeter corresponded to 112. The amylose thus prepared by the Ling and Nanji method is not pure but a mixture of amylose and amylopectin.

Properties of the Amylopectin.—The amylopectin formed a viscous opalescent solution in water and had a specific rotation (*c*, 1) in 1 *N* sodium hydroxide $[\alpha]_D$ +161°. Its hydrolysis with β-amylase ceased when 54% was converted to maltose. The color produced with iodine was blue and the reading on the photocolorimeter was 50.

Methylation of Amylose.—The amylose used for methylation was first acetylated in the usual manner.[9] Thirty grams of the amylose triacetate was dissolved in 300 cc. of acetone and simultaneously deacetylated and methylated at 55° with 150 cc. of methyl sulfate and 450 cc. of 30% sodium hydroxide. The reagents were added in 10 equal portions at ten-minute intervals with vigorous stirring. This procedure was repeated twice. Seven subsequent methylations were then carried out by dissolving the partially methylated amylose in 250 cc. of acetone and methylating with 100 cc. of methyl sulfate and 300 cc. of 30% sodium hydroxide. A yield of 91% of the theoretical was obtained. The final product was insoluble in both cold and hot water. It had a specific rotation (*c*, 1) in chloroform $[\alpha]_D$ +213°. The methoxyl content, OCH_3 was 44.8% (calculated for $(C_6H_7O_2(OCH_3)_3)_n$, 45.6%). The specific viscosity, η sp., at 23° of a 0.4% solution of the methylated amylose in *m*-cresol was 0.22. This corresponds to an apparent molecular weight of 50,000 determined by Staudinger's formula with $K_m = 10^{-3}$. This constant was used by Haworth[4] in the case of synthetic starch possessing a linear molecular structure.

Hydrolysis of Methylated Amylose.—Ten grams of the methylated amylose was boiled for eight hours with 350 cc. of methanol, containing 1.7% of dry hydrogen chloride under a reflux condenser. The solution was neutralized with lead carbonate, filtered and evaporated to dryness. The residue was extracted with chloroform and, after removal of the solvent by evaporation, 10.93 g. of material was obtained (95% yield). The methylglucosides were fractionally distilled from a flask fitted with a vacuum-jacketed fractionating column at a temperature between 90 and 180° and 10^{-4} mm. pressure. The fractions obtained are collected in Table I.

Hirst and Young's method[10] was used for the quantitative determination of mixtures of tetramethyl- and trimethylmethylglucoside. Fraction I contained 0.032 g. of tetramethylmethylglucoside by this method. Its methoxyl content was 54.0% and agreed with the value 13.5% tetramethylmethylglucoside found from the rotation and index of refraction.

The five subsequent fractions contained only trimethylmethylglucoside. Their methoxyl contents agreed well with the theoretical OCH_3 content of 52.6%. The identity of 2,3,6-trimethylmethylglucoside was confirmed by isolation of crystalline 2,3,6-trimethylglucose from the hydrolysis product of combined fractions II to VI.

The amount of 0.032 g. tetramethylmethylglucoside (end group), obtained from a 95% yield of the theoretical total methylglucosides, was originally derived from 10 g. of the methylated amylose. On this basis, applying a correction factor, the amount of tetramethylmethylglucoside becomes 0.034 g., which is equivalent to 0.032 g., or 0.32% tetramethylglucose. This proportion of "end group" corresponds to an approximate chain length of 350 glucose units.

Methylation of Amylopectin.—Methylation of the acetylated amylopectin was carried out as in the case of amylose. The yield was 84% of the theoretical. The methylated product was soluble in cold water but insoluble in hot. Its specific rotation (*c*, 1) in chloroform was $[\alpha]_D$ + 217°. The methoxyl content, OCH_3 was 44.6% (calculated for $(C_6H_7O_2(OCH_3)_3)_n$, 45.6%). The specific viscosity, η sp.. at 23° of a 0.4% solution of the methylated amylopectin in *m*-cresol was 0.29. This corresponds to an apparent molecular weight of 92,000. In this case Carter and Record's[11] modified constant $K_m = 1.6 \times 10^{-4}$ for Staudinger's formula was used. When this constant is

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TABLE I

HYDROLYSIS PRODUCTS OF METHYLATED AMYLOSE

Fraction	Wt., g.	$\eta^{16}D$	Constant[a]	"Tetra," %	"Tetra," g.	"Tri," g.	"Di," %	"Di," g.
I	0.234	1.4558	(a) 1.4430	13.5	0.032	0.202	...	...
			(b) 1.4578					
II	1.000	1.4580	(a) 1.4432	...	...	1.000	...	...
			(b) 1.4580					
III	2.963	1.4586	(a) 1.4435	...	...	2.963	...	...
			(b) 1.4585					
IV	2.349	1.4588	(a) 1.4436	...	...	2.349	...	...
			(b) 1.4587					
V	2.244	1.4588	(a) 1.4438	...	...	2.244	...	...
			(b) 1.4589					
VI	1.614	1.4588	(a) 1.4439	...	...	1.614	...	...
	10.404		(b) 1.4590		0.032	10.372		
			HYDROLYSIS PRODUCTS OF METHYLATED AMYLOPECTIN					
I	0.223	1.4462	(a) 1.4432	79.7	0.178	0.045	...	...
			(b) 1.4580					
II	0.407	1.4540	(a) 1.4440	31.5	0.133	0.274	...	...
			(b) 1.4586					
III	1.550	1.4572	(a) 1.4433	6.1	0.094	1.456	...	...
			(b) 1.4581					
IV	2.471	1.4589	(a) 1.4438	...	...	2.471	...	...
			(b) 1.4590					
V	2.712	1.4591	(a) 1.4438	...	...	2.712	...	...
			(b) 1.4592					
VI	1.623	1.4606	...	...	...	1.282	21	0.341
	8.986				0.405	8.240		0.341

[a] (a) and (b) are the $\eta^{16}D$ values of the "tetra" and the "tri" portions, respectively, present in these fractions, as estimated from rotational data.

applied, the molecular weight of natural starch, possessing a branched structure, corresponds to that obtained by osmotic pressure measurements.

Hydrolysis of Methylated Amylopectin.—Nine grams of methylated amylopectin was hydrolyzed with 300 cc. of 1.7% methyl alcoholic hydrogen chloride and the methylglucosides estimated as previously described. The methylglucosides (yield 91%) were fractionally distilled at 10^{-4} mm. pressure and 90 to 190° into fractions shown in Table I. Methoxyl determinations were carried out as checks on the six fractions; the results agreed well with those obtained from rotational data and indices of refraction.

The relative proportions of the hydrolysis products, represented as percentage of the total recovery, were as follows: 2,3,4,6-tetramethylmethylglucoside, 4.5%; 2,3,-6-trimethylmethylglucoside, 91.7%; dimethylmethylglucoside, 3.8%. Considering the fact that only 91% of the theoretical value of methylglucosides was obtained, the amount of tetramethylmethylglucoside, 0.405 g., becomes 0.445 g., which is equivalent to 0.421 g. or 4.67% tetramethylglucose. This proportion of "end group" corresponds to a repeating chain length of 25 glucose residues.

The identity of 2,3,4,6-tetramethylmethylglucoside and 2,3,6-trimethylmethylglucoside from the hydrolysis of the methylated amylopectin was established by isolation from the respective fractions of crystalline 2,3,4,6-tetramethylglucose and 2,3,6-trimethylglucose as indicated in a previous publication.[9]

The authors are grateful to Mr. W. H. Dore for his interest and suggestions during the course of the investigation.

Summary

Potato starch consists of two distinct fractions, amylose (approximately 20%) and amylopectin, which differ in their physical properties, molecular constitution and behavior toward β-amylase.

On hydrolysis of the methylated amylose, followed by a quantitative separation of the cleavage products, 0.32% of tetramethylglucose (end group) was obtained. This proportion of "end group" corresponds to a chain length of approximately 350 glucose units. It is, therefore, concluded that the amylose is made up of long non-branched chains of glucopyranose units (300 to 400) joined by α-glucosidic linkages between the first and fourth carbon atoms.

The methylated amylopectin yielded on hydrolysis 4.67% tetramethylglucose (end group) which corresponds to a repeating chain length of 25 glucose units. These relatively short chains are combined to form a branched structure.

The difference in behavior of β-amylase toward

amylose and amylopectin is in accord with the differences in their chemical constitutions: the amylose, which is completely hydrolyzed to maltose by this enzyme possesses a non-branched long chain structure, while the incompletely hydrolyzed amylopectin is identified with a branched-chain structure.

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[CONTRIBUTION FROM THE PHYSICO-CHEMICAL LABORATORY OF THE NEW YORK STATE EXPERIMENT STATION]

The Reaction of Formaldehyde with $l(+)$-Aspartic and $l(+)$-Glutamic Acids[1]

BY D. C. CARPENTER AND F. E. LOVELACE

The polariscopic method has been employed in the work herein described to give information concerning the reaction of formaldehyde with $l(+)$-aspartic and $l(+)$-glutamic acids as their respective di-sodium salts. A similar study has shown[2a] that $l(-)$-asparagine with an equivalent of base reacts with one mole of formaldehyde at the α-amino group to give a fairly stable compound designated by Schiff[2b] as methylene asparagine. At higher aldehyde concentrations, a second compound of highly unstable character was produced which could not be isolated or identified. It was suggested before that the second mole of formaldehyde perhaps reacted with the amide group of asparagine inasmuch as many acid amides are known to react with aldehyde giving comparatively unstable derivatives. From the work here reported it is clear that α-amino acids react with formaldehyde at the amino group, binding first one mole and then a second mole as the concentration of the latter is increased. The first mole of aldehyde reacts readily but the second one unites with difficulty and the latter product readily decomposes. From the equilibrium constants found for the latter reaction, it is clear that for any arbitrarily selected ratio between the concentrations of methylol amino acid and its final reaction product, the free aldehyde concentration required for methylol $l(+)$-aspartic acid is about 3.5 times and for methylol $l(+)$-glutamic acid about 7.7 times that required for methylol $l(-)$-asparagine. Under the experimental conditions the amide group of the α-amino acid previously examined ($l(-)$-asparagine) does not react with formaldehyde and the reaction with aldehyde is confined to the α-amino group.

We have employed melting point and amino nitrogen content of our acids as criteria of purity as well as specific rotation. Whether an impurity may be a foreign amino acid or the *dl*-form of the acid, the above criteria should give a reliable clue as to purity. In comparisons of specific rotations we have referred only to rotations reported on preparations in which the *d*- and *l*-isomers were actually separated from one another as crystalline salts of benzoyl derivatives of the respective acids. The above criteria indicate a high degree of purity of our preparations.

Preparation of Materials

$l(+)$-Aspartic Acid.—This was prepared from a sample of recrystallized $l(-)$-asparagine that we have used before, by boiling for six hours under reflux with two moles of normal hydrochloric acid solution. The solution was then cooled and the calculated amount of normal sodium hydroxide added with good stirring and then set away in the cold for the aspartic acid to crystallize. It was recrystallized four times from hot water and twice from fifty per cent. ethyl alcohol; m. p. 283° (cor.). Amino nitrogen by the Van Slyke method 10.43% (found); 10.52% (calcd.). A solution of $l(+)$-aspartic acid, 5.320 g. containing two equivalents of sodium hydroxide and diluted to 100 ml., gave a rotation of −0.17° (2 dm.); $[\alpha]^{20}D$ −1.60°. Fischer[3] reports $[\alpha]^{20}D$ −1.90 for the di-sodium salt (c = 6.0).

A solution (d = 1.032) of our $l(+)$-aspartic acid weighing 7.8993 g. and containing 0.3311 g. of aspartic acid dissolved in three equivalents of hydrochloric acid solution gave a rotation of +1.10° (1 dm.); $[\alpha]^{20}D$ +25.43°. This is in close agreement with $[\alpha]^{20}D$ −25.35° reported by Fischer[3] for a similar solution of the antipode (c = 4.15).

$l(+)$-Glutamic Acid.—This was prepared from a commercial sample, precipitating as the hydrochloride and decomposing the hydrochloride with the calculated amount of aniline[4] and allowing to crystallize overnight in the cold. The crystals were filtered off and washed with 95% ethyl alcohol until free from chloride and dried. The separation and decomposition of the hydrochloride was re-

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