175. *The Enzymic Synthesis and Degradation of Starch. Part XIII. Improved Methods for the Fractionation of Potato Starch.*

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Modification and improvement of the published methods for the preparation of the two starch components, amylose and amylopectin, are described. Amylose is prepared by a combination of the methods using aluminium hydroxide and thymol (*J.*, 1949, 1; 1948, 1687), and amylopectin by further fractionation with methanol of amylopectin prepared by the thymol method. Properties of typical preparations of these components are described.

The methods which have been used for the fractionation of starch have been critically reviewed by Schoch (*Adv. Carbohydrate Chem.*, 1945, **1**, 259) and by Kerr (" Chemistry and Industry of Starch," Academic Press, New York, 2nd Edn., 1950, pp. 179—244). Amongst the earliest successful methods was that of electrophoresis devised by Samec and Haerdtl (*Kolloid-Beih.*, 1920, **12**, 281) which was, however, limited in its applicability. Aqueous leaching of starch grains at temperatures of 70° and upwards has also been used as a preparative method and for the subfractionation of starch components (see Kerr, *loc. cit.*). The most widely used method is precipitation of the amylose component as an insoluble complex with alcohols or other substances, amylopectin remaining in solution. The amylopectin is isolated, after removal of the amylose, by precipitation with excess of ethyl or methyl alcohol. Thus, Schoch used butanol and later " Pentasol " (*loc. cit.*), and Bourne, Donnison, Haworth, and Peat (*J.*, 1948, 1687) used thymol and *cyclo*hexanol as amylose precipitants. Whistler and Hilbert (*J. Amer. Chem. Soc.*, 1945, **67**, 1161) employed nitroparaffins, esters, and thiols as precipitants. Later Bourne, Donnison, Peat, and Whelan (*J.*, 1949, 1) reported an alternative method of fractionation in which the amylopectin component was precipitated as an insoluble complex with aluminium hydroxide. This method yields amylose fractions which are consistently less contaminated with branched polysaccharide than are those prepared by the published thymol method.

It may be helpful to other workers in this field if we put on record an account of the modified procedures we have devised for the fractionation of starch and which we have used with good results for more than a year. The percentage overall yields by these methods are low (for amylose 40% of theory, for amylopectin, 8%) but compare favourably with those given by other and more tedious methods designed to yield components of comparable purity.

The modified method for the preparation of amylose is a combination of the aluminium hydroxide and the thymol method (*loc. cit.*). The complex of aluminium hydroxide and amylopectin is first precipitated by mixing aluminium nitrate and aqueous ammonia in the presence of the dispersed starch. It is important to ensure that an excess of ammonia is not added. Failure to observe this precaution causes the amylose finally isolated from the supernatant liquid to be contaminated with amylopectin. It may be that the hydroxide–amylopectin complex is somewhat soluble in an excess of ammonia, although the formation of aluminate ion is slight even in the highest concentrations of ammonia. After " ageing " at 30° for 3 days the mixture is centrifuged and the supernatant amylose solution dialysed to remove salts, which otherwise remain associated with the polysaccharide when it is precipitated with alcohol. Thymol is then added and the amylose complex separates, the precipitation

being complete in 48 hours. Thymol is removed from the complex when the precipitate is washed with ethanol. Both the thymol complex and the thymol-free amylose isolated by this method are crystalline and form rosettes such as are depicted by Kerr (*loc. cit.*) who first reported the isolation of crystalline potato amylose. When examined under polarised light both the thymol complex and the amylose exhibited weak birefringence. The consistent yields of amylose of high blue value are illustrated by the data of Table I where the properties of six successive batches of amylose prepared by this method are listed. The average carbohydrate content (measured by acid hydrolysis) is 98·4% and the average ash content is 0·86%. That the amylose specimens are of a high purity with respect to freedom from amylopectin-like substances may be shown from a consideration of the action of β-amylase on them.

Peat and his co-workers (*Nature*, 1949, **164**, 499; *Biochem. J.*, 1950, **47**, xl) have shown that potato and other amyloses appear to contain branch linkages, as shown by the failure of crystalline sweet potato and purified soya-bean β-amylases to achieve complete conversion of amylose into maltose (see Table I).

TABLE I.

Properties of amyloses prepared by the aluminium hydroxide–thymol method.

					Action of β-amylase.			
					Stock soya.		Purified soya.	
No.	Blue value.	Ash content (%).	Conversion into glucose by acid (%).	Yield (g.).	% Conv.	A.V.	% Conv.	A.V.
1	1·47	0·51	99·4	9·4	96·8	0·056	67·5	0·411
2	1·47	1·84	98·5	8·2	95·5	0·061	—	—
3	1·46	0·98	97·1	8·6	91·5	0·097	—	—
4	1·45	0·46	98·2	9·6	95·9	0·069	70·8	0·381
5	1·49	0·56	99·3	7·3	92·0	0·058	67·5	0·369
6	1·46	0·78	99·0	9·9	96·0	0·045	68·1	0·371

% Conv. = % conversion into maltose; A.V. = absorption value (680 mμ.) of iodine-stained residual polysaccharide; Stock soya = soya-bean preparation containing β-amylase and Z-enzyme; Purified soya = soya-bean β-amylase preparation free from Z-enzyme. Values are based on dry weight of polysaccharide.

Complete conversion of amylose by β-amylase is only approached in the presence of another enzyme, Z-enzyme, which hydrolyses the branch linkages. The stock soya-bean preparation contains both enzymes and the conversion of the potato amyloses by this preparation is only 4% (average) below the percentage conversion into glucose by acid hydrolysis (Table I). If the failure to achieve complete β-amylolysis were due to the presence of amylopectin, only half of which is converted into maltose by β-amylase, it would be necessary to assume that the amylose preparations still contained about 8% of amylopectin. This explanation cannot, however, account for the intensity of the iodine stain of the polysaccharide remaining after hydrolysis by the stock β-amylase preparation. The final average A.V. (680 mμ.) is 0·064. Eight per cent. of amylopectin, giving rise to 4% of limit β-dextrin, of known blue value (0·160), would contribute only 0·006 to this final A.V. It seems more likely that a small amount of the amylose is removed, by spontaneous precipitation (retrogradation), from the sphere of action of the β-amylase, thus causing an apparent lower degree of conversion into maltose. When such retrogradation occurs, the precipitated but finely dispersed amylose exhibits almost the same intensity of iodine stain as it does in solution. The final A.V. (680 mμ.) would in this case be 4% of the original blue value, or 0·058, which is in good agreement with the average value determined experimentally.

The method for the preparation of amylose-free amylopectin consists in the further fractionation of the crude amylopectin prepared by the thymol method (*J.*, 1948, 1687) by the addition (to its aqueous solution) of methanol to a concentration of 17% (by volume). At this concentration the whole of the amylose impurity is precipitated together with some amylopectin. After removal of the precipitate the polysaccharide remaining in solution is precipitated by the addition of excess of methanol. The addition of sodium chloride is necessary to cause coagulation of the polysaccharide. The temperature of precipitation is critical. At 20° the first addition of methanol brings about no precipitation although at 0° the whole of the polysaccharide is precipitated and no fractionation is achieved. The most suitable temperature for fractionation was found to be 15°. Properties of an unfractionated amylopectin and of two purified amylopectins obtained from it are listed in Table II.

Solutions of the methanol-fractionated amylopectins are water-clear, whereas solutions of thymol-amylopectins are opalescent. Other changes taking place in the physical properties of amylopectin preparations on subfractionation with methanol are consistent with the view

that the process is one of removal of a linear, amylose-type component and not merely a separation, according to molecular size, of amylopectins of similar degrees of branching. If

TABLE II.

Properties of amylopectins prepared by the thymol–methanol method.

Method of fraction.	Blue value.	Ash content (%).	Conversion into glucose by acid (%).	Yield (%).*	Action of β-amylase.			
					Stock soya.		Purified soya.	
					% Conv.	A.V.	% Conv.	A.V.
Thymol	0·221	0·73	94·0	—	54·1	0·090	53·1	0·108
Thymol–MeOH	0·165	0·20	98·0	7·1	51·7	0·084	51·4	0·088
Thymol–MeOH	0·170	2·23	98·4	8·4	—	—	—	—

* As % of original thymol–amylopectin. For the explanation of other abbreviations see Table I.

the subfractionation were of the latter character it would be conditioned only by molecular weight, whereas it is actually found that the blue values and the degrees of hydrolysis by stock and purified β-amylases of methanol-fractionated amylopectins are lower than the corresponding values for the unfractionated material (Table II). Such changes are to be expected if it is amylose which is being removed by the treatment with methanol.

EXPERIMENTAL.

Analytical Methods.—(*a*) For the methods used in determination of (i) iodine stains of polysaccharides, see Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924), (ii) reducing sugar, starch polysaccharides by acid hydrolysis, and ash content, see Pirt and Whelan (*J. Sci. Food Agric.*, 1951, in the press), and (iii) β-amylase activity, see Part X (*J.*, 1950, 3566).

(*b*) *β-Amylolysis of starch polysaccharides.* Stock soya-bean β-amylase which was prepared as in Part II (*J.*, 1945, 882) contains Z-enzyme. Purified soya-bean β-amylase, which was prepared by a method to be published in a later paper of this series, was free from Z-enzyme. The dried polysaccharide (*ca.* 15 mg.), contained in a 25-c.c. flask, was wetted with alcohol and dissolved in 0·012N-sodium hydroxide (10 c.c.) by intermittent heating on a boiling water-bath and neutralised to phenolphthalein with N-sulphuric acid. Acetate buffer (0·2M.; 3 c.c.; pH 4·8) was added, followed by the stock or purified β-amylase solution (equivalent to 800 units of activity), and the whole was diluted to 25 c.c. The digest was incubated at 35° together with a control digest, not containing polysaccharide, which was used for measurements of the reducing power of the enzyme solution. Measurements of reducing sugar, as maltose, were made after 3 and 5 hours, on 5-c.c. portions of the digests. Measurements of A.V. (680 mμ.) were also made by the standard method, referred to above, with a volume of digest equivalent to 1 mg. of original polysaccharide. The conversion limit was always reached within 3 hours.

Preparation of Amylose by Treatment of Starch with Aluminium Hydroxide and Thymol.—(a) *Precipitation of the aluminium hydroxide complex.* Potato starch (100 g.; dry wt.) was creamed with water (500 c.c.) and added, during 15 minutes, to boiling 0·1% sodium chloride solution (3·5 l.), contained in a 5-l. bolt-head flask, with vigorous stirring. Boiling and stirring were continued for a further 45 minutes, whereafter the paste was cooled rapidly in running water to 30°. Aluminium nitrate [$Al(NO_3)_3,9H_2O$; 48·25 g.] dissolved in the minimum quantity of water was stirred into the paste, and ammonia solution (*d* 0·88) was added slowly from a burette, with mechanical stirring, until the mixture was just alkaline to phenolphthalein. The flask was stoppered and the solution allowed to "age" for 3 days at 30° before the aluminium hydroxide complex was removed on the centrifuge (2500 r.p.m. for 30 minutes). The supernatant liquid, usually *ca.* 900 c.c., was dialysed against running tap water for 2 days.

(b) *Precipitation of thymol complex and isolation of amylose.* If precipitation occurred during dialysis the solution was centrifuged and the supernatant liquid acidified by the addition of a few drops of N-sulphuric acid (alkalinity was due to the tap water). Finely powdered thymol (0·132 g./100 c.c. of solution) was added with shaking, and the vessel stoppered and kept at 30° for 48 hours. The amylose–thymol complex was sedimented on the centrifuge and washed twice by shaking it with thymol-saturated water, centrifuging between each washing. Thymol was removed by grinding the precipitate twice with absolute ethanol and washing it twice with ethanol and then ether. Ether and moisture were removed by storage in a vacuum over phosphoric oxide at room temperature, the sample being subsequently stored in a closed bottle.

Fractionation of Thymol-amylopectin with Methanol.—Thymol-amylopectin was prepared by the method of Bourne, Donnison, Haworth, and Peat (*J.*, 1948, 1687). The amylopectin (*ca.* 7·4 g., dry wt.) was moistened with ethanol and partly dispersed in water (800 c.c.) by being heated to boiling with vigorous stirring and boiled for 1 minute. The hot dispersion was autoclaved at 20 lb. pressure for 1 hour and then cooled to 15°. Sodium chloride (1 g.) was added and the solution diluted to 1 l. Methanol (200 c.c.) was added dropwise with rapid stirring and the precipitate which formed was removed on the centrifuge. To the supernatant liquid was added a further quantity (1 g.) of sodium chloride, followed by absolute ethanol (2 volumes). The precipitate was allowed to coagulate and was removed on the centrifuge. It was dried by washing it with ethanol and ether as in the preparation of amylose above.

The authors thank Mrs. O. Owen, working under the auspices of the Colonial Products Research Council, and Miss G. J. Thomas for assistance in the preparation of the starch fractions.

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[*Received, December 27th*, 1950.]