The Fractionation of Normal and Mottled Wheat Starch by Elution in the Absence of Oxygen. Viscometric Properties of the Amylose Fraction.

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Wheat starch is shown to be extracted easily in high yield from grains which have been softened without chemical treatment by 2 minutes' heating in water. Undried starch, freshly prepared in this way, is dispersed in water at 95° for 10 min. in a stream of nitrogen which, besides excluding oxygen, stirs the suspension and generates foam which removes surface-active impurities. Centrifugation of the cooled dispersion leads to a fractionation of the starch into *ca.* 25% by weight of amylose of " blue value " 1·1—1·2 and 75% by weight of amylopectin of " blue value " 0·15. The intrinsic viscosity of the acetylated amylose in chloroform solution at 20° is found to be 4·8. There appears to be no difference between the properties of normal and mottled wheat starch.

Two principal methods are available for the fractionation of starch. In the first to be discovered (A) the starch is extracted for a few moments with hot water which elutes amylose from the granules and leaves amylopectin as an undissolved gel. In the other method (B) the starch is heated in water or autoclaved until it appears to be completely dissolved, and then a selective precipitant is added which forms an insoluble complex with the amylose and leaves the amylopectin in solution. These methods are reviewed in detail by Schoch (in Radley, " Starch and its Derivatives," Chapman and Hall, London, 1953, Vol. I, p. 123). Method (A) has had a long history. Its use is described by Maquenne (*Compt. rend.*, 1908, **146**, 542) in observations on a note by Gatin-Gruzewska (*ibid.*, 1908, **146**, 540) concerning the fractionaction of alkali-dispersed starch. Maquenne boiled potato starch in various salt solutions and separated the dissolved amylose from the residual amylopectin by filtration. Since as much as 60% of the granule was dissolved and so assessed as amylose, only a very poor fractionation could have been achieved. Improved results were obtained by Tanret (*ibid.*, 1914, **158**, 1353) by restricting the time of extraction to a few moments, 73% of potato starch then remaining undissolved, compared with 40% if extraction was continued for an hour.

Schoch (*op. cit.*, p. 136) has concluded that methods of fractionation depending upon leaching fail to be efficient owing to the concurrent leaching of amylopectin, and to retrogradation within the granule of approximately half the amylose which thereby becomes included in the amylopectin fraction.

Method (B), discovered by Schoch (*Cereal Chem.*, 1941, **18**, 121) and independently by Weigel (*Z. phys. Chem.*, 1941, **188**, *A*, 137), requires both fractions to be dissolved. Vigorous stirring of the dispersion for several hours at 92° is said to achieve this for cereal starches (Schoch, *op. cit.*, p. 146). Higginbotham and Morrison (*Shirley Inst. Mem.*, 1948, **22**, 148) find that potato starch requires 1—2 days at 90° with moderately vigorous stirring. Addition of butanol or pentanol to such dispersions leads on cooling to the precipitation of amylose as a complex, and a practically complete separation from amylopectin is attained after one or two repetitions of the process.

Clearly one essential difference between the two methods is the state of subdivision of the amylopectin, and the question has to be asked whether the amylopectin becomes dissolved owing to degradation or to disaggregation.

Oxygen causes the slow degradation of amylose in hot neutral solution (Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541), and may be assumed to do the same to amylopectin. After this had been noted, an attempt was made to prepare undegraded fractions of potato starch by method (B) using rigorously oxygen-free conditions of dispersion (Baum and Gilbert, *ibid.*, 1954, pp. 489, 490). The attempt failed because treatment at 100° for several hours did not dissolve any of the amylopectin. This, however, provided ideal conditions for the application of method (A), since amylose dissolved

immediately. Centrifugation in the presence of sodium chloride led to fractionation comparable in effectiveness with method (B), giving amylose of " blue value " 1·2—1·3 and amylopectin of " blue value " 0·2. The improvement in efficiency of method (A) compared with the earlier work criticised by Schoch cannot be attributed to the use of anaerobic conditions alone. Of equal importance may have been the use of undried freshly prepared starch and the absence of mechanical stirring. The part played by each of these factors and the effects of variety of potato and season are under investigation. Starches from different botanical sources are also being studied, and this paper describes the application of the method to wheat starch.

Ripe wheat sometimes shows a defect in the grain which has led to the term " mottled wheat " because some or all of the grains in an ear are then relatively soft, opaque, and paler yellow than the normal hard and translucent grains. The work of one of us (H. L. W., in the press) had shown that there were significant differences between mottled and normal wheat grains in nitrogen and moisture content and in ash composition, and advantage was taken of the present investigation to compare the starch of the two forms. Only one variety of wheat was examined, namely " Charter " from Queensland, Australia, harvested in November, 1953.

EXPERIMENTAL

Isolation of the Starch from the Grain.—It is necessary to soften the grain before starch can be extracted efficiently, but any chemical treatment which might degrade the starch has to be avoided. It was found sufficient to heat the wet grain for 2 min. in a boiling-water bath. Simultaneously the enzyme activity of the grain was reduced or destroyed. For the softening process, about 50 g. of washed grain were placed in a container connected to a gas-train (nitrogen or hydrogen purified over hot copper). After evacuation at a water-pump for 10 min. to remove air from the vacuoles, the grain was flooded with air-free water from the previous vessel in the train, and gas passed through the mixture for a further 20 min. A boiling-water bath was then placed round the container for 2 min. with the gas still passing. After being cooled, the grain was removed and washed well with distilled water.

The softened grains were crushed individually by a hammer (rollers could be substituted for larger-scale work) and blended for 3 min. in a high-speed " Atomix " blender containing 1% sodium chloride solution. The blend was poured through muslin to remove husks, bran, and coarse material, and the filtrate centrifuged to sediment the starch. The starch was washed three times in the centrifuge with ice-cold water, this serving also to coagulate the protein accompanying the starch. The starch was removed from the coagulum by gentle swirling of the ice-cold water, followed by filtration through muslin, the process being repeated if necessary. After being allowed to settle, the starch was kept at 0° under water. A high yield of starch was obtained (visual estimate) compared with a very low yield if the initial softening process was omitted.

Dispersion and Fractionation.—Dispersion was carried out in distilled water at approx. 95° in a fast stream of nitrogen, which had the dual purpose of preventing oxidation and of providing stirring without damage to the soft gel particles of amylopectin. The dispersion vessel was made by sealing a length of glass tubing (15 × 4 cm.) to the top of a Grade-3 sintered glass filter-funnel (plate diam. 4 cm.), the stalk of which was turned up to serve as a gas inlet. The vessel was capped by a B45 ground joint which led to a water bubbler to exclude air. Gas was passed first through the empty vessel, and then a slurry of fresh undried starch granules in 100 ml. of water was added (0·5% w/v final concentration of starch) and the gas stream continued in the cold for 30 min. The vessel was then heated in boiling water for 10 min. Frothing began after about 1 min. and continued for about a further 4 min. The foam passed right out of the vessel, carrying with it surface-active materials, and thereby freeing the starch of most of its protein and fatty impurities. The starch dispersion was next cooled to about 30°, removed, and centrifuged. It separated readily into a clear supernatant liquid and a tightly packed gel. The supernatant liquid was centrifuged a second time if any floating gel particles were noticed. It was then filtered (G4 filter).

The gel of amylopectin was washed on the centrifuge with distilled water until the washings gave no colour with iodine, and then redispersed in distilled water at *ca.* 95° for 10 min. as before. It was then cooled, centrifuged, and washed.

The amylose can most simply be recovered from solution by precipitation with butanol. No further fractionation occurs, but the amylose is obtained in a form suitable for storage under butanol-saturated water. In the present experiments a much more elaborate treatment with butanol was undertaken in order to ensure the removal of any residual fatty materials by extraction with hot butanol.

The amylose solution plus butanol (50 ml.) was put back in the dispersion vessel, de-aerated, and then heated in a water-bath to *ca.* 60° until the opalescent solution became clear. The gas speed was next increased until the two layers became completely mixed, in order to extract fatty materials into the butanol phase. After 2 min. the rate of passage of gas was decreased to allow the layers to re-form, the vessel opened, and the hot butanol layer removed by pipette so far as possible. The surface of the amylose solution was washed with a little fresh butanol, which was removed, and the solution was cooled. The whole process was repeated, and then the amylose solution was cooled in the presence of excess of butanol, and the precipitate centrifuged and stored. No iodine-staining material was present in the supernatant solution and therefore no further fractionation was occurring. As a further precaution in the present experiments the above series of extractions with butanol was repeated before characterisation of the amylose.

Characterisation of the Fractions.—In the initial stages of a study of a procedure for fractionation, the most useful properties to follow are the iodine stains of the two fractions and the intrinsic viscosity of the amylose. The iodine stain is a sensitive measure of the degree of separation attained, and the viscosity indicates whether breakdown has been extensive or not.

The fractions were stained under the conditions described by McCready and Hassid (*J. Amer. Chem. Soc.*, 1943, **65**, 1154) to obtain " blue values " as defined by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924). It is usual to regard pure amylose as having a " blue value " of 1·4 and pure amylopectin a value between 0·1 and 0·2. Before estimation of its " blue value," the amylopectin was refluxed twice with 85% methanol for 2 hr., the hot methanol being decanted each time. The centrifuged suspension was then dried to constant weight *in vacuo* in a drier jacketed by chloroform vapour, and a known weight dispersed in 0·5N-sodium hydroxide. For the " blue value " of whole wheat starch, a dispersion of the starch in hot water was extracted three times with hot butanol as described above for amylose, and then the homogeneous suspension consisting of amylose complex and amylopectin was freeze-dried and dried *in vacuo* in a chloroform-drier. Weighed amounts were dispersed in 0·5N-sodium hydroxide, neutralised, and stained with iodine.

The intrinsic viscosity of amylose was determined under four sets of conditions: (*a*) on undried amylose in 0·5N-sodium hydroxide, (*b*) on freeze-dried amylose in 0·5N-sodium hydroxide, (*c*) on acetylated amylose in chloroform, and (*d*) on deacetylated amylose acetate in 0·5N-sodium hydroxide.

Details.—Viscosity measurements were made at 20° in a modified Ubbelohde viscometer (Davis and Elliott, *J. Colloid Sci.*, 1949, **4**, 313) which allowed dilution *in situ*. No kinetic-energy corrections were necessary.

The sample of amylose stored under butanol–water was centrifuged and drained. A portion of the wet paste was dissolved in N-sodium hydroxide at room temperature and diluted to half this concentration before the measurement of the intrinsic viscosity. To find the concentration of amylose in this solution, the solution was neutralised and stained with iodine in the normal way, and the optical density compared with the known " blue value " of the sample.

Another portion of the paste was dispersed in hot water, butanol added to precipitate the amylose, and the suspension cooled quickly, frozen, and freeze-dried. The resulting powder, which contained about 5% of moisture, was further dried in a chloroform-drier in a vacuum to constant weight. Samples were used for determination of intrinsic viscosity and " blue value " as above.

A third portion of the paste was acetylated by the method of Higginbotham and Morrison (*loc. cit.*). The paste was washed with dry butanol and then dry pyridine. After 24 hours' storage in pyridine the amylose was shaken at room temperature for 24 hr. with a mixture of equal volumes of pyridine and acetic anhydride. After filtering, the solution was run in a fine stream into ice-water, and the precipitated fibre simultaneously spun on to a glass rod. The fibre was washed well with water, and then with dry ethanol, and finally stored in a vacuum-desiccator over solid potassium hydroxide.

Weighed quantities of the acetate were used for measurements of intrinsic viscosity in chloroform and for deacetylation. Deacetylation was carried out by shaking the acetate for 24 hr. at 0° in N-sodium hydroxide. The solution of amylose formed was diluted to 0·5N-sodium hydroxide for its intrinsic-viscosity determination.

The viscosity of the amylopectin was not determined because of the difficulty of dealing with a suspension of particles with a strong tendency to become oriented in the stream-lines and to settle during the measurement.

Control Experiments.—To determine whether granule size influenced the molecular size of the amylose, a suspension of starch granules in water was allowed to settle for 5 min. and then a sample of the smaller granules which had still not settled was obtained from the upper layer and dispersed and fractionated as before. This amylose sample is marked U_6 in Tables 1 and 2.

The influence of butanol on the viscosity of amylose in alkali was found by determining the intrinsic viscosity in 0·5N-sodium hydroxide containing various concentrations of butanol.

A comparison was also made between the intrinsic viscosity of amylose dissolved in the form of its butanol paste in sodium hydroxide, and amylose from which the butanol had first been removed by steam-distillation under nitrogen.

Storage in neutral solution in the presence of salt had been found to reduce the intrinsic viscosity of potato amylose as measured in alkali (Baum and Gilbert, *loc. cit.*). Some samples of amylose were therefore made 1% in sodium chloride and left for a short time before being made alkaline for viscosity determinations.

Results

The results of six independent fractionation experiments are collected in Table 1. The letter M is used to indicate mottled wheat and U normal wheat; *a*, *b*, *c*, and *d* refer to the conditions in which the viscosities were determined (p. 4049).

Table 1.

Amylose sample	" Blue value "	Intrinsic viscosity at 20°				
		Undried amylose in NaOH (*a*)	Dried amylose in NaOH (*b*)	Amylose acetate in $CHCl_3$ (*c*)	Deacetylated in NaOH (*d*)	Ratio (*c*) : (*d*)
M_1	1·17, 1·25 *	2·8	3·6	4·8	3·0	1·6
U_2	1·10	2·5	3·7	4·8	3·0	1·6
U_3	1·22	—	4·3	4·8	3·0	1·6
M_4	1·10	2·5	3·5	—	—	—
M_5	1·18	—	4·5	—	—	—
U_6	1·17, 1·18 *	—	2·9	3·8	2·4	1·6

* " Blue values " determined on solutions of weighed amounts of amylose acetate in NaOH.

The " blue values " of amylopectin of two samples obtained from independent experiments were both 0·15. No protein was detected in the amylose or the amylopectin fraction. Normal and mottled wheat starch had the same " blue value " within experimental error (0·41 and 0·40 respectively).

Apart from the very small amount of amylose which was discarded in the washings of the amylopectin, losses were merely those due to handling and transference, and were small. If the " blue value " for whole starch of 0·41 is combined with that of 0·15 for amylopectin and 1·18 for amylose (the average value in Table 1), the percentage of the starch soluble in hot water and assessed as amylose can be calculated to be 25%, which may be compared with the estimate of 26% given by Schoch (*op. cit.*) for the amount of amylose in wheat starch.

Results of Control Experiments.—Addition of 1% sodium chloride to solutions of amylose had no effect on the intrinsic viscosity in alkali.

Steam-distillation, under nitrogen, of the wet paste of amylose, sample M_1, to remove butanol, left the intrinsic viscosity in 0·5N-sodium hydroxide unchanged at 2·8. This proved that traces of butanol accompanying amylose when solutions in sodium hydroxide were prepared from the wet paste had no effect on the intrinsic viscosity. Larger quantities of butanol had the effect shown in Table 2, in which the code used is as for Table 1. The intrinsic viscosity has been determined in 0·5N-sodium hydroxide in the presence of the listed amounts of butanol. It is seen that in every case, except for amylose prepared by deacetylation, butanol decreases the viscosity. The decrease occurs during the addition of the first 0·5% of butanol, after which the intrinsic viscosity remains constant. Allied with this is a decrease in the interaction of the

amylose molecules with one another, which is shown by a decrease in the slope of the plot of $\eta_{sp.}/c$ against c as the concentration of butanol is increased, until with 5% of butanol $\eta_{sp.}/c$ has become independent of c.

TABLE 2.

Amylose sample *	Concentration of butanol (% v/v)				
	0·0	0·5	1·0	2·5	5·0
	Intrinsic viscosity in 0·5N-NaOH at 20°				
M_1 (*b*)	3·6	3·0	3·0	3·0	3·0
M_1 (*a*)	2·8	2·3	2·3	—	—
M_1 (*a*) †	2·8	2·3	2·3	—	—
U_6 (*b*)	2·9	2·2	—	—	—
U_6 (*d*)	2·4	—	2·4	—	—

* For meaning of *a*, *b*, and *d*, see p. 4049.
† Wet butanol paste of amylose steam-distilled before being added to NaOH.

DISCUSSION

These results show that the simple elution procedure which was effective for potato starch works equally well for wheat starch. There is a slight difference in that salt is not needed as wheat amylopectin settles readily in distilled water, but as before only fresh undried starch has been used and mechanical stirring has been avoided. The action of oxygen, which rapidly lowers the viscosity of a hot dispersion of potato starch (Baum and Gilbert, *loc. cit.*), has not yet been studied for cereal starches.

It will still be necessary to rely for fractionation on the selective precipitation of amylose (Lansky, Kooi, and Schoch, *J. Amer. Chem. Soc.*, 1949, **71**, 4066) where prior treatment of the starch, as for instance in the production of some commercial starches, has rendered the amylopectin soluble or has insolubilised some of the amylose within the granule. It should be mentioned, however, since it has a bearing on the true molecular size of amylopectin, that Bechtel (*Cereal Chem.*, 1951, **28**, 29) showed that apparently clear solutions of starch prepared by vigorous methods of dispersion contain large numbers of undissolved fragments of granule which can be seen by means of the phase-contrast microscope.

The dependence of the intrinsic viscosity of alkaline solutions of amylose on the method of preparation of the solution (Table 1) lends emphasis to the idea that amylose can be obtained in different configurations in solution (see Husemann and Bartl, *Makromol. Chem.*, 1953, **10**, 183). However, the acetate has a reproducible intrinsic viscosity of 4·8 in chloroform, and on deacetylation gives a reproducible value of 3·0 in 0·5N-sodium hydroxide. Under such conditions, therefore, conclusions can be drawn from intrinsic viscosity as to relative molecular size. The respective values of 4·8 and 3·0 may be compared with the corresponding values of 6·2 and 4·1 obtained in earlier unpublished work for potato amylose (Baum, Bottle, and Gilbert). Wheat amylose has therefore a somewhat smaller molecule than potato amylose. The results for the sample U_6 lead to the conclusion that the more slowly sedimenting granules of wheat contain amylose of lower average molecular weight.

Throughout no difference has been found between the starch of mottled and of normal wheat, and the duplication of results has served rather to illustrate the reproducibility of the methods and measurements.

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