XXVII. THE DETERMINATION OF STARCH IN PLANT TISSUE, WITH PARTICULAR REFERENCE TO THE APPLE FRUIT.

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THE quantitative determination of starch in plant tissues remains a difficult matter in spite of the numerous analytical methods described. Although two recent procedures [Denny, 1934; Sullivan, 1935], based on preliminary extraction of the starch in boiling concentrated CaCl_2 solution [after von Fellenberg, 1916], have attained a high degree of selectivity for true starch, both methods are laborious. In the present paper a relatively simple procedure is described which appears to be strictly specific for starch and to give unusually exact values. With certain precautions its application to tissues other than the apple should present little difficulty.

The starting material for starch determination, as in most methods, is the residue left after thorough extraction of the finely-divided tissue with hot 70–80 % alcohol. The particular advantages of the method are due to the two following innovations: (1) The tissue residue is subjected to a treatment which converts native starch into "soluble" starch; (2) use is made of β -malt-amylase (from ungerminated barley) as a selective hydrolysing agent.

(1) The "solubilising" treatment is based on the observation [Small, 1919] that hot dilute alcoholic HCl converts starch into a highly soluble form, with minimum production of reducing products.

A suspension of the material in boiling 95 % alcohol is treated with 1/100 vol. of conc. HCl and vigorous boiling is continued for 12 min. under a reflux condenser. After rapid cooling, the residue is drained on a suction filter and washed by gradual addition of an equal volume of 95 % alcohol. In practice, weights of apple residue up to 1.5 g. ($\approx$ about 50 g. fresh weight) were treated with 100 ml. 95 % alcohol *plus* 1 ml. HCl ; for larger amounts proportionately larger volumes were used to ensure adequate mixing by the boiling of the alcohol.¹

After this standard solubilising treatment starch can be removed quantitatively from the residue by hot water extraction. In addition, solubilisation facilitates rapid action of amylase on starch.

(2) The second innovation, namely the use of β -malt-amylase, is largely responsible for the improved selectivity. Highly active preparations are readily obtainable (Appendix I) which contain probably no other carbohydrase.

A further advantage is that the reducing products of the action of this enzyme consist almost exclusively of maltose, a characteristic which is the basis of its use in the standard method of preparing maltose [Harding, 1923; cf. Hanes, 1935].

The degradation of starch by β -amylase proceeds to a definite "hydrolysis limit" which is independent of enzyme concentration and is only slightly

¹ Note added January 8th, 1936. Attention is called to the recent paper by Niemann *et al.* [1935, *Plant Physiol.* 10, 579] in which a preliminary solubilising treatment with alcoholic HNO_3 is described.

influenced by variation in the method of solubilising the substrate.¹ The value is remarkably constant when the latter is controlled (*cf.* Table I).

In order to convert into its equivalent of starch an observed increase in reducing power resulting from the action of the enzyme on a tissue extract, a knowledge of the hydrolysis limit for the particular variety of starch is necessary. Starches from potato and apple after the standard solubilising treatment are found to give almost identical values, the increase in reducing power corresponding to 0.603 and 0.600 mg. maltose, respectively, per mg. starch. It is probable from van Klinkenberg's [1932] results that similar values hold for wheat, arrow-root and buckwheat starches [*cf.* Hanes, 1935]. However, since some starches may differ in this respect, it is advisable to make fresh determinations for each individual variety.

EXPERIMENTAL.

1. *Technique of determination.*

The same procedure was followed in determining maltose production by the action of β -amylase on solutions of purified apple starch (control starches) and on filtrates from apple tissue.

Values for increase in reducing power are based on differences in reduction values of parallel digests with active and "killed" enzyme (inactivated by holding at 90° for 10 min.), the conditions and duration of reaction being such as to ensure attainment of the hydrolysis limit.

Digests were maintained at the optimum acidity by the addition of acetate buffer [Cole, 1933] of p_H 4.63 (final concentration $N/70$ to $N/90$), at a temperature of 25°, the final starch concentration not exceeding 0.25%. Pipettes were plugged with cotton-wool to exclude traces of saliva, and toluene was added as a preservative.

It is convenient to use a concentration of enzyme considerably in excess of that actually required to accomplish the hydrolysis within the selected reaction period. (This avoids the need for more than occasional examination of its activity, which for the present purpose is best done by ascertaining that no maltose is produced during the final quarter of the period.) Analysis of digests in the subsequent experiments was done after approximately 42 hours' reaction. In general, digests were of 28 ml. final volume, comprising 25 ml. substrate (control starch or tissue filtrate), 2 ml. $N/5$ acetate buffer and 1 ml. stock β -amylase solution (Appendix I), but in some cases 2 ml. enzyme were used.

In general an alkaline ferricyanide reagent² [Hanes, 1929] was used for maltose determinations on 5 ml. (occasionally 3 ml.) digest samples, but in addition a copper reagent, Reagent 60 of Shaffer and Somogyi [1933], was used for comparison, after standardisation against maltose.

¹ Maltose production ranging from 60 to 67% theoretical has been reported for this enzyme by different investigators. Part of the variability arises from the custom of expressing the hydrolysis limit in terms of final reduction value without correction for initial reducing power of starch and enzyme. In addition to maltose a non-reducing dextrin (erythrogranulose) is produced. These points are discussed elsewhere [Hanes, 1935].

² With certain batches of potassium ferricyanide (supplied as A.R. grade) deviations from the normal linear standardisation curve for maltose have been observed, but after purification by twice recrystallising the salt from solutions saturated at 50° the discrepancy has disappeared. This purification has accordingly been adopted as a routine procedure. With this reagent 1 ml. $N/100$ thiosulphate $\equiv$ 0.413 mg. anhydrous maltose.

2. *Determination of hydrolysis limit on control starches.*

Substrate solutions were prepared in a standard manner from the five specimens of soluble apple starch described in Appendix II. A weighed amount of starch, suspended in cold water, is washed into a larger volume of boiling water. The suspension is boiled 3 min., held at 100° on a steam-bath for 20 min. longer, then cooled and brought to volume. Concentrations are given in terms of anhydrous starch.

In a number of experiments the effect of varying enzyme concentrations was studied. The results showed that, provided sufficient enzyme is added, it may be widely varied without affecting the final value for maltose production. For example, in a series of digests (final volume 29 ml., containing 4.11 mg. control starch/5 ml.) the additions of enzyme were 0.1, 0.2, 0.3 and 0.5 ml., respectively, of stock enzyme. The final increases in reducing power indicated the production of 2.42, 2.46, 2.45 and 2.46 mg. maltose/5 ml.

In the experiments summarised in Table I the enzyme concentration was most commonly double the maximum amount above, but in some cases four times that amount.

The hydrolysis limit has been determined both with dry enzyme and, as recommended for routine use, with preparations stored in solution.

Table I. *Observed hydrolysis limits for soluble apple starch.*

S, mg. anhydrous starch/5 ml. digest.

M, increase in reducing power as mg. maltose/5 ml.

M/S, mg. maltose produced from 1 mg. starch.

1. Using stock β -amylase stored in solution

Starch A	S	2.045	2.045*	4.09	4.09*	10.21
	M	1.25	1.24	2.49	2.46	6.10
	M/S	0.611	0.606	0.609	0.601	0.597
Starch B	S	0.80	3.99	3.99*	—	—
	M	0.47	2.385	2.38	—	—
	M/S	0.589	0.598	0.597	—	—
Starch C ₁	S	2.015	4.03	4.03*	6.045	—
	M	1.29	2.415	2.42	3.66	—
	M/S	0.612	0.600	0.601	0.606	—
Starch C ₂	S	2.06	2.06*	4.12	4.12*	6.18
	M	1.26	1.22	2.46	2.47	3.72
	M/S	0.611	0.592	0.597	0.599	0.6015
Starch D	S	1.255	4.11	4.11	4.11	6.18
	M	0.76	2.46	2.45	2.46	3.72
	M/S	0.606	0.598	0.596	0.598	0.601

2. Using dry β -amylase preparation

Starch B	S	1.96	3.26	3.26*	3.91	—
	M	1.15	1.945	1.95	2.36	—
	M/S	0.588	0.596	0.599	0.600	—

* In these cases reduction values were by the copper reagent, all others by ferricyanide reagent.

The *M/S* values are closely reproducible both with varying starch concentrations and with different starch specimens. The average value of maltose production by the completed action of the enzyme in the 26 cases given is 0.600 mg. maltose/mg. starch, the extremes being 0.588 and 0.612. The different specimens were prepared from fruit picked throughout the growing season and the average sizes of the starch grains in the different cases ranged from 5.0

to 9.2μ in diameter (C_1 and C_2 , respectively). It is apparent that neither of these factors influences the hydrolysis limit by β -amylase.

Moreover, the good agreement observed by the use of the ferricyanide and the copper reagents is convincing evidence of the homogeneity of the reducing material produced, since these reagents were selected as giving widely different reducing ratios for glucose and maltose.

3. *Extraction of starch from tissue residues.*

Starch determinations were made usually on 1 g. portions of alcohol-extracted residue ($\equiv 22\text{--}33$ g. fresh apple tissue). The residue is subjected to the standard solubilising treatment (p. 168), after which the alcohol is allowed to evaporate. The product, suspended in about 110 ml. water, is boiled 3 min. and held at 100° for a further 20 min. It is then cooled and diluted to 200 ml. The suspension is filtered with suction (ordinary non-hardened paper) and 25 ml. portions of the filtrate are used as substrate in digests with active and "killed" enzyme (*cf.* p. 169).

This technique, adopted as a routine for investigating apple tissue, would require appropriate modification for other tissue with different starch content.

That the whole of the starch contained in solubilised residues was dissolved by treatment with hot water was shown as follows. Residues were extracted with 3 and 4 successive lots of boiling water and the enzyme allowed to act both on portions of the combined filtrates, and on suspensions of the extracted residues. In all cases the whole of the starch was present in the filtrates, there being negligible amounts of hydrolysable material left in the extracted residues.

The validity of the standard technique for preparing filtrates is attested by the following experiment.

Four portions of a solubilised residue were extracted as follows:

1. 1 g. residue, suspended in water, heated in the standard way and cooled, was made up to 100 ml. before filtration.

2. 1 g. residue treated as in 1, but diluted to 200 ml. before filtration (*i.e.* the standard extraction).

3. 1 g. residue extracted with four successive portions of water, the filtrates being made to 200 ml.

4. 0.486 g. residue subjected first to standard heating in 75 ml. water, then extracted 4 hours in a Soxhlet apparatus. Final volume 71 ml.

The results of β -amylase action are given in Table II.

Table II.

Extract	Tissue equivalent to 5 ml. final digest		Maltose production* mg./5 ml.	$\equiv$ starch† mg.	Starch per g. mg.
	Fresh wt. mg.	Residue mg.			
1	392	17.9	1.825 - 0.605	2.04	5.19
2	392	17.9	1.83 - 0.62	2.015	5.14
3	392	17.9	1.835 - 0.635	2.005	5.11
4	267	12.2	1.525 - 0.795	1.38	5.16

* Reducing values of digests with active and "killed" enzyme are given.

† Obtained by dividing "maltose production" by 0.600 (the hydrolysis limit).

There is good agreement in the values obtained with the different extraction methods. The slightly higher value for Extract 1 may be due to the volumetric error (from the presence of solids) being significant when the suspension was diluted to only 100 ml. before filtration.

4. *Recovery of added starch.*

Starch added to residues before the solubilising treatment is determined quantitatively by the method. The results are given of an experiment carried out with virtually starch-free apple tissue.

Five 1 g. portions of residue ($\equiv 31.5$ g. fresh weight) were used. To four of these were added amounts of native apple starch as follows—89.1, 222.9, 357.1 and 444.8 mg. The residues were then subjected to the standard solubilising and extraction procedure.

The observations are given in Table III.

5 ml. digest equivalent to		Maltose production mg./5 ml.	$\equiv$ starch mg.	Increase mg.	% recovery
mg. fresh weight	mg. added starch				
704	—	0.675 - 0.62	0.09	—	—
704	1.99	1.88 - 0.64	2.07	1.98	99.5
704	4.97	3.73 - 0.705	5.04	4.96	99.8
704	7.96	5.71 - 0.75	8.25	8.16	102.4
704	9.93	6.87 - 0.77	10.17	10.08	101.5

The results illustrate both the accuracy with which added starch is recovered and the specificity of the method since for the first residue (without added starch) a value approaching zero was obtained.

Results (not given under experimental).

Analysis of apples during storage. Analyses were made on a series of residues (from an experiment conducted by Dr F. Kidd) for which determinations of material hydrolysed by takadiastase were available.¹ The series represents a succession of samples of young apples (Bramley's Seedling) removed from a population stored at 15° from the time of picking.

By the 10th day slices treated with I-KI appeared starch-free macroscopically, but under the microscope showed traces of starch. By the 190th day the tissues were virtually starch-free, only a few minute grains being seen in a large number of slides examined.

Days at 15°	5 ml. digest $\equiv$ mg. fresh wt.	Maltose production mg./5 ml.	$\equiv$ starch mg./5 ml.	mg. "starch" per g. fresh weight		Difference mg./g.
				β -Amylase method	Taka- diastase method	
0.5	486	2.36 - 0.72	2.74	5.64	9.20	3.56
1	500	2.02 - 0.695	2.21	4.42	7.93	3.51
2	510	1.99 - 0.74	2.08	4.08	7.58	3.50
3	552	1.545 - 0.67	1.455	2.63	5.59	2.96
4	559	1.37 - 0.73	1.07	1.92	4.09	2.17
6	582	0.99 - 0.775	0.36	0.62	2.41	1.79
8	623	0.795 - 0.695	0.17	0.27	1.60	1.33
10	625	0.73 - 0.69	0.07	0.11	1.55	1.44
190	686	0.64 - 0.61	0.05	0.07	1.37	1.30

¹ I am indebted to Dr Kidd for allowing me to use the takadiastase data. In these determinations (which were made by Miss D. G. Griffiths and Miss N. A. Potter) an excess of takadiastase was allowed to act to completion on the unfractionated residues after suspension in water and heating at 100° for 30 min. The resulting reducing material was considered, for convenience in expressing the results, to be glucose produced from starch hydrolysis, and was converted into a starch value by a factor obtained from the action of the enzyme on starch paste.

Observations on filtrates from these samples are given in Table IV. Throughout the series 5 ml. digest correspond to 22.3 mg. residue, and the equivalent fresh weights are given in the table.

The values obtained by the two methods are plotted in Fig. 1, upper graph, and the differences in the lower graph.

Fig. 1. Upper: determinations by β -amylase and takadiastase methods on apples stored at 15°. Lower: takadiastase value - β -amylase value.

By the 10th day the values by the β -amylasemethod, initially considerably smaller than the takadiastase values, fall to a low level approaching zero. The takadiastase values fall to about 1.5 mg. per g. and remain at about this level during subsequent storage although the true starch content falls to zero.

The lower graph suggests that two distinct classes of material are hydrolysed by takadiastase but not by β -amylase, one of which disappears during storage and so is presumably metabolised, whilst the other persists. That two non-starch fractions hydrolysable by takadiastase are present was suggested by the

Table V.

	5 ml. digest mg. fresh weight	Maltose production mg./5 ml.	$\equiv$ starch mg./5 ml.	mg. "starch" per g. fresh weight	
				β -Amylase method	Takadiastase method
Onion	2230	1.06 - 0.99	0.11	0.05	—
	1115	0.72 - 0.70	0.035	0.03	—
Wheat leaf	370	0.60 - 0.575	0.04	0.1	7.1*
	351	0.52 - 0.48	0.08	0.2	6.9
Wheat sheath	342	0.53 - 0.48	0.08	0.25	12.1
	418	0.595 - 0.54	0.09	0.2	10.0

* These samples were kindly supplied by Dr H. R. Barnell, School of Agriculture, Cambridge, who had previously made takadiastase determinations on the same residues and concluded on other grounds that the material hydrolysed was not starch.

further observation that about 1/3 of the "extra starch" by takadiastase was not extractable by hot water, whilst 2/3 was present in the filtrates, when a portion of the 0.5 day residue was so fractionated.

Analysis of other tissues. The selectivity of the method was further confirmed in an examination of two types of tissue in which starch is either completely absent (the onion) or is present only in traces in stomata (leaves and leaf sheaths of wheat). The observations are given in Table V.

These examples demonstrate further the selectivity of the method.

SUMMARY.

A method for the determination of starch in plant tissues is described which appears to combine the advantages of selectivity, accuracy and relative technical simplicity.

After preliminary extraction of the tissue with 70–80 % alcohol the residue is boiled in dilute alcoholic hydrochloric acid to convert native starch into soluble starch. The starch is then completely extractable in hot water. Soluble starch in the extract is next selectively hydrolysed by β -malt-amylase (from ungerminated barley). Various advantages conferred by the use of this enzyme are discussed, in particular its specificity as a starch "reagent", the production of maltose as the almost exclusive reducing product and the existence of a definite hydrolysis limit.

Before applying the method to apple tissue a number of specimens of apple starch were isolated in the form of starch grains. Throughout the season, apple starch is hydrolysed to the same extent by β -amylase, the increase in reducing power being equivalent to the production of 0.600 mg. maltose per mg. starch. This value agrees with that for potato starch under similar treatment.

The method is illustrated by a number of analyses on apple and other tissues.

I wish to acknowledge the careful technical assistance of Mr Eric Whitmore.

APPENDIX I.

Preparation of enzyme.

Ungerminated barley of good malting quality (preferably pale in colour) is ground to a fine flour. Metal-free water (from an all-pyrex still) is used in the preparation.

200 g. of the flour are extracted with 750 ml. 50 % ethyl alcohol for 2 hours with stirring. After centrifuging, the residue is re-extracted (400 ml. 50 % alcohol; 0.5 hour) and again centrifuged. The supernatant liquors are combined and after filtration the enzyme is precipitated by increasing the alcohol concentration to 80 %.

The precipitate, after removal on the centrifuge, is well drained on a suction filter and suspended in 450 ml. water. Toluene is added and the solution is stored in the dark at 1°. (It may require filtration after several days to remove a flocculent precipitate.)

With reasonable precautions against heavy metal contamination (from dust and unclean pipettes) such preparations may be used for several months; inactivations of the order of 20 % are to be expected after 3–4 months' storage.

For routine purposes these solutions are preferable to dry preparations since the latter, although more stable, are extremely difficult to dissolve.

APPENDIX II.

Preparation of control starches from apples.

Five specimens of apple starch (in the form of starch grains) were prepared from fruit (Worcester Pearmain) picked at different stages of maturity from July 11th to Sept. 2nd.

Apples were frozen at -20° soon after picking; the peel was removed and the flesh ground to a powder while still frozen. The powder was suspended in a large volume of water at 1° with stirring. (For Specimens A and B the powder was dehydrated and then boiled in 95% alcohol before suspension in water; for Specimen D, 10 mg. KCN per litre were added to the water.) The suspension was then filtered through fine silk which allowed the finer particles, including starch grains, to pass. The washing of the residue was repeated several times.

Isolation of the starch from the filtrates was now carried out on the centrifuge. By repeated fractional sedimentation in water and 70% alcohol a fine grey sludge was ultimately removed. The pure product was then washed in 95% alcohol, absolute alcohol and ether.

Specimens C_1 and C_2 are two fractions of a single preparation, fine-grained and large-grained, respectively. The separation was accomplished by 24 hours' sedimentation of the partially purified product (about 12 g.) in 6 litres of water. The finer grains, removed from the supernatant liquor on the centrifuge, and the larger grains, which had settled, were then worked up separately.

The range of diameter of starch grain and the average diameter (from 100 random measurements) for the different specimens were as follows (in μ): A, 2-9, 5.5; B, 2-14, 6.5; C_1 , 3-7, 5.0; C_2 , 5-13, 9.2; D, 3-13, 7.1.

The control starches were prepared from these specimens of native apple starch by subjecting portions (2-5 g.) to the standard solubilising treatment. No loss of dry matter occurred during this process.

Determinations of moisture were made by drying to constant weight at 110° in a high vacuum in a vessel with one limb containing P_2O_5 at room temperature.

The solubilised starches had a small reducing power which ranged from 0.7 to 1.4% of the reducing power of maltose.

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