A METHOD FOR THE DIRECT AND QUANTITATIVE STUDY OF AMYLOCLASTIC ACTIVITY OF AMYLASES

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(Received for publication, July 11, 1935)

A new method for the measurement of amylase activity is presented briefly here. It has been developed to meet the need for a more direct measure of the amylolytic activity of amylases than has hitherto been possible. This need has long been evident (1) and has been emphasized by recent work with the amylases of barley malt (2).

The method to be described is based upon the direct determination of residual starch or amylase at any stage in its hydrolysis and depends upon its quantitative precipitation, free from dextrans and maltose, by ethyl alcohol. The difference between the weight of original amylase and the weight of residual amylase gives a measure of the amylolytic activity of the enzyme analogous to the measurements of its saccharogenic activity which are based upon determination of reducing sugar formed (3-5).

The new method is practicable, accurate, and adapted for use with different amylases. It offers a more direct measure of amylolytic activity than is afforded by viscosity measurements (6, 7) and makes possible the study of earlier stages in the hydrolysis of amylases than methods like that of Wohlgemuth (8, 9) which inherently depend upon the complete disappearance from the hydrolysis mixtures of products which give a blue color with iodine. It does not necessarily supplant these methods, however, as there are indications that they also yield information concerning amylase action which would not otherwise be available. It is, in fact, becoming increasingly evident that studies of the nature of amylases and their action should include quantitative comparisons of different kinds of activity measurements.
This point has also been stressed recently by Northrop and Kunitz (10) in work with proteases.

**EXPERIMENTAL**

*Procedure*—The general procedure developed for the determination of starch or amylose, either alone or in the presence of dextrins and maltose or maltose alone, may be briefly described as follows:

To the sample of starch or amylose dispersion, sufficient alcohol is added to give the desired final concentration¹ (55 per cent), and the mixture is stirred vigorously by hand. In most cases, stirring for 30 seconds to 1 minute brings about the appearance of a white flocculent precipitate. This is allowed to settle and the supernatant liquid is decanted through a Gooch crucible containing a thin mat of asbestos² and half filled with fluffy asbestos (11), which with the crucible has previously been heated to constant weight in a muffle furnace. As much as possible of the precipitate is retained in the beaker and is washed by decantation, twice with 55 per cent and twice with 70 per cent alcohol, with vigorous stirring after each addition of alcohol. The washed precipitate is finally transferred quantitatively to the crucible with a jet of 96 per cent alcohol and washed with a mixture of equal volumes of 96 per cent alcohol and ether. The crucible is then dried to constant weight in an oven at 105°, cooled in a desiccator over sulfuric acid, and weighed in a closed vessel, with care to expose the crucible to the air as little as possible. The difference in weight is taken as amylose. After ignition in a muffle furnace at a low red heat for 3 hours, the crucible is again cooled and weighed and may be used for another determination without further treatment.

*Accuracy*—The accuracy of the above method was established by a series of experiments in which the starch was determined, after precipitation, both by direct weight and by acid hydrolysis. In the latter case, after the addition of the calculated volume of alcohol, the mixture was centrifuged, the supernatant liquid decanted off, the residue ground under 96 per cent alcohol, again centrifuged and decanted, and the starch remaining determined by acid hydrolysis according to the method of the Official Agricul-

¹ Concentrations of alcohol refer in all cases to per cent by volume.
² The asbestos was alkali- and acid-washed.
tural Chemists (12). The data, which are omitted for the sake of brevity, show that the differences in the values obtained for starch by filtration and weighing and by centrifuging and acid hydrolysis were no greater than differences found in duplicate determinations by either method alone. The direct weighing procedure may, therefore, be used for quantitative measurements in place of the more time-consuming method of analysis by acid hydrolysis.

Factors and Conditions Studied—In order to determine whether the proposed method was generally applicable, studies were carried out with different concentrations and different samples of soluble potato starch (13) and with corn β-amylose (14). These were made up and adjusted to the conditions of hydrogen ion activity and electrolyte concentration which favor the action of different amylases including those of barley malt (15, 2), of Aspergillus oryzae (16, 17), and pancreatic amylase (18).

The desired weight of starch or amylose was stirred with a small amount of cold water, poured into water at 100°, boiled exactly 3 minutes, and cooled rapidly. Sufficient salt solutions were added to give the desired final concentration and hydrogen ion activity and the mixture made up to volume. Dispersions prepared for experiments with pancreatic amylase (or representing conditions for its action) were adjusted to pH 7.0 and contained 0.01 M phosphate and 0.025 M sodium chloride (18). Similarly, those for the amylases of barley malt and of Aspergillus oryzae were adjusted to pH 4.5 and 5.5 respectively, and contained 0.01 M acetate (15–17).

The precipitation of the amyloses (starch) was studied, with the amyloses alone, with mixtures of the amyloses and known concentrations of dextrins and maltose, and, finally, with reaction mixtures at various stages of the hydrolytic action of the different amylases.

Concentration of Alcohol—In order to find the minimum concentration of alcohol which would give a quantitative precipitation of starch or amylose under any given conditions, different volumes of alcohol were added to equal aliquot volumes of starch or amylose dispersion and the precipitated amyloses (starch) determined by direct weight, according to the method described above.

Alcohol concentrations were established by adding the same
volumes of 96 per cent alcohol at the same temperature to volumes of distilled water equal to those of the amylose dispersions used and taking the specific gravities of the alcohol-water mixtures by means of a hydrometer.

Temperature—A series of comparable experiments carried out at 21°, 26°, and 31° with the same starch dispersion showed that it is not necessary to include strict control of the temperature of precipitation as one of the conditions of the method. Quantitative precipitation of the amylose was obtained at each of these temperatures in the presence of 55 per cent alcohol. It is therefore possible to obtain comparable and reproducible results, under otherwise constant conditions, at ordinary (21–31°) laboratory temperatures, provided, of course, the effect of temperature upon the actual concentration of alcohol is taken into account.

Results

Influence of Concentration of Alcohol upon Extent of Precipitation of Amylose or Starch The influence of the concentration of alcohol upon the extent of precipitation of amylose or starch is best described by the data summarized in Figs. 1 and 2. These show that 55 per cent alcohol is sufficient to bring about the complete precipitation of soluble potato starch and also of corn β-amylose when the starch or amylose dispersions are adjusted to several different hydrogen ion activities (pH 7.0, 4.5, and 5.5) and contain different concentrations of electrolytes (0.01 M phosphate and 0.025 M sodium chloride or 0.01 M acetate) selected to correspond to the conditions which favor the action of the different amylases (15–18).

The data summarized in Fig. 2 show further that 55 per cent alcohol is sufficient to bring about the complete precipitation of potato β-amylose (19) from dispersions which contain it in different concentrations, 1, 2, and 4 per cent.

Moreover, 55 per cent alcohol was found to give quantitative precipitation of the starch when three samples of soluble potato starch which had been purified in this Laboratory by different workers over a period of 15 years were similarly treated.

Separation of Amyloses from Known Mixtures with Dextrins and Maltose—Amyloses may also be quantitatively recovered from their mixtures with dextrins and maltose by adjusting the alcohol
concentration to 55 per cent as described above. This was shown by a study of the weight and properties of the material thus precipitated from such mixtures. An example is discussed briefly here.

2 per cent soluble potato starch brought to the conditions which favor the action of pancreatic amylase (18) was combined in known proportions with a mixture\(^3\) of dextrins and maltose and adjusted to 55 per cent alcohol.

\(^3\) The mixture of dextrins and maltose was obtained by the hydrolysis of starch by pancreatic amylase. When the point was reached at which the reaction mixture gave a clear red and no blue color with iodine, the action of the amylase was stopped by placing the reaction flask in a large volume of alcohol.

![Diagram](http://www.jbc.org/)
The weights of precipitated material recorded in Table I show satisfactory recovery of the starch and indicate that it was not appreciably contaminated by dextrins or maltose. If these had been precipitated with, or adsorbed by, the starch to any significant extent, one would expect a progressive increase in the weights of the precipitates with increasing proportions of dextrins and maltose. No such increases were observed, however, even when the combined weights of dextrins and maltose in the mix-

rapidly boiling water, where it was held with vigorous shaking for 5 minutes. The reducing action of the solution was determined by a gravimetric Fehling's method (4) and calculated as maltose. The difference between the maltose and the total solids of the solution was taken as "dextrins." It is significant to note that this hydrolysis mixture alone gave no precipitate with 55 per cent alcohol.
ture amounted to more than 5 times the weight of the starch. On the other hand, when the concentration of alcohol was raised above 55 per cent, such progressive increases were readily demonstrated. They were markedly noticeable in the presence of 59 per cent alcohol.

**TABLE I**

*Precipitation of Soluble Potato Starch, in Presence of Dextrins and Maltose, from 55 Per Cent Alcohol*

2 per cent starch; pH 7.0 to 7.1; 25°; 0.025 M sodium chloride; 0.01 M phosphate.

<table>
<thead>
<tr>
<th>Original mixture</th>
<th>Dextrins*</th>
<th>Maltose</th>
<th>Starch recovered</th>
<th>mg.</th>
<th>mg.</th>
<th>mg.</th>
<th>per cent</th>
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</thead>
<tbody>
<tr>
<td>Starch</td>
<td></td>
<td></td>
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<tr>
<td>100</td>
<td>10.9</td>
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<tr>
<td>100</td>
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<td>495.4</td>
<td>495.4</td>
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<td></td>
</tr>
</tbody>
</table>

Average recovery.......................... 99.31

* As explained in the text, the "dextrins" were determined by difference between total solids and maltose in a hydrolysis mixture obtained from starch by the action of pancreatic amylase. The hydrolysis mixture alone gave no precipitate with 55 per cent alcohol.

Further evidence that the material precipitated from known mixtures of amyloses, dextrins, and maltose in the presence of 55 per cent alcohol was not appreciably contaminated with maltose or dextrins was provided by a study of its properties. Portions of such precipitates were tested for reducing action, both by a gravimetric Fehling's method (4) and by iodometric titrations (5), for optical activity, and for alkali lability (20). The
latter is an exceedingly sensitive property of amyloses. As defined by Taylor and Salzmann (20), alkali lability is measured by the difference between the reducing value obtained before and after a specified treatment with hot aqueous alkali. In these studies, the alkali lability obtained after holding the material in 0.10 N sodium hydroxide for 1 hour at 100° was 7.6 mg. of iodine per 100 mg. of original starch and also 7.6 mg. of iodine per 100

Table II

Comparison of Reducing Values of Original and of Residual Amyloses Obtained throughout Their Hydrolyses by Different Amylases*

<table>
<thead>
<tr>
<th>Time of hydrolysis (min.)</th>
<th>Reducing value of pptd. amylose, mg. I per 100 mg.; hydrolysis by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight of pptd. amylose</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>171.7</td>
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<td>10</td>
<td>152.7</td>
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<td>15</td>
<td>134.2</td>
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<tr>
<td>20</td>
<td>93.6</td>
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<td>54.0</td>
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<td>30</td>
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<td>40</td>
<td>4.13</td>
</tr>
<tr>
<td>45</td>
<td>0.00</td>
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<tr>
<td>Original amylose</td>
<td></td>
</tr>
</tbody>
</table>

* Hydrolyses at 40° of 2 per cent soluble potato starch adjusted to: (a) 0.025 M sodium chloride, 0.01 M phosphate, pH 7.0 (18); (b) 0.01 M acetate, pH 4.5 (15); (c) 0.01 M acetate, pH 5.5 (16, 17). In the last case, adjusting the mixture to 0.025 M sodium chloride before the precipitation by alcohol made the precipitates easier to work with.

mg. of precipitated material. Data for the reducing values and optical activities are omitted for the sake of brevity. In each case, however, the values obtained with the precipitated material agreed with those given by the original amylose or starch.

Precipitation of Residual Amyloses at Different Stages in Their Hydrolyses by Amylases--A similar series of experiments, carried out with aliquot portions removed from hydrolysis mixtures at
frequent intervals throughout the course of the hydrolyses of soluble potato starch, by different amylases (15–18), shows that precipitation from 55 per cent alcohol according to the method outlined here may be used for the quantitative determination of residual amylose (starch) at any stage in its hydrolysis by amylases.

Part of the evidence for this statement is summarized in Table II which shows that the reducing value of the precipitated material obtained throughout the course of the hydrolysis of soluble potato starch by different amylases agrees with that of the original unhydrolyzed starch and, therefore, gives no indication of contamination with dextrins or maltose even under conditions in which dextrins of relatively high molecular weights might be expected to be present in, and more readily precipitated from, the reaction mixtures.

Moreover, extraction of the precipitated material for 1 hour with boiling 80 per cent alcohol (21) failed to give evidence of the presence of maltose or dextrins. The extracts, after the removal of the alcohol, showed no measurable reducing action and gave no weighable residues. This is convincing evidence, especially in view of the fact that the process had previously been found to cause the quantitative extraction of dextrins and maltose from known mixtures with starch.

The only difficulties in applying the method, as outlined, to the recovery of residual amyloses (starch) from hydrolysis mixtures containing the different amylases were encountered in those cases in which commercial taka-diastase was used as a source of the amylase of *Aspergillus oryzae*. Under these conditions, the precipitates tended to come down in a finely divided, gummy form which was difficult to wash and transfer to a crucible. Small additional amounts of electrolytes, introduced after the action of the amylase had been stopped but before treatment with alcohol, removed this difficulty. Thus, adjusting the mixture to 0.025 M sodium chloride just prior to the addition of the alcohol gave satisfactory results.

SUMMARY

A new method has been developed for the measurement of amylase activity. It depends upon the determination of residual starch or amylose at any stage of its hydrolysis by amylases.
through its quantitative precipitation by ethyl alcohol. The precipitates obtained under the specified conditions are not appreciably contaminated by dextrins or maltose. The method appears to offer a more direct measure of the early stages of the amylolytic activity of amylases than is afforded by other methods previously available. It is practicable, accurate, and adapted for use with different amylases.

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