THE EFFECT OF THE HYDROGEN ION CONCENTRATION 
UPON THE STARCH-LIQUEFYING ACTIVITY OF THE 
AMYLASE OF ASPERGILLUS ORYZÆ. 

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In order to determine by viscometric methods (1), the effect of the hydrogen ion concentration of starch substrates upon the activity of the amylase of _Aspergillus oryzae_ (taka-diastase), it was first necessary to ascertain the effect of acid and alkali alone upon the viscosity of starch solutions. 

_Effect of Hydrogen Ion Concentration upon the Viscosity of Unbuffered Starch Solutions._—10.2 gm. of commercial soluble starch (Merck), made according to Lintner's method (2), were thoroughly mixed in 500 cc. of distilled water, boiled for 1 minute, and then autoclaved at a pressure of 18 pounds for 15 minutes. After cooling to 34°C., the solution was made 500 cc. with distilled water and filtered through muslin. 49 cc. of this solution (containing 1 gm. of soluble starch) were placed in each of nine 50 cc. volumetric flasks. The amount of HCl or NaOH required to adjust the reaction of each flask to the desired pH was added and the total volume was made 50 cc. with distilled water. The approximate amounts of acid or alkali required are indicated in Table I. These amounts differ for different lots of starch. The hydrogen ion concentration was determined by colorimetric methods (3). The viscosity of each of these nine solutions was determined in large Ostwald viscosimeters at 34°C. with accurate stop-watches. These results are expressed in Table II in terms of relative viscosity; i.e., the observed viscosity divided by the viscosity of water for the particular viscosimeter employed. A viscosity approximately two and a half times that of water is the most suitable for accurate measurements and this was produced
Activity of Amylase

by a 2 per cent solution of the lot of starch employed. These boiled and autoclaved solutions of starch in water were colorless and 100 cc. would reduce only 127 mg. of copper (Bertrand method (4)). As this amount is equivalent to 0.1 per cent maltose or 0.07

TABLE I.
Approximate Amounts of HCl or NaOH Required to Adjust the Reaction of 50 Cc. of 2 Per Cent Boiled and Autoclaved Starch Solution to pH 1 to 9 Inclusive.

<table>
<thead>
<tr>
<th>Desired pH of 2 per cent starch solution</th>
<th>HCl</th>
<th>NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.66 cc. 10 N</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5 &quot; N</td>
<td>0.5 &quot; N/10</td>
</tr>
<tr>
<td>3.0</td>
<td>0.5 &quot; N/10</td>
<td>0</td>
</tr>
<tr>
<td>4.0</td>
<td>0.1 &quot; N/10</td>
<td>0</td>
</tr>
<tr>
<td>5.6</td>
<td>0</td>
<td>0.16 cc. N/100</td>
</tr>
<tr>
<td>6.0</td>
<td>0</td>
<td>0.166 &quot; N/10</td>
</tr>
<tr>
<td>7.0</td>
<td>0</td>
<td>0.22 &quot; N/10</td>
</tr>
<tr>
<td>8.0</td>
<td>0</td>
<td>0.43 &quot; N/10</td>
</tr>
<tr>
<td>9.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE II.
Effect of the Hydrogen Ion Concentration upon the Viscosity of 2 Per Cent Starch Solutions at 31°C.

<table>
<thead>
<tr>
<th>pH of solution</th>
<th>Relative viscosity (observed viscosity / viscosity of water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>2.22</td>
</tr>
<tr>
<td>2.0</td>
<td>2.27</td>
</tr>
<tr>
<td>3.0</td>
<td>2.41</td>
</tr>
<tr>
<td>4.0</td>
<td>2.64</td>
</tr>
<tr>
<td>5.6</td>
<td>2.61</td>
</tr>
<tr>
<td>6.0</td>
<td>2.55</td>
</tr>
<tr>
<td>7.0</td>
<td>2.57</td>
</tr>
<tr>
<td>8.0</td>
<td>2.59</td>
</tr>
<tr>
<td>9.0</td>
<td>2.58</td>
</tr>
</tbody>
</table>

per cent dextrose it is improbable that very much hydrolysis occurred as a result of this treatment. However, if starch is excessively heated with acid or alkali, hydrolysis and caramelization are produced. This made it necessary to cool the aqueous
solutions before acid or alkali was added. Inasmuch as it has been reported (5) that the addition of small amounts of various salts to substrates made with commercial soluble starch, which already contains a certain amount of salts, does not affect the saccharogenic activity of Aspergillus oryzae (taka-diastase), salts were not added to our starch preparations.

As may be seen from Table II, reactions from pH 3.0 to pH 9.0 had but little effect upon the viscosity of starch solutions. At pH 1.0 and 2.0 partial precipitation of the starch occurred. This may perhaps explain the reduction of viscosity, for suspensions of undissolved starch have practically the same viscosity as water. The viscosity of starch solutions at pH 3.0 to pH 9.0 did not appreciably change after standing at 34°C. for 24 hours. The viscosity of the starch solutions at pH 1.0 and 2.0, however, decreased slightly after 24 hours, probably because of the precipitation which had very noticeably increased. It has since been found (6) that although the initial viscosity of solutions at pH 3.0 to 9.0 made with this same lot of starch after it had been kept at room temperature for several months had not changed, yet the viscosity was unstable and decreased progressively at 34°C. Solutions made with unstable starch are, of course, useless for the viscometric determination of enzyme action. The instability of the viscosity of the solutions made with this sample of starch did not depend upon the water content of the starch, for at the time the starch produced stable solutions it contained 9.2 per cent moisture, while 5 months later, when it was unstable, it contained 9.4 per cent. Other samples of starch which have been kept bottled for several months did not become unstable.

Effect of Hydrogen Ion Concentration of Unbuffered Starch upon the Starch-Liquefying Activity of Amylase (Taka-Diastase).—10 cc. of a 2.0 per cent starch solution at pH 1.0, prepared as described above, were placed in a large Ostwald viscosimeter at 34°C. and the initial viscosity determined. 0.1 cc. of a 0.2 per cent solution of commercial taka-diastase in distilled water was then added to the 10 cc. of starch solution in the viscosimeter, thoroughly mixed by shaking, and the time noted. The viscosity was determined at intervals of 3 or 4 minutes for a period of 1 hour. The viscosimeter was then cleaned with boiling water and dried with alcohol and ether. The same procedure was repeated with 2.0 per cent
starch solutions at pH 2.0, 3.0, etc. The hydrogen ion concentration was determined after the period of incubation and found to be the same as at the beginning of the experiment.

The results of the determinations are expressed in Fig. 1. The abscissae represent the elapsed time (in minutes) after adding the enzyme. The ordinates represent the observed viscosities expressed as percentages of the initial viscosity (1). The viscosity of starch solutions containing boiled inactivated enzyme remained constant for 24 hours or more. These curves represent the averages of four experiments done at different times in which the determinations at each hydrogen ion concentration were made in duplicate.

Fig. 1. The effect of the reaction of the substrate (2 per cent starch solution) upon the starch-liquefying activity of amylase (*Aspergillus oryzae*, taka-diastase).
tion upon the starch-liquefying activity of the amylase of taka-diastase, illustrated in Fig. 1, can also be expressed in numerical

### TABLE III.

Effect of Hydrogen Ion Concentration of the Substrate (2 Per Cent Starch Solution) upon the Starch-Liquefying Activity of the Amylase of Aspergillus oryzae at 34°C. in Units per Gm. of Dried Taka-Diastase (6.3 Per Cent Moisture).

| pH of substrate | Time required for 20 per cent reduction of initial viscosity by 0.1 cc. of 0.2 per cent taka-diastase in 10 cc. of substrate (Fig. 1) | Units per amylase per gm. of dried taka-diastase =
|                | min.                                                                 | \[
|                |                                                                     | \[
| 1.0            | \infty                                                             | \[
| 2.0            | \omega                                                              | \[
| 3.0            | 4.7                                                                 | 68,121
| 4.0            | 9.1                                                                 | 35,183
| 5.6            | 10.6                                                                | 30,205
| 6.0            | 7.1                                                                 | 45,094
| 7.0            | 12.6                                                                | 25,410
| 8.0            | 5.6*                                                                | 18,295
| 9.0            | 10.6†                                                               | 4,229

* Indicates time required for 10 per cent reduction in initial viscosity.
† Indicates times required for 5 per cent reduction in initial viscosity.

Units are calculated on basis of \( \frac{19.2}{T} \).
Units are calculated on basis of \( \frac{8.4}{T} \).

1 A unit of amylase is that amount of enzyme which requires 60 minutes to reduce the initial viscosity 20 per cent. It was found that if 0.1 cc. of an enzyme preparation required 60 minutes to reduce the initial viscosity 20 per cent, 0.2 cc. would reduce it a similar amount in 30 minutes. If the initial viscosity was reduced 20 per cent in 10 minutes, the enzyme preparation was assumed to contain \( \frac{5}{6} \) or 6 units, etc. It has also been noted in a large series of amylase reactions that, except in those of great rapidity, the amount of enzyme which required 60 minutes to reduce the initial viscosity 20 per cent would reduce it 10 per cent in an average of 19.2 minutes and 5 per cent in 8.4 minutes. Therefore, if the reaction has been so slow that the initial viscosity has not been reduced 20 per cent during the period of observation, the number of units can be determined from the time required to produce a 10 or 5 per cent change.
Activity of Amylase

the initial viscosity of 2 per cent starch at pH 3.0, 20 per cent (Fig 1). Therefore, 0.1 cc of 0.2 per cent taka-diastase contains \frac{60}{4.7} or 12.77 units. 1.0 gm. of the dried product (6.3 per cent moisture) would contain $12.77 \times \frac{10 \times 500}{0.937}$ or 68,121 units. Fig. 2 illustrates the effect of the hydrogen ion concentration of the substrate upon the starch-liquefying activity of amylase, expressed in units per gm. of dried taka-diastase.

![Graph](image)

**Fig. 2.** The effect of the reaction of the substrate (2 per cent starch) upon the starch-liquefying activity of the amylase of *Aspergillus oryzae*, in units of amylase per gm. of dried taka-diastase.

It is apparent from Figs. 1 and 2 that the optimal reaction for the starch-liquefying effect of taka-diastase upon a 2 per cent starch solution without additional buffer, as determined by viscometric methods, was at pH 3.0 and that the limits of activity were between pH 2.0 and pH 9.0. With an exception at pH 6.0, the activity of amylase decreased progressively from pH 3.0 to pH 9.0. We are unable at present to explain this exception at pH 6.0. At pH 1.0 and 2.0, the enzyme was completely destroyed, for the addition of sufficient alkali to readjust the reaction to the optimum failed to reactivate the enzyme. The exposure of taka-diastase for 5 minutes to sufficient HCl to produce a reaction of pH 1.0 and 2.0 also completely destroyed the amylase.

**Effect of Hydrogen Ion Concentration of Starch Solutions containing Universal Buffer Solution upon the Starch-Liquefying**
Activity of Amylase (Taka-Diastase).—In the preceding experiments no buffer was added because the hydrogen ion concentrations of the solutions of commercial Lintner starch were stable and did not change even after 20 hours hydrolysis. However, in order to study the effect of additional buffer, a boiled and autoclaved 4 per cent solution of starch was made. 25 cc. of this solution were added to each of six flasks containing 25 cc. of normal universal buffer solution (a mixture of phosphate, formate, acetate, sulfonate, and thymol) at pH 2, 3, 4, 5, 6, and 7. The hydrogen ion concentration of the resulting 2 per cent starch solutions containing N/2 universal buffer solution were in each instance the same as that of the original normal buffer solution.

The rate at which 0.1 cc. of 0.1 per cent solution of commercial taka-diastase in distilled water reduced the initial viscosity of 10 cc. of each of these solutions was then determined as previously described. These determinations were made in duplicate on five different occasions. Reactions similar to those illustrated by the curves of Fig. 1 were obtained except that the rate of hydrolysis was the most rapid at pH 4.0 and that at pH 3.0 as well as at pH 2.0 the enzyme was destroyed. The relative rates at pH 4, 5, 6, and 7 were in the order named but the differences between the rates at these hydrogen ion concentrations were not as great as those illustrated in Fig. 1 for unbuffered starch solutions. Furthermore, the same amount of enzyme can apparently reduce the viscosity of starch solutions containing universal buffer more rapidly than that of unbuffered starch solutions. It is possible that one or more of the constituents of the universal buffer may act as an accelerator in this enzyme reaction.

DISCUSSION.

In viscometric determinations at 34°C. the optimal reaction for the hydrolysis of unbuffered starch solutions by the amylase of Aspergillus oryzae (taka-diastase) was pH 3.0 and of starch solutions buffered with N/2 universal buffer pH 4.0. Of course, to determine the optimal reaction accurately, the hydrolysis of solutions whose hydrogen ion concentrations varied only 1/10 of a pH should be studied. However, this change of the optimum from pH 3.0 to pH 4.0 after the addition of this buffer mixture was so striking that one is led to believe with Wistätter and his co-
workers (8) that the optimal reaction for an enzyme instead of being specific for that enzyme may be dependent upon the chemical substances present in the enzyme-substrate mixture. He found that the optimum for a crude solution of lipase was from pH 5.5 to 6.3 and for a preparation purified with kaolin from pH 7.1 to 7.9. However, different pH optima with different buffers may not be real, for true hydrogen ion values are not always obtained by the ordinary methods of measurement.

The optimal hydrogen ion concentration for an enzyme reaction also depends upon the type of buffer solution used. Michaelis and Pechstein (9) found that the optimal reaction for saliva diastase was pH 6.1 to 6.2 in phosphate, acetate, and sulfate mixtures and pH 6.9 in chloride and nitrate mixtures. Hahn and Meyer (10) reported that the optimal pH for saliva diastase was 6.6 in a phosphate mixture and 5.6 in an acetate mixture, while the corresponding optima for pancreatic diastase were pH 7.2 and 5.6.

The optimal reaction for an enzyme also depends upon the temperature at which the hydrolysis is determined, for Compton (11) found that the amount of maltose converted by the maltase of *Aspergillus oryzae* (taka-diastase) reached a maximum at pH 3.0 when determined at 32.5°C. and at pH 7.2 at 47°C. Sörensen (12) found that pH 4.4 to 4.6 was the optimum for yeast invertase at 52.1°C. and Michaelis and Davidsdohn (13) reported that pH 4.2 was the optimum at 22.3°C. Olson and Fine (14) found that the optimal reaction for malt diastase was pH 4.3 at 25°C. and pH 6.0 at 69°C. These differences in optimal hydrogen ion concentrations at different temperatures may be fictitious as Thomas suggested. In all the cases cited, the pH values were measured at room temperature but the true pH value at the temperature of the experiment may have been quite different.

It is possible that differences in the chemical content of the enzyme-substrate preparation as well as in the temperature at which the amylase activity was studied are responsible for the discrepancies between the optimum of pH 3.0 for taka-diastase which we found with unbuffered starch, pH 4.0 with buffered starch, and pH 4.8 reported by Sherman, Thomas, and Baldwin (15). Sherman, Thomas, and Baldwin added a solution of purified

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*We wish to express our thanks to Dr. A. W. Thomas for many helpful suggestions.*
taka-diastase to a 2 per cent solution of Lintner starch at various hydrogen ion concentrations and after 30 minutes of incubation at 40°C. determined the amount of reducing sugar with Fehling's solution. They found that the greatest enzymatic activity was at pH 4.8 and the limits were between pH 2.6 and 8.0.

Differences in the methods used may also have been responsible for the difference between our results and those of Sherman, Thomas, and Baldwin. The changes in the starch which cause a decrease in viscosity may not be the same as those which produce an increase in the reduction of copper. When two different methods are used to measure the activity of an enzyme, especially methods in which the rate of the disappearance of the substrate and of the formation of end-products is determined (as in diastatic enzymes), distinctly different optima may be observed. For instance, Sjöberg (16) reported that the optimal zone for the activity of Phaseolus diastase was at pH 4.0 to 6.0 if the rate of the disappearance of starch was measured but at pH 5.0 to 5.5 if the rate of the formation of reducing sugars was determined. Gore (17), with the polarimetric method, found practically the same optimal pH range for malt amylase which Sherman, Thomas, and Baldwin reported for this enzyme with the copper reduction method. Both of these methods measured the saccharogenic action of the enzyme while the viscometric method we used measured the starch-liquefying power.

Different criteria for enzyme activity may also be responsible for differences in results. We have adopted the time required to effect a definite amount of change in the substrate as our standard while others have determined the amount of change in the substrate in a definite period of time. It has been shown (1) that in viscometric determinations the former is proportional to the activity of the enzyme employed while the latter is not.

The optimal reaction for an enzyme apparently depends upon so many factors that it is not surprising that the optima for the digestive and other body enzymes as determined by laboratory methods are sometimes different from the reactions at which these enzymes must function in the body.
92 Activity of Amylase

CONCLUSIONS.

1. The viscosity of 2.0 per cent starch solutions was only slightly affected by changes of reaction from pH 3.0 to 9.0. Starch was partially precipitated at pH 1.0 and 2.0.

2. The optimal reaction for the starch-liquefying activity of the amylase of Aspergillus oryzae (taka-diastase) was at pH 3.0 and the limits of activity were between pH 2.0 and pH 9.0 when determined with unbuffered starch solutions by the viscometric method at 34°C. The optimum was at pH 4.0 when the starch solutions were buffered with N/2 universal buffer.

3. The amylase was completely destroyed at pH 1.0 and 2.0 with unbuffered starch and at pH 2.0 and 3.0 with starch buffered with N/2 universal buffer.

4. It is worthy of comment that the optimal pH for amylase both with unbuffered and buffered starch was close to the reaction at which the enzyme was destroyed.

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