ENZYMICALLY INDUCED CHANGES IN THE TURBIDITY OF STARCH SOLUTIONS*

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The usual methods for detecting the action of amylolytic enzymes on starch are confined to three types of measurements: (1) increase in reducing action; (2) decrease in viscosity; and (3) decrease in the color of the resulting dextrin-iodine complex. Optical methods based upon the changes in optical rotation (1) and upon the refractive indices of the individual split-products (2) have been less frequently employed. Yet one of the most characteristic properties of starch solutions is the fairly intense Tyndall effect they exhibit, especially some time after their preparations. This opalescence can be ascribed to the light-scattering effect of the high molecular constituents of the substrate, the amylopectin moiety probably contributing a major portion. When amylolytic enzymes are added to such solutions, certain well defined changes in the opalescence or turbidity are observed. As will be shown in this paper, each type of amylase used gives a characteristic turbidity pattern. A display of these patterns, as well as some of the pertinent factors affecting the turbidity changes, is presented.

Methods and Materials

Enzymes—The α-amylase used was a preparation of malt α-amylase crystallized three times (3). Crude α-amylase was prepared from a concentrated malt extract by heating and ammonium sulfate fractionation. The β-amylase preparation was a commercial preparation used for the determination of α-amylase. It was presumably prepared by acid treatment and ammonium sulfate fractionation, and was evidently free of traces of α-amylase. "Crude" Bacillus macerans amylase was prepared from the culture fluid of B. macerans by concentration and ammonium sulfate fractionation (4). The "purified" B. macerans amylase was prepared from the crude enzyme by adsorption on starch (5), treatment with periodate, and ammonium sulfate fractionation, as described by Hale and Rawlins (4). The unit of enzyme activity for both malt α-amylase and B. macerans amylase is based upon the rate of decrease in the

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color of the digest after the addition of iodine (4, 5). The unit for \( \beta \)-amyylase activity is based upon the reducing action of the starch digest (6). 1 unit of \( \alpha \)-amyylase or \( \beta \)-amyylase activity will hydrolyze about 5 per cent of the glycosidic linkages in the starch under the conditions employed. \( B. \) macerans amylase does not create appreciable amounts of reducing groups.

Determination of Turbidity Changes—1 ml. of enzyme solution was added at 26° to 10 ml. of 2 per cent Lintner soluble starch, pH 4.75, in an Evelyn photoelectric colorimeter tube. After mixing, the tube was immediately placed in the colorimeter and the per cent transmission read off at frequent time intervals with a light filter which absorbed maximally at 400 \( \mu \). The optical density (corrected, when necessary, for the blank due to the enzyme) times 100 is defined as the “turbidity” of the digest.

Absorption Spectra of Iodine Complexes—7.5 mg. of each sample of carbohydrate were dissolved in 4 ml. of 0.5 N NaOH, neutralized with 0.2 ml. of glacial acetic acid (final pH 4.75), and diluted to 40 ml. 0.5 ml. of this solution was added to 5 ml. of 0.0035 M \( I_2 \) containing 0.25 M KI. After adding 10 ml. of \( H_2O \), the absorption of the iodine complex with various light filters was determined in an Evelyn photoelectric colorimeter.

Results

Digestion with \( \alpha \)-Amylase—As the digestion of soluble starch proceeds, there is an initial drop in the turbidity to a minimum value. The turbidity then increases at an accelerating rate until an apparent maximum is reached, after which it decreases quite slowly (Fig. 1). Increasing the enzyme concentration does not appreciably affect the minimum turbidity value, but does decrease the time required to reach this point. The time required to achieve maximum turbidity is also decreased and the actual turbidity attained is higher (Table I). Aging the starch solution prior to the addition of the enzyme increases the maximum turbidity value, but does not greatly affect the time required to reach it. Increasing the starch concentration either conceals or prevents the initial decrease in turbidity, but does not change the time required to reach maximum turbidity. Although the maximum turbidity is greater with increased concentrations of starch, the increase is not proportional to the starch concentration (see Table I). Use of crude enzyme in intermediate concentrations gives characteristic turbidities and times which are not out of line with those obtained with crystalline enzyme.

The substance responsible for the final turbidity eventually settled out of the digestion mixture as a flocculent precipitate and was thus easily isolated. About 100 mg. (dry) of this dextrin were obtained from an
Fig. 1. Changes in the turbidity of starch solutions as affected by \( \alpha \)-amylase concentrations, starch concentrations, and aging of the starch solution. The values next to each curve give starch and enzyme concentrations, respectively. The conditions of assay and the expression of the turbidity units are described in the text.

**TABLE I**

*Factors Affecting Turbidity Constants of Malt \( \alpha \)-Amylase-Starch Digest*

<table>
<thead>
<tr>
<th>Enzyme units</th>
<th>Starch</th>
<th>Turbidity characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Age</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>2</td>
<td>Fresh</td>
</tr>
<tr>
<td>1.5</td>
<td>2</td>
<td>Aged</td>
</tr>
<tr>
<td>3.5 (Crude enzyme)</td>
<td>2</td>
<td>&quot;</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>Fresh</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td>15</td>
<td>5 (55°)</td>
<td>&quot;</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>( \beta )-Limit dextrin</td>
</tr>
</tbody>
</table>

* With the exception indicated, the system consisted of crystalline enzyme dissolved in 1 ml. plus 10 ml. of soluble starch buffered at pH 4.75 at 26°. Fresh starch was less than 2 hours old; aged starch was between 3 and 4 days old.

† Time and constants at which the increased turbidity was observed to fall off from linearity, after which it continued to rise.

‡ Turbidity at 19 minutes.
α-amylase digest of 20 gm. of aged Lintner soluble starch (0.5 per cent yield). Its properties were compared with that of authentic samples of amylose and amylopectin. Like amylose, this "dextrin" was insoluble in hot or cold water. Upon addition of iodine to a small solid sample, it at first seemed to become soluble but eventually became insoluble, thus again resembling amylose rather than amylopectin. High concentrations of α-amylase will digest a suspension of the dextrin as well as a suspension of amylose. The color of the solid iodine-dextrin complex was, however, more red than blue, thus tending to resemble amylopectin but not amylose (which gives a blue-black color). The absorption spectrum of the material compared with potato amylose and with soluble starch is shown in

\[\text{Fig. 2\ (Fig. 3)}\]

\[\text{Fig. 2. Comparison of the absorption spectrum of the "turbidity" dextrin-iodine complex with that of amylose and soluble starch. The concentrations of the reagents are given in the text.}\]

\[\text{Fig. 3. Changes in the turbidity of starch by β-amylase in the presence and absence of α-amylase.}\]

Fig. 2. In shape and intensity, the curve resembles that of amylose at wave-lengths shorter than 580 mμ, and more closely that of soluble starch at longer wave-lengths.

When α-amylase is allowed to act on a β-limit dextrin solution (prepared by allowing wheat β-amylase to act on soluble starch until no further detectable change occurs, and then precipitating and drying the dextrin), there is an immediate small increase in turbidity (Table I), followed by a slow decrease. The turbidity is due entirely to an extremely small amount of an insoluble highly flocculent precipitate, since the supernatant from a centrifuged digest showed no turbidity whatsoever, being identical with the blank solution.
Digestion with \( \beta \)-Amylase—In the presence of \( \beta \)-amylase, the turbidity of a soluble starch solution decreases smoothly until about 67 per cent of the original turbidity is reached, after which there is no apparent change in turbidity (Fig. 3). No flocculent precipitate appeared, as in the case of \( \alpha \)-amylase above. Increasing the \( \beta \)-amylase concentration did not change the minimum turbidity value.

Addition of \( \beta \)-amylase to \( \alpha \)-amylase does not greatly affect the time or value of minimum turbidity, although there apparently may be slight retardation of the rate of decrease in turbidity. After the point of minimum turbidity is reached with \( \alpha \)-amylase, the action of added \( \beta \)-amylase resembles the effect of adding more \( \alpha \)-amylase; i.e., the time required to reach maximum turbidity is decreased and the turbidity value at this point is somewhat increased. The usual flocculent precipitate observed above with \( \alpha \)-amylase also settled out after a while.

Digestion with \( B. \) macerans Amylase—The turbidity changes in a fresh starch solution to which purified \( B. \) macerans amylase was added are shown in Fig. 4. The effect of a solution of \( \alpha \)-amylase at exactly the same apparent enzyme concentration is shown for comparison. As can be seen, the rate of decrease of the turbidity, unit for unit, is much slower in the presence of the \( B. \) macerans enzyme compared with malt \( \alpha \)-amylase. Furthermore, with the purified enzyme, at least, there is no subsequent immediate increase in turbidity, which decreases to a constant value at about 40 per cent of the initial turbidity. Upon prolonged digestion, there is a slight gradual increase in turbidity, although very little visible pre-
Turbidity of Starch Solutions

Precipitate usually appeared in the digest. The same was true when an aged starch solution was used. As Fig. 5 shows, increasing the concentration increased the rate and extent of turbidity decrease, and also magnified, to a slight extent, the apparent tendency for the digest to increase subsequently in turbidity. In contrast, the use of "crude" preparations of B. macerans enzyme shows an almost identical turbidity pattern with that of malt \( \alpha \)-amylase. The main difference was that, unit for unit, the B. macerans enzyme went through the characteristic changes at a much slower rate. Addition of maltose to the crude B. macerans enhanced the rate of increase of turbidity, but not the final value obtained (see Fig. 5). As with \( \alpha \)-amylase, a flocculent precipitate eventually settled out of solution.

Discussion

It would seem that each enzyme investigated presents a typical change in turbidity pattern. Thus \( \alpha \)-amylase goes through a fast decrease, a fast increase, and finally a slow decrease in turbidity. \( \beta \)-Amylase will cause a decrease to about two-thirds of the original turbidity, independent of concentration, whereas purified B. macerans amylase will cause a decrease to about 40 per cent of the original value, depending upon the enzyme concentration. The pattern obtained by a given enzyme preparation might be of value in detecting the presence of other amylolytic enzymes. Thus an \( \alpha \)-amylase pattern in an apparently enzymically pure \( \beta \)-amylase preparation would show up fairly small amounts of the \( \alpha \)-enzyme. Again, the \( \alpha \) pattern occurs strongly in crude B. macerans amylase, but only weakly or not at all in purified preparations.

One may postulate three possible mechanisms to account for the observed results. \( \alpha \)-Amylase may possess a synthetic action, building up insoluble polysaccharide from the oligosaccharide formed by hydrolysis. Secondly, there may be present in solution an enzyme-resistant "preformed" colloid, which is maintained in suspension by the amyllopectin (supporting colloid), as suggested by Hanes (7). The preformed colloid may be incompletely dispersed starch (8) or a complex of amylose with fatty acid (9), fiber, or protein. Thirdly, the destruction of enough amyllopectin to render it incapable of acting as a "supporting colloid" may cause retrogradation of some of the incompletely hydrolyzed amylose.

Although the data presented here are not extensive enough to permit assigning the proper importance of these three mechanisms, it does not seem that the data support the possibility of synthetic action. Thus the aging of a starch solution would hardly increase its susceptibility to enzymic synthesis. Furthermore, the minimum turbidity occurs when only about 0.5 to 2.0 per cent of the total glycosidic bonds is hydrolyzed.
If the reappearance of turbidity were due solely to the precipitation of preformed colloid, one would assume that (a) there would not be much of a decrease in initial turbidity and (b) the "yield" of maximum turbidity should be strictly proportional to the initial concentration of the starch. The data obtained do not fit rigorously these requirements. Hence the third mechanism may be at least partially responsible for the turbidity pattern of α-amylase. The fact that only a small amount of the amylose has escaped hydrolysis is in accord with the observation that amylose is hydrolyzed at a maximum rate about double that of amylopectin (10), and that maximum rate of retrogradation occurs at an optimum (not at the longest) chain length (11).

Since β-amylase does not destroy the colloidal character of starch solutions (digesting the amylose completely, but leaving the amylopectin as β-limit dextrin, a residue of large molecular weight), no increase in turbidity occurs during the course of digestion. The addition of β-amylase to the α enzyme does not speed up the loss of turbidity, and may even lower it to some extent. This indicates that the β enzyme (since it is an "end-group" enzyme and the number of end-groups in amylopectin is much greater than in amylose) would delay to a certain extent the extensive α-amylolytic depolymerization of the amylopectin fraction. The results with the β-limit dextrin can best be explained by assuming that it contains a trace of amylose. Upon adding α-amylase, the hydrolysis of a few bonds in the β-limit dextrin would cause the latter to lose its colloidal character, and the trace of amylose would then retrograde from solution.

The absorption spectrum of the dextrin responsible for the turbidity indicates that some non-amylose material is associated with it. This may be due to the "aggregation of amylopectin" which occurs in the staling of bread and starch pastes, as discussed by Schoch and French (12). Undoubtedly the process of retrogradation drags down some of this aggregated amylopectin.

The effect of maltose on the crude B. macerans amylase is in accord with the ability of this sugar to undergo transglycosidation when added to a mixture of dextrins containing the B. macerans amylase (4, 13).

SUMMARY

The turbidity changes which occur when a starch solution is digested by amyloytic enzymes have been investigated. It was found that α-amylase, β-amylase, and B. macerans amylase each give a typical turbidity pattern. Some of the more pertinent factors affecting these patterns were also investigated. A tentative explanation for the appearance of turbidity maxima and minima with crystalline α-amylase, though not with β-amylase or purified B. macerans amylase, is advanced. It is based
partly upon the formation of a small amount of retrograded amylose after the partial disappearance of amylpectin fractions of high molecular weight.

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