Beta-amylase action on high molecular weight maltosaccharides

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BETA-AMYLASE ACTION ON HIGH MOLECULAR WEIGHT MALTOSACCHARIDES

by

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INTRODUCTION

The subject of this thesis is the study of the action pattern of beta-amylase on the maltosaccharides. The endwise attack of the enzyme has been well established but considerable controversy has arisen over the number of maltose units removed from the starch molecule upon each encounter with the enzyme. In past studies, natural starch or amylose, a linear fraction, was used as the substrate. Unfortunately both of these are complex mixtures of many molecules that vary in their degree of chain length and to a lesser extent in their degree of branching.

In order to determine the number of maltose units removed upon each encounter with the enzyme, it is necessary to measure the initial changes in the average degree of polymerization of the substrate. This was usually done by some physical measurement. From experiments of this type two theories originated to explain the action pattern of beta-amylase. In the first, multichain action, the enzyme is considered to remove only one maltose unit upon each encounter with the substrate. In the second, single chain action, the enzyme is considered to completely degrade a linear substrate molecule during a single encounter. It is apparent that these theories are diametrically opposite and inconsistent with each other.

Recently a third theory has been proposed to explain the results obtained when a synthetic dextrin of a narrow chain
length distribution was used as the substrate. This theory, the multiple attack theory, states that there is a certain probability that the enzyme will attack the substrate molecule more than once during each encounter.

Now that the high molecular weight linear maltosaccharides are available, it should be possible to use these specific compounds as a substrate for beta-amylase. The initial products of this reaction can be separated chromatographically and quantitatively determined. If a maltosaccharide containing an odd number of glucose units is used, one can measure the number of completely degraded chains by the amount of maltotriose formed. This method should clearly differentiate between the three existing theories and it should be possible to test the reported changes in the action pattern of the enzyme under unfavorable conditions.

Although this controversy has existed, beta-amylase has been an important enzyme tool in the study of the structure of starch. If the action pattern of this enzyme on the maltosaccharides were better understood, it would be possible to gain a better understanding of how the enzyme degrades starch. This would make beta-amylase an even more useful tool in the structural study of starch.
LITERATURE REVIEW

Beta-amylase

In 1877 Marker (1) reported that by varying the temperature of malt digests of starch, two different types of action were present. At low temperatures, saccharogenic action, mainly maltose was produced, while at high temperatures, dextrinizing, mainly dextrin was produced. From this he deduced that there were two types of amylases present in malt. In 1924 Kuhn (2) classified these two enzymes as alpha and beta-amylases. The alpha-amylase formed products (alpha configuration) which mutarotated downward, while the beta-amylase formed maltose (beta configuration) which mutarotated upward. Ohlsson (3) was able to separate the two enzymes and concluded that beta-amylase attacked the non-reducing end of the chain, splitting off maltose units.

In 1948 Balls et al. (4) were successful in obtaining the first crystalline beta-amylase. Sweet potatoes were used as the source. Crystalline beta-amylase has now been obtained with malt, wheat and soy beans. Sweet potato or soy bean beta-amylase has been used in most of the recent studies due to its ease in isolation and to the kindness of Dr. A. K. Balls and his associates in making the enzyme available. In general the action pattern of the enzyme from different sources has been considered to be the same, but this has
never been proven.

Amylases are of considerable industrial as well as academic interest, therefore there is a large volume of literature on the subject. Summer (5) has reviewed the subject with regard to the action pattern of beta-amylase on branched oligosaccharides. Recently French (6) has completely reviewed the subject of beta-amylase.

**Action pattern**

The endwise action of beta-amylase is well established but considerable discussion has arisen over whether the enzyme degrades linear starch molecules (amylose) by a single-chain or multichain mechanism. In the single chain mechanism the enzyme is considered to degrade completely one substrate molecule before it attacks a second molecule. In the multichain mechanism the enzyme removes only one maltose unit from the substrate and then attacks a second molecule, degrading all the substrate molecules more or less uniformly. The evidence for or against these theories has in general dealt with some measurement of the change in the average D. p. (degree of polymerization) of the substrate. If the substrate has a narrow D. p. distribution and the average D. p. does not change throughout the course of the reaction, then the mechanism must be single chain. The multichain mechanism would require that the average D. p. of such a substrate would
gradually decrease during the reaction. Inferences regarding the D. P. of the substrate have generally been drawn from the iodine spectrum or from physical methods. In a few cases the action pattern has been studied using oligosaccharides as substrate.

In 1948 Swanson (7) suggested that since no shift was found in the iodine spectrum during a beta-amylase digest the enzyme must be acting in a single chain fashion. In this same year Cleveland and Kerr (8) and later Kerr (9) also concluded that the mechanism is of the single chain type. Later in 1950 French et al. (10) added support to this theory by using maltoheptaose as a substrate. The products of the hydrolysate were examined by electrophoresis and no appreciable amount of maltopentaose was found. Although this was not a very sensitive method for maltopentaose, the results indicated that the enzyme was acting by a single chain mechanism. In alkaline solution and at higher temperatures the presence of maltopentaose was found indicating a possible shift to multichain action.

In 1948 Hopkins et al. (11, 12) suggested a multichain action from similar information. Later Bird and Hopkins (13) studied a series of linear dextrins with average D. P. of 16 to 30, isolated from a hydrolysate, and maltohexaose. This work also indicated a multichain action. The maltohexaose experiment yielded large quantities of maltotetraose.
Recently, French (14) has reported that it is not possible to differentiate between the two theories when the distribution of the substrate chain length is the most probable distribution. Most amylose preparations and fractions are considered to correspond to the most probable distribution. Husemann (15) has recently reported that the action of beta-amylase on a synthetic polysaccharide with a fairly sharp chain length distribution seems to differ from the action of the enzyme on natural amylose. This fact tends to substantiate French's theory.

Examining changes in the iodine spectrum, Bailey and Whelan (16) have tested the action pattern of the enzyme on a phosphorylase synthesized dextrin of D. P. 49. They found that the enzyme's action pattern was temperature dependent. At normal temperatures (20° to 50°C) the action pattern of the enzyme was interpreted in terms of a combination of the single-chain and the multichain action. At higher or lower temperatures the action appeared to become more of the multichain type. They also used maltohexaose and maltoheptaose as substrates under normal conditions. The first gave 56% multichain action while the second gave 26%. At pH 8.0 maltoheptaose gave an increase to 35% multichain action. At 70°C maltohexaose again gave 56% multichain action while at 0.5°C it gave 98% multichain action. Their definition of the percent of multichain action assumed first-order kinetics.
Bailey and French (17) have proposed a multiple attack theory. Using a terminally labeled phosphorylase-synthesized dextrin (of D. P. 44), they found that an average of 4.3 maltose units was removed from the substrate during each effective encounter with the enzyme. An encounter was defined as the sequence of events during the lifetime of the enzyme-substrate complex.

This average value of 4.3 maltose units should not be taken to mean that the probability is very high for the enzyme to release 4.3 maltose units. If 100 molecules of substrate were attacked by the enzyme, 24 would not be hydrolyzed; 18 would have 1 unit of maltose removed; 13 would have 2 units of maltose removed; 9 would have 3 units and only 6 would have 4 units removed. The probability that the enzyme will hydrolyze the substrate n times is: \[ P_n = (1-f)f^n \] where \( f = 0.76 \). The average value of n for this most probable distribution is 4.3. The authors very carefully differentiated between a random attack (normal type distribution) and the multiple attack (most probable or skewed type).

**Active site**

Thoma and Koshland (18) have reported the presence of three different functional groups at the active site of sweet potato beta-amylase. The pK's of these groups are 3.7, 6.4 and 7.0 according to their data. The Michaelis con-
stant is reported to be relatively constant but the $V_m$ is very pH dependent. A plot of the log $V_m$ versus pH has two breaks, the first at pH 3.7 (a carboxyl group) and the second at pH 7.0 (a sulfhydryl group) in the native enzyme. When the sulfhydryl groups of the native enzyme are blocked with iodoacetamide, the second break in the pH plot shifts over to pH 6.4. This group is thought to be an imidazole group.

Ito and Yoshida (19) have reported that sweet potato beta-amylase contains eight titratable sulfhydryl groups per molecule. They oxidized the sulfhydryl groups with iodoacetoic acid and they were able to show that 75% of the activity was dependent upon 50% of the sulfhydryl groups; while the remaining 25% of the activity depended upon the remaining 50% of the sulfhydryl groups. The oxidized enzyme was not reactivated by mercaptoacetic acid.

**Maltosaccharides**

The maltosaccharides are linear saccharides formed by the hydrolysis of amylose. The first two members of this homologous series, glucose and maltose, are very well known. Whistler (20) has been able to isolate maltosaccharides as large as six glucose units in length by repeated adsorption chromatography.

In 1958, Thoma (21) developed a gradient elution system for the separation of compounds as high as 15 glucose units.
By paper chromatography he was able to show that these amorphous compounds were pure up to the one containing 12 glucose units. The larger compounds were contaminated with their next lower homologs. A new composition pattern for the gradient elution system was developed (22). This work has extended the range of the maltosaccharides isolated to those containing 18 glucose units. All compounds containing nine or more glucose units were crystallized. All the compounds containing five or more glucose units formed crystalline iodine complexes.
METHODS AND MATERIALS

Carbohydrates

Amylodextrin

Nageli amylodextrin was prepared by Dr. Phillip Nordin and Mrs. Valerie Yount in this laboratory. The dextrin had an average degree of polymerization of about 25, as estimated by reducing tests.

Maltosaccharides

The maltosaccharides were separated on a cellulose powder column. The method used has been previously described (22). Maltohexaose will be referred to as G₆, Maltoheptaose as G₇, etc.

Amylase

Three times reocrystallized sweet potato beta-amylase was purchased from Wallerstein Chemical Company. The enzyme was prepared by the method described by Balls et al. (4).

Chromatographic Techniques

Multiple ascent paper chromatography was used in this experiment (22). Whatman number 1 paper was used. A 16" high by 19" wide sheet of paper was first stapled into a cylinder
and placed into a chromatography jar with water as the solvent. A two inch strip was then cut off the top of the dried sheet. The compounds were then spotted or banded on the paper on a line one inch from the bottom. The paper was then developed in a number 14' solvent (25 parts by volume water, 40 parts of absolute ethanol and 35 parts of nitromethane). Four ascents were used to separate G_6 and G_7, five ascents for G_8 and G_9 and six ascents for G_{12} and G_{13}.

The positions of the spots were located by using the silver dip (21). The chromatograms were first dipped in an acetone solution of AgNO_3, dried, and then dipped in methanolic NaOH. After the spots appeared, the dried papers were then dipped in a Na_2 S_2O_3 fixing bath (Kodak F-24) and washed with water. This procedure is sensitive to less than one microgram of maltose.

Analytical

Two standard reducing tests were used, the Nelson's test and the dinitrosalicylic acid test. The first test is almost independent of chain length and therefore preferred, but the second is much less time consuming and therefore better for velocity measurements. In measuring the initial velocity the chain length dependence is not very critical because a difference is measured.
Nelson's test

The method as described by Nelson (23) was used. Some modifications were made to increase the sensitivity. The optical density of the samples was read at 750 millimicrons and sufficient glucose was added to the copper reagent so that the water blank would read as 0.01 mg. of glucose. This latter change was made to eliminate an abnormality in the standard curve at low concentrations. With these modifications the test covered the range of 0.01 to 0.15 mg of maltose. The range could be extended to 0.5 mg. by diluting the developed samples 1 to 5. Standards must be run with each set of samples.

Dinitrosalicylic acid test

The dinitrosalicylic acid test was used as described by Schwimmer (24). A 2 ml. sample was added to 2 ml. of the reagent. The samples were heated in a boiling water bath for 5 minutes and the color intensity was measured in a Klett-Summerson colorimeter with the number 54 filter. The standard curve is very reproducible and the range covered is from 0.1 mg. to 1.0 mg. per ml. of standard.

Quantitative determinations

The phenol method as described by Dubois et al. (25) was used. One tenth of a ml. of 80% phenol is added to a
1 ml. sample, and then 3 ml. of conc. H$_2$SO$_4$ is pipetted directly into the solution. This is allowed to stand for 10 minutes and then cooled. The samples were read in a Spectronic "20" spectrophotometer at 490 millimicrons. The range covered is 5 to 40 micrograms. The range can be extended to 200 micrograms by reading the samples in a Klett-Summerson colorimeter with a number 42 filter. By diluting with conc. H$_2$SO$_4$ the range can be extended even higher. The standards must be run with the samples and must be treated in the same manner as the samples.
Stability of Beta-amylase

Preliminary experiments with beta-amylase have shown that the enzyme, with amylodextrin as a substrate, loses its activity rapidly as the reaction proceeds. This loss makes the measurement of the initial velocity experimentally difficult. Therefore, it was necessary to investigate the effect in order to see if there was some means of stabilizing the enzyme. The standard conditions adopted for the velocity measurements were: 0.05% amylodextrin, 0.02 M pH 4.8 acetate buffer, 1 ml. of enzyme, all in a total volume of 20 ml. at 35°C. Six or seven 2 ml. samples were removed at intervals and assayed by the dinitrosalicylic acid method. The velocity was then measured as the slope of the line and reported as mg. of maltose produced per minute. Special care was taken to make sure that at least the first four points would lie in a straight line. This usually meant that the measured portion of the reaction would only proceed to about 10% completion. The enzyme was able to hydrolyze only a fraction of the substrate before it became inactive, usually 20% to 30%. When higher concentrations of the enzyme were used the enzyme was able to completely hydrolyze the substrate, but because of the speed of the reaction the initial velocity could not be measured. The amount of hydrolysis before
inactivation seemed to be proportional to the amount of enzyme present. This could be due to the instability of the enzyme or the inhibition of the enzyme by some agent present. Also, product inhibition could be an explanation.

The enzyme was studied for product inhibition by the addition of various amounts of maltose equal to that produced by 5, 10 and 20% hydrolysis. No effect was found. The components of the mixture were then checked for contamination. The water was redistilled with basic potassium permanganate. The concentration of buffer was varied over a wide range (1 M. to 0.005 M.) and the substrate was recrystallized three times from pure water. None of these changes had any effect.

In order to test the stability of the enzyme, the enzyme was held at 35°C in the presence of 0.02 M buffer and samples were assayed at different time intervals. The velocity was then plotted versus time. The activity of the enzyme dropped off rapidly during the first 20 minutes to about 1/3 of its original velocity and the remainder then dropped off at a much slower rate, for the next two hours. The enzyme is definitely unstable under these conditions. The enzyme was then tested at temperatures from 0°C to 35°C. As expected, the effect was more pronounced at the higher temperature, but it was still present to a lesser extent at 0°C.

It has been reported that wheat and malt beta-amylase have a sharp break in their Arrhenius plots at 20°C. There-
fore, an Arrhenius experiment was run (0°C to 50°C) using fresh enzyme and enzyme that had been held at 35°C for one hour. The slopes of these lines were essentially identical and the lines were continuous through the entire range. At higher temperatures (above 35°C) it was very difficult to measure the initial velocity, but it could be shown that no break occurs. The value obtained was 15 ± 1 kilocalories; similar to the reported value of 16.2 kilocalories found from 0°C to 20°C for malt beta-amylase. The fresh enzyme was held again at 35°C for one hour, cooled overnight and then held at 35°C with samples taken out at 5 minute intervals. These results were identical to those obtained by holding fresh enzyme at this temperature except less activity was found.

The activity versus holding time curves are very similar to those obtained by Ito and Yoshida (19) when they held the enzyme in the presence of iodosobenzoic acid. Under their conditions the sulfhydryl groups were specifically oxidized. Therefore, the fresh enzyme was held at 35°C as before but in the presence of a sulfhydryl protecting reagent, 10⁻³ M. mercaptoacetic acid, with the proper control. Under these conditions the enzyme was stabilized. It lost less than 5% of its activity after one hour. The holding experiment was then repeated by replacing the mercaptoacetic acid with 0.2 M. versene and then by purging the system of air with
nitrogen. It was hoped that if this instability was caused by the presence of metal ions or air oxidation of the sulfhydryl groups one might expect some stabilization, but none could be measured. The mercaptoacetic acid will not activate the enzyme before or after it has been partially inactivated by being held at 35°C for one hour.

The addition of $10^{-3}$ M. mercaptoacetic acid to the system raises the background reducing value. This makes it more difficult to measure the initial velocity because dilutions are necessary. If the temperature is lowered to 25°C, $10^{-4}$ M. mercaptoacetic acid will stabilize the enzyme. The initial velocity can be measured and the mercaptoacetic acid will have only a minor effect on the reducing value.

**Action Pattern of Beta-amylase on Maltosaccharides**

**Enzyme activity**

In order to follow the action pattern of beta-amylase by chromatography a high concentration of substrate must be used. If this is not done the samples must be concentrated and then desalted. The concentration of 2% substrate was chosen. Further experiments have proven that the concentration of substrate has very little effect, if any, on the action pattern of the enzyme.

Since only small quantities of the maltosaccharides are available, the required enzyme concentration was first esti-
mated by using amylodextrin as a substrate under standard conditions at 25°C with 10^{-4} M. mercaptoacetic acid. Unfortunately at 2% amylodextrin, the amylodextrin appears to inhibit the enzyme. If this effect is ignored and the velocity is extrapolated from lower concentration to a 2% concentration a very good approximation of the proper enzyme concentration can be made. The activity of the stock enzyme solution with the amylodextrin was determined as the production of maltose in mg. per minute. The amount of this stock solution of enzyme necessary to degrade the given weight of maltosaccharide to maltose in 20 minutes was then calculated. The ratio of this volume of enzyme to the total volume of digest was determined. This ratio was then used in the maltosaccharide digests.

**Digests**

The reaction was run in a total volume of 0.3 ml. using 6 mg. of maltosaccharide. Because of the small volumes used it was necessary to run the reaction in a paraffin block. Indentations were melted into the block by means of a small hot test tube. One large hole was made at one end for the reaction mixture and smaller holes were made along the sides to contain the removed samples. A typical digest consisted of: 6.0 mg. of maltosaccharide, 0.03 ml. of 0.2 M. pH 4.8 acetate buffer, 0.240 ml. of water and 0.030 ml. of enzyme.
Samples of 0.030 ml. were taken out at 1, 3, 5, 7, 10, 15, 20, and 30 minute time intervals. The enzyme was inactivated with 0.010 ml. of $10^{-3}$ M. mercuric chloride solution. The samples were then placed on a chromatogram by means of a capillary melting point tube and capillary action. The paraffin block allowed quantitative removal of the sample by this method. Variation of this digest consisted of the addition of more or less enzyme and the addition of 0.030 ml. of $10^{-2}$ M. mercaptoacetic acid.

Digests of the following compounds were run: $G_7$, $G_9$, $G_{11}$, $G_{13}$, $G_{15}$, and $G_{17}$. Odd numbered chains were used so that the end products from the reducing end of the substrate would be $G_3$ rather than maltose. The digests were chromatographed in the #14 solvent with three to six ascents and the spots were located by the silver dipping method. If the enzyme acts in a pure single chain fashion on $G_9$ the initial products would be only maltose and $G_3$. No other products should be found during the course of the reaction. If the enzyme acts in a multichain pattern the initial products should be only maltose and $G_7$. $G_5$ and $G_3$ should then appear subsequently as the reaction continues. If the enzyme is attacking the substrate in the multiple attack, all the possible products should be found initially. Maltose will be found in the highest concentration: the remaining products in much lower concentrations. If it is assumed that
only 10% of the substrate is attacked then the mole percent of each would be 78.0% G9; 1.7% G7; 1.1% G5; 3.8% G3; 15.2% G2. Calculated on the basis of product formation this would amount to about 6% hydrolysis.

All the chromatograms from the above digests were found to be essentially the same except for the number of spots present. Even when smaller quantities of enzyme were added the initial products were the same; see Fig. 1. The addition of mercaptoacetic acid had no visible effect except to stabilize the enzyme. All the possible products were formed immediately. Initially maltose was found in the highest concentration while the other compounds all appeared to be in about equimolar quantities as indicated by their spot intensities. As the reaction continued the maltose and the G3 spots became more intense while the intensities of the other spots seemed to increase at much slower but equal rates.

These chromatograms seem to rule out pure multichain attack and pure single chain attack. The products found are almost identical to those one would expect if the enzyme were acting in a multiple attack action pattern.

Action Pattern of Beta-amylase Under Less Favorable Conditions

The digests in this section were all run under similar conditions to those previously described, except in some cases inhibitors were added and in others the pH was changed. Under
Figure 1. Chromatogram of beta-amylase acting on 2% Gg, at pH 4.8
alkaline conditions the reactions were run in 0.02 M tris buffer.

pH variation

In order to measure the initial products 1/2 of the normal enzyme concentration was used. Chromatograms of G₉ and G₁₃ at pH 3.0, 7.0, 8.4 and 10.0 are shown in Fig. 2 and 3. A gradual change from the usual action pattern at pH 4.8 over to an almost pure multichain action at pH 10.0 was found. The chromatogram at pH 7.0 is almost identical to the one at pH 4.8 except that less G₃ is formed. At pH 8.4 the action is more that of multichain. Very little G₅ was formed as compared to the G₇ formed. Thoma and Koshland (18) have found two pH dependent, functional groups of the enzyme in this pH region; the sulfhydryl at about pH 7.3 and the imidazole at about pH 6.7. A chromatogram at the unfavorable acid condition of pH 3.0 is identical to those at pH 4.8.

Denaturation

In order to see if there is any change in the action pattern due to partial denaturation of the enzyme, the following reaction was run. The enzyme was held at 35°C until its activity was reduced to 1/10 of the original activity. A digest was then run with G₉ and chromatographed. The results were identical to those using fresh enzyme. No
Figure 2. Chromatogram of beta-amylase acting on 2% G9

Top: at pH 7.0
Bottom: at pH 8.4
Figure 3. Chromatograms of beta-amylose acting on 2% maltosaccharides

Top: on G9 at pH 3.0
Bottom: on G13 at pH 10.0
change in the action pattern was found.

Cupric ion

A digest of G₁₁ at pH 4.8 was run in the presence of $10^{-3}$ M copper sulfate, see Fig. 4. The initial products on this chromatogram are very similar to those produced without copper. As the reaction progresses larger quantities than expected of G₉ are being produced as compared to the other products. This could indicate a slight shift toward a multi-chain action. If this shift is correct then this might imply that the blocking of the sulfhydryl group causes the shift. Unfortunately copper also forms many other complexes with proteins.

Temperature variations

Bailey and Whelan (16) have reported a shift in the action pattern at high and low temperatures. Chromatograms of G₉ were run at 0°C, 20°C and 35°C. They all seemed identical to those run at 25°C. Due to the stability of the enzyme and due to the evaporation from the small volume of digest used, it was impossible to run the digest at 60°C to test this high temperature effect. These results indicate that there is no large low temperature shift in the action pattern with the maltosaccharides.
Figure 4. Chromatograms of beta-amylase acting on 2% G11

Top: in $10^{-3}$ CuSO$_4$
Bottom: in 5% maltose
Dilute substrate

Since one might expect the action pattern of the enzyme to be dependent upon the concentration of the substrate, a 0.05% digest of Gg at pH 4.8 was run. The samples were then lyophilized and chromatographed. The chromatogram was identical to those run at 2%. The aesthetic value of the chromatogram was not good, due to the presence of the concentrated salts, but one was still able to compare the results.

Maltose

Maltose is being produced by the reaction. It is conceivable that the maltose produced may have some effect on the action pattern. Therefore, a 2% digest of Gg at pH 4.8 was run in the presence of 5% maltose; see Fig. 4. Except for the maltose spot, this chromatogram was identical to one run without maltose. No effect was found.

Quantitative Chromatography

A visual study of the chromatograms is a very easy method for distinguishing between pure multichain and pure single chain action. It also yields, by visual comparison, a rough estimate of the relative intensities of the spots. Since beta-amylase appears to be acting in a multiple attack pattern, it is necessary to determine more quantitatively the rate of product formation.
Digest

The digests were run as previously described but in the presence of $10^{-3}$ M. mercaptoacetic acid. For G7 and G8 a digest of 2% substrate in a total volume of 1 ml. was used. Samples of 0.10 ml. were used. For G9 the total volume and the volume of the samples were increased by a factor of 0.25. For G11 they were increased by a factor of 0.50. These factors were used to compensate for the extra compounds formed. The reaction was then stopped in each case with 0.05 ml. of $10^{-2}$ mercuric ion.

Separation

The reaction mixtures were separated by paper chromatography. The compounds were located by using 0.025 ml. of the sample as an indicator strip. The bands of compounds were then cut out and eluted with $10^{-4}$ M. HgCl₂ into 5 ml. volumetric flasks. For the elution, a small tray of the eluting solution was placed upon a Lab Jack. One end of the paper strip was cut to a point and the other end was placed between two microscope slides. One end of the slide was immersed in the solution while the other end protruded over the edge with the strip hanging vertically. The height of the Lab Jack was regulated so that the tips would drip directly into the 5 ml. volumetric flask. The tray and the flasks were then covered with an inverted chromatography jar. About 1 ml. of sample
was collected after three hours. The samples were then diluted to volume with $10^{-4}$ M HgCl₂ and analyzed using the phenol method.

Results

The results were plotted as moles per mole of substrate added versus the extent of the reaction; see Fig. 5 to 8. The extent of the reaction was determined on the basis of the amount of maltose produced, assuming a theoretical yield. It was not particularly informative to plot the amount of each compound formed in terms of the mole fraction because the total number of moles present is continuously increasing due to hydrolysis. Therefore, the amount of each compound formed was plotted per mole of substrate used. The amount of maltose produced was not plotted, because by definition it would be linear. The maltose line would start at zero and extend toward an integral multiple of the final G₃ concentration (the exact value would depend upon the substrate used). The plots themselves are self explanatory.
Figure 5. A plot of product formation when beta-amylase acts on G7
MOLES PER MOLE OF SUBSTRATE

EXTENT OF REACTION

0.0
0.25
0.5
0.75
1.0

0.5
1.0
Figure 6. A plot of product formation when beta-amylase acts on $G_3$
MOLES PER MOLE OF SUBSTRATE

EXTENT OF REACTION
Figure 7. A plot of product formation when beta-amylase acts on $G_{11}$
MOLES PER MOLE OF SUBSTRATE
Figure 8. A plot of product formation when beta-amylase acts on $G_8$. 
MOLES PER MOLE OF SUBSTRATE

EXTENT OF REACTION

MOLES PER MOLE OF SUBSTRATE

G

G

G

G

G

G
DISCUSSION

Stabilization

The activity of beta-amylase is unstable in dilute solution. This instability appears to be dependent upon the temperature of the digest and the concentration of the enzyme. No reference toward this instability of the dilute crystalline enzyme could be found in the literature. Beta-amylase is known to be a sulfhydryl enzyme, and sulfhydryl groups are very sensitive to oxidation and to heavy metal ions.

Ito and Yoshida (19) have reacted the enzyme with a specific sulfhydryl reagent, iodosobenzoic acid. They found that 75% of the activity is dependent upon 25% of the sulfhydryl groups present. Upon plotting the activity of the enzyme versus the loss of titratable sulfhydryl during this reaction they found a rapid decrease to 25% of the original activity followed by a slower decrease as the remaining 25% of the sulfhydryl groups are oxidized. This two step process is similar to the instability of beta-amylase at 35°C. In both cases the enzyme is stabilized but not reactivated by mercaptoacetic acid.

The heat dependence of this instability might imply that this is heat inactivation. If this were true the small amount of mercaptoacetic acid used should not protect the enzyme. Another explanation could be that this
instability is being caused by trace heavy metal ions or metal ion catalyzed oxidation. The heat dependence could then be due to the heat dependence of this reaction. But the sulfhydryl groups in general, are the most sensitive groups of the enzymes to heavy metal ions or oxidation, so they again would be implicated.

It has not been proven that the sulfhydryl groups cause this instability but it seems likely that they are involved. The most important fact obtained about this instability is that the enzyme can be stabilized.

Action Pattern on the Maltosaccharides

One should not expect that the action pattern of beta-amylase on the smaller maltosaccharide will be exactly the same as it is on native amylase. The chain length of course, is not the same. But it should be possible to study the action pattern of the enzyme on a series of maltosaccharides of increasing size and from this obtain some idea of what might be happening on larger substrates.

Three theories have been proposed to explain the action pattern of the enzyme. In the first, single chain action, the enzyme combines with the substrate and completely degrades it to maltose before attacking the next substrate molecule. In the second, multichain action, the enzyme combines with the substrate and removes one maltose unit and then attacks a
new substrate molecule. This reduces all the substrate chains uniformly. Both of these theories have been previously tested with amylose as a substrate by studying the average chain length of the remaining substrate molecules. There is positive evidence for and against both theories. Recently, French (14) has reported that it is not possible to differentiate between the two theories unless the distribution of the substrate chain length is known to be normal. Most amylose fractions are considered to correspond to the most probable distribution. A third theory is the multiple attack theory. In this case the enzyme combines with the substrate and the most probable event that happens is that the enzyme releases the substrate without attacking it. The next most probable event is that the enzyme will attack the substrate once. The next the enzyme will attack it two times, three times, etc.

The chromatographic experiments indicate that neither of the first two theories is correct under normal conditions. Single chain action is not correct since the only products formed from GI1 would be maltose and G3. Multichain attack would yield only maltose and the substrate molecule minus one maltose unit initially. Even a combination of these two theories will not explain the presence of G5 as an initial product, when G9 is used as a substrate. A mixed action might be expected if there were two active sites each having
a different action pattern.

The multiple attack theory explains the results very well. From this theory, the probability that the enzyme will attack the enzyme n times is given by the following equation: \( P_n = (1-f) f^n \) where \( f \) is equal to 0.76. If one calculates the probability of the formation of each of the products when \( G_9 \) is used as a substrate, the probability that \( G_7 \) is formed is 0.18, \( G_5 \) is 0.13 and \( G_3 \) is 0.45. The probability that \( G_3 \) is formed is very high because in this case we must consider all the values of \( n \) equal to or greater than 3. This high value for \( G_3 \) makes these chromatograms appear to be like a single-chain action but the presence of the intermediates disqualify this idea.

When an attempt was made to compare the results of the quantitative experiments to those predicted by the probabilities of the multiple attack theory, the comparison was very favorable. Unfortunately there is a 10% error in the values given for \( G_5 \) and \( G_7 \) because of the analytical method.

It can be seen from the multiple attack theory that there will be an end-effect with these maltosaccharides. These chains are not infinite in length. Therefore the probability that the \( G_3 \) will be formed must be a summation of all the values of \( n \) greater than those possible with the compounds used. This high value for \( G_3 \) gives a simulated single chain action. There is another possible end-effect.
If the smaller compounds were more resistant to the enzyme than the larger compounds, then one might expect a simulated multichain action. This would be the case with G₅, because there is not any other way that it can be degraded. There is also reason to believe that G₆ is degraded by a simulated multichain because G₄ seems to be degraded at a slower rate than G₅. G₅ is degraded at about the same rate as G₇ and G₉. This can be seen visually on the chromatograms or from the quantitative data. Therefore, this simulated multichain end-effect does not extend to compounds as large as G₇ but it may explain why workers using G₆ always find a higher proportion of multichain action.

It has been previously reported (10) that there is a shift toward a multichain action of beta-amylase as the pH of the digest is increase. These experiments definitely bear this out. In fact, the present experiments indicate that the action is almost entirely multichain at pH 10.0. If the affinity of the enzyme for the substrate were reduced as the pH was increased, a simple explanation could be presented for the results obtained. The Michaelis constant, however, does not change at pH 6.0 or above when starch is used as a substrate.

The cupric ion experiments indicate that the change in the action pattern at alkaline pH's may be caused by a sulfhydryl group. Thoma and Koshland's (18) experiments
proved that the sulfhydryl groups are not essential for the activity of beta-amylase but their loss results in a large reduction in activity. This information might imply that the sulfhydryl groups are not the groups being blocked by heating at 35°C, because there is no change in the action pattern. This is not necessarily the case. If one attack by the sulfhydryl enzyme could produce 10 maltose molecules, while one attack by the enzyme with the sulfhydryl groups blocked could produce only one maltose unit, it would be very hard to demonstrate the action pattern of the enzyme with the sulfhydryl group blocked in the presence of a small amount of sulfhydryl enzyme.

The quantitative results, see Fig. 5 to 8, substantiate the fact that only very small amounts of the intermediates are formed in this reaction. Much larger quantities would be expected if the multichain process dominated at optimal conditions as well as at high pH's. If a single chain action pattern were present the line that represents the formation of G3 and the one that represents the loss of substrate would be linear. This is not the case. Due to the errors of ± 1 mg. in the 5 mg. range of this experiment, very little can be said about the initial rates of formation of these compounds.

Under optimal conditions beta-amylase acts in a multiple attack pattern. It has been again confirmed that this is a true multiple attack pattern. The action of the enzyme
does change over to a multichain action as the pH becomes more alkaline. The action pattern of the enzyme is not appreciably affected by acid pH or the presence of maltose.

A Possible Model for the Enzyme Site

Beta-amylase is an enzyme that attacks very large substrate molecules and is very specific for the non-reducing end. Yet it has a very high turnover number (moles of product per mole of enzyme per minute). This could be explained by the enzyme having many sites or by its having a mechanism by which the substrate molecule could feed into one or two sites.

One way of describing the action of beta-amylase would be to try to picture what a site would have to be like in order to explain the results found.

First, let us consider the hydrolytic site itself. It must be very specific for the non-reducing end of the linear starch molecule. It probably has a very efficient means of eliminating the maltose produced. If this were not the case then the maltose would build up and interfere with the enzyme or when a small amount of maltose is added the activity would be reduced.

Secondly, there could be some sort of a "feeder" site adjacent to the active site. This site must have a strong affinity for the substrate and be able to feed it into the active site without allowing it to diffuse very far away.
If this were not true, one would expect to find a dilution effect on the action pattern or the maltose should tend to displace the substrate and change the action pattern toward the multichain type. A sulfhydryl group is probably implicated but not necessarily located here. This feeder site is not necessary for activity but when it is operative the activity of the enzyme is greatly increased. The mechanism of this feeding action possibly consists of a series of binding sites. As the substrate moves along these sites toward the active site it is probably going from a higher energy complex to one of lower energy. This would explain the strong tendency to give a multiple attack pattern instead of the multichain attack. A larger molecule may not be as free to feed into this site as the smaller molecules. It would have to use the energy gained by moving down this feeder site to drag the rest of the chain behind it. Therefore, the value of $f$ (multiple attack theory) may be a function of chain length.
SUMMARY

1. The normal action pattern of beta-amylase on the malto-
saccharides in the range of G7 or above is of the multiple
attack type.

2. As the pH of the digest is increased to an unfavorable
level the action pattern changes over to the multichain
type.

3. The addition of certain inhibitors seems to have little
or no effect upon the action pattern of beta-amylase.
However, the addition of cupric ion seems to shift the
action pattern toward multichain in the later stages of
the reaction.

4. A model of the active site was proposed to explain
variations observed in the action pattern of beta-amylase.
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