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John H. Pazur
Iowa State College

Dexter French
Iowa State College

Doris W. Knapp
Iowa State College

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By John H. Pazur, Dexter French and Doris W. Knapp

The action of salivary amylase on amylo-type compounds is interpreted by most investigators as a random hydrolysis of the α-1, 4-glucosidic linkages. By this mode of action, intermediate reaction products which would have physical and chemical properties characteristic of the reaction mixture may be formed. The nature of these intermediates has been deduced from measurements of osmotic pressure, viscosity, iodine coloration, reducing power, etc. generally on crude enzymic digests. Investigations of this type which form the basis of the concept of random action are excellently reviewed by Hanes (9).

According to Hanes' postulation of the action of the enzyme, an amylose-salivary amylase digest would contain high molecular weight reducing dextrins, low molecular weight reducing dextrins, hexasaccharide fragments and eventually maltose during the course of the reaction. These products would account for the progressive decrease in the chain length of the reaction products and would be formed by a random cleavage of the amylose chains. Additional evidence for such a mechanism is presented in the recent studies with purified enzymes and substrates by Bernfeld and Studer-Pecha (3) and by Alfin and Caldwell (1).

It should be emphasized that the specific intermediates of the reaction, as for example, "hexasaccharide" of Hanes (9) were not isolated in any of these experiments and also that random hydrolysis fails to explain the presence of relatively large amounts (20%) of low molecular weight sugars (including maltose) in the early stages of reaction as was observed by Freeman and Hopkins (6).

Evidence for Non-Random Hydrolysis

While investigating the initial hydrolytic products of the Schardinger dextrins (8) we became interested in the resolution of the linear amylooligosaccharides by the paper chromatography technique. The compounds of this series up to the dodecasaccharide have been resolved by the chromatographic procedure described in the experimental section, and such a mixture is routinely used as a control for the identification of the reaction products. Using this method the identification of the intermediates in a salivary amylase-starch digest becomes possible.

The first experiments in this direction were performed with amylodextrin as a substrate. This compound was chosen in the study for several reasons. It is a linear, crystalline, starch degradation product having an average chain length of *ca* 23 glucose units. It readily dissolves in hot water to give an optically clear solution even at concentrations of 20%. Furthermore, in an enzymic digest of this substrate, all the possible short chain intermediate compounds should be readily identifiable. When the course of the reaction of salivary amylase and amylodextrin was followed, the first and only detectable intermediates were the di-, tri- and tetra-saccharides (see Figure 1). Other intermediates, i.e., pentasaccharide, hexasaccharide, etc., which would be expected if random action occurs, were not present or at most present in very low concentrations. The tri- and tetra-saccharides though hydrolyzed at a
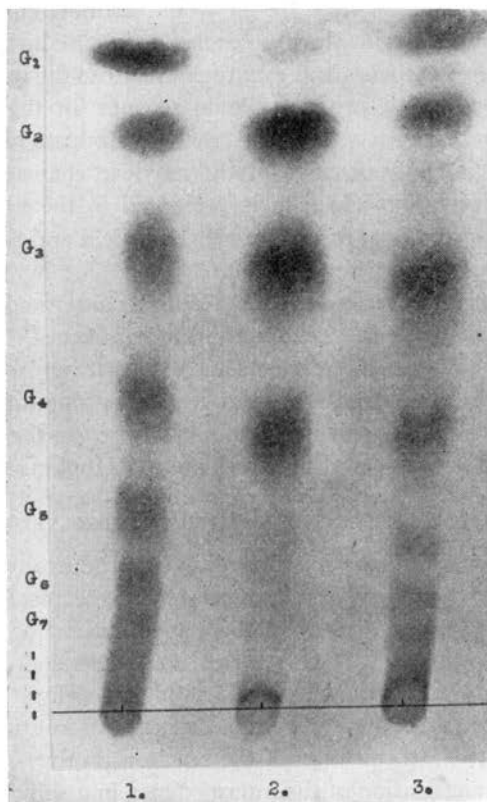

Fig. 1. Paper Chromatogram of Salivary Amylase-Amylodextrin Digests. 1. Reference oligosaccharide mixture. 2. Digest at pH 7.0. 3. Digest at pH 10.3. G_1, G_2, G_3, — indicates glucose, maltose, amylotriose.

much slower rate are, nevertheless, eventually converted into one molecule of maltose and one molecule of glucose or two molecules of maltose, respectively. Similar observations have been obtained with amylose and starch as substrates for the enzyme. As the present mechanism for the action of the enzyme does not explain these results, other mechanisms must be considered.

ROLE OF DIFFUSION IN SALIVARY AMYLASE ACTION

That diffusion may be rate controlling process in enzyme reactions has been mentioned by a few investigators, but, in general, its importance has been minimized. A mechanism in which diffusion is an important process and, perhaps a rate controlling process under certain conditions, is proposed for the action of salivary amylose. The evidence which is presented in support of this theory is qualitative in nature. Nevertheless, with this ·theory we have been able to (1) explain the results of the preceding section, and (2) to predict and then verify that the course and the intermediate products in an enzymic reaction may be altered by varying certain reaction conditions.

The essential details of our theory follow. The substrate molecule in diffusing through the solution collides with an enzyme molecule. If the resulting complex is in an "activated state" reaction occurs and a linkage in the starch chain is broken. Under favorable conditions and before the molecules separate by diffusion, further reaction ensues with the production of a low molecular weight compound. After two or three fissions of 1-4 linkages in a localized region, the substrate molecules diffuse out of the sphere of influence of the enzyme. This same substrate molecule can collide with the enzyme molecule at a later time and the chain is broken at another point. Once more there is a production of several short chain molecules. By this mechanism the viscosity and iodine coloration changes can be correlated with the formation of low molecular weight reducing sugars.

SUPPORTING EVIDENCE

If the reaction is proceeding according to this theory, then reducing the number of "activated collisions" should effect the course and intermediate products of the reaction. Under such conditions, amylopentaose, amylohexaose, etc., should be present in salivary amylase-amylodextrin digests. The number of "activated collisions" has been reduced by working at pH conditions which are unfavorable for the action of the enzyme. In view of the results of Bernfeld, Staub and Fischer (2) on the effect of pH on the stability of the

enzyme, pH values of 4.5 and 10.3 were selected for study. A typical chromatogram of a digest of unfavorable pH is shown in figure 1. Reaction products maltose (G_2) up to amyloheptaose (G_7) can be identified on the chromatogram. Higher molecular weight saccharides were also present but these were not resolved on this chromatogram. The series of linear oligosaccharides can result if the enzyme breaks only one linkage at a time, which, in turn, is quite probable since under the unfavorable conditions the substrate molecules diffuse away from the enzyme before a second fission can occur.

In an amylose-salivary amylase digest, low molecular weight products were not observed as would be expected since the enzyme hydrolyzes only one linkage at a time and whence low molecular weight compounds would be formed in very small quantities and only from the ends of the amylose chains. That reaction was ocuring in these experiments has been checked. The changes in absorption of the iodine complexes and reducing power of the digests as a function of time have been obtained for pH values of 7.0 and 10.3.

Although reducing compounds other than maltose are present, the total reducing power is expressed in milligrams of maltose which would have the same total reducing power. The rate of change in these properties for the two digests can be compared from a plot of the data in table 1. Since the optical density of iodine complex in the alkaline digest dropped at about $\frac{1}{5}$ the rate of that at the neutral pH, and the reducing power increased by only 1/11, different reaction routes are indicated. The greater increase in reducing power of the neutral digest can be attributed to formation of low molecular saccharides. The presence of these compounds was established by paper chromatograms.

Table 1

The reducing power and iodine coloration of amylose-salivary amylase digests as a function of time.

Time	Mgs. of "Maltose" per 20 mls.		Optical Density*	
Minutes	pH 7.0	pH 10.3	pH 7.0	pH 10.3
2	3.89	0.72	0.92	1.30
5	8.30	1.05	0.38	1.14
15	16.3	3.08	0.02	0.56
30	25.9	4.88	—	0.32
60	33.0	7.04	—	0.04

* Optical density values are for wave lengths of maximum absorption and on 1 ml. aliquots diluted to 50 mls. and final iodine concentration of .0002 M.

It is informative to examine the two reaction mixtures at the iodine achomic stage. At this point there are no starch fragments which form a color complex with iodine. One would expect that the two digests should contain the same intermediates. This was not the case. The reducing power of the neutral digest was approximately twice that of the alkaline digest due to the larger quantity of low molecular weight saccharides in the digests of neutral pH.

The results of these experiments were predicted and are explained by our qualitative theory on the action of salivery amylase. However, quantitative interpretation is not possible at present. Experiments, in progress, on the effect of unfavorable pH or temperature on the amylase action may elucidate the exact role of diffusion in these reactions. In addition, information on the relationship of diffusion, affinity constants, enzyme-substrate disintegrating rate constants, under favorable and unfavorable reaction conditions previously indicated (7) may also be obtained.

EXPERIMENTAL

Amylodextrin. Amylodextrin was prepared by an extended action of cold 15 per cent sulfuric acid on defatted corn starch. The resulting insoluble residue was recrystallized several times from hot aqueous solution to which an equal volume of methanol was added. To reduce its molecular size, the crystalline amylodextrin was refluxed in .001 N hydrochloric acid for several hours. The recovered amylodextrin was recrystallized twice more from aqueous-methanol solution. The average molecular size of the product calculated from its reducing power (5) was twenty-three glucose units.

Amylose. A sample of potato amylose prepared by alcohol precipitation procedure (10) and recrystallized three times from n-butanol was available in our laboratory.

Paper Chromatography. Droplets of *ca* 0.01 ml. of the substances being tested were placed at intervals of 1 inch along a line ruled 1 inch from one edge of a rectangle, generally 8 in. by 9 in., of filter paper. After drying, the paper was rolled into a cylinder and held in this form by a wire staple at each end, thus giving a cylinder capable of supporting itself even when wet with solvent. The cylinder, with the sample spots near the bottom, was then placed in a shallow layer of solvent (3 parts water, 4 parts pyridine, 6 parts *n-butanol* by volume) (4) such that the sample spots were above the solvent level. The solvent vessel was kept away from marked thermal disturbances, out of direct light, and tightly closed. After the solvent had climbed by capillary attraction to the

top of the cylinder, it was removed from the vessel, air dried and oven dried, then returned to the solvent for one or more additional climbs. For the resolution of oligosaccharides in the hexasaccharide range from six to ten climbs may be needed. Finally the dried cylinder was sprayed lightly with alkaline copper reageant 60 without KIO_3 and KI (11) heated in an over at 105°C. for about five minutes, then sprayed with phosphomolybdic acid reagent (12) to locate the areas in which reduction of the copper reagent had taken place. The reducing carbohydrates show up as blue spots against a white background. For best photographic prints, the chromatograms should be photographed soon after drying since the background gradually becomes blue.

Amylodextrin Digests. The stock solution of salivary amylase was prepared as needed by diluting one ml. of filtered saliva to 25 mls. with appropriate buffer, either .08 M Na_2CO_3 and .02 M $NaHCO_3$ buffer of pH 10.3 or .06 M K_2HPO_4 and .04 M KH_2PO_4 of pH 7.0. One ml. of the enzyme solution was added to 4 mls. of a 2% amylodextrin and .02 M sodium chloride solution which had been previously boiled to expell methanol of crystallization then cooled to 40°C. The digest was kept at 40°C. in a constant temperature water bath. One ml. samples were withdrawn at specified times, concentrated to ½ the original volume and examined chromatographically for intermediate products. Typical chromatograms at a stage just prior to the achromic point are shown in figure 1, for the two pH values.

Amylose Digests. One hundred and thirty-five mls. of a .5% amylose solution, 3 mls. of 1 M NaCl, and 10 mls. of phosphate buffer (pH 7.0 and .1 M total phosphate) were mixed and allowed to equilibrate in a temperature bath of 40°C. To this solution 2 mls. of salivary amylase solution, prepared by diluting 1 ml. of filtered saliva to 10 mls. with water, were added. At specified time intervals the following aliquots were removed. A twenty ml. sample was pipetted into 25 mls. of ferricyanide reagent and the reducing power of the aliquot was determined (5). One ml. of the digest was added to 49 mls. of iodine solution of final iodine concentration of .0002 M. The optical density in the visible range of these samples was measured in a spectrophotometer. These values as well as the reducing values expressed as milligrams of maltose are recorded in table 1. One ml. samples were concentrated by boiling to ½ ml. in order to inactivate the enzyme. The latter samples were examined for low molecular weight saccharides by the paper chromatography method.

A similar experiment at equivalent concentrations and at unfavorable pH was carried out. Ten mls. of .08 M Na_2CO_3 and .02 M $NaHCO_3$ buffer of pH 10.3 and 2 mls. of filtered saliva were the buffer and enzyme employed in this experiment. The results are also given in Table 1.

SUMMARY

1. The intermediate compounds in salivary amylase reactions at pH conditions favorable and unfavorable for optimum enzyme activity have been investigated by a new method — paper chromatography, as well as by two conventional methods — reducing power and iodine coloration.

2. In salivary amylase action at optimum conditions, the conversion of a large portion of the substrate into low molecular weight compounds, namely di-, tri- and tetra-saccharides is interpreted as evidence for non-random hydrolysis.

3. Salivary amylase acts by hydrolyzing several linkages of the substrate molecule in a localized region before the enzymesubstrate complex dissociates by diffusion.

Literature Cited

1. Alfin, R. B., and Caldwell, M. L., J. Am. Chem. Soc., 71, 128 (1949).
2. Bernfeld, P., Staub, A., and Fischer, E. H., Helv. Chim. Acta, 31, 2165 (1948).
3. Bernfeld, P., and Studer-Pecha, H., Helv. Chim. Acta, 30, 1895 (1947).
4. Chargaff, E., Levine, C., and Green, C., J. Biol. Chem., 175, 67 (1948).
5. Farley, F. F., and Hixon, R. M., Ind Eng. Chem., Anal. Ed., 13, 616 (1941).
6. Freeman, G. G., and Hopkins, R. H., Biochem. J., 30, 442 (1936).
7. French, D., Knapp, D. W., and Pazur, J. H., in press, J. Am. Chem. Soc.
8. French, D., Knapp, D. W., and Pazur, J. H., To be submitted for publication in J. Am. Chem. Soc.
9. Hanes, C. S., New Phytol., 36, 189 (1937).
10. Schoch, T. J., in Advances of Carbohydrate Chemistry, Vol. 1, pp. 247-277. Academic Press: New York (1945).
11. Shaffer, P. A., and Somogyi, M., J. Biol. Chem., 100, 695 (1933).
12. Tauber, H., and Kleiner, I. S., J. Biol. Chem., 99, 249 (1932).

CHEMISTRY DEPARTMENT

IOWA STATE COLLEGE

AMES, IOWA