TRANSFERASE ACTIVITY IN MALT AMYLASE PREPARATIONS[1]

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ABSTRACT

Barley malt contains a $1\rightarrow4{:}1\rightarrow4$ transferase which accompanies alpha-amylase during classic purification steps (i.e., heat-treatment and glycogen precipitation). Previous results on the action and specificity of malt alpha-amylase must be re-evaluated as possibly arising from the combined action of alpha-amylase and transferase. Effects of transferase are most important on G_6 and lower oligosaccharides upon which pure malt alpha-amylase itself acts exceedingly slowly if at all.

In a paper dealing with alpha-amylases, it was reported by Bird and Hopkins (1) that maltohexaose (G_6) was cleaved by malt alpha-amylase to give G_4 plus G_2. Such a result is not in harmony with the action pattern of *Bacillus subtilis* alpha-amylase (1,2), which in many respects resembles malt alpha-amylase. Moreover, with higher oligosaccharides and branched polysaccharides, malt alpha-amylase has an overwhelming propensity for leaving unattacked the last four to five glycosidic links at the nonreducing end of the chain. Thus, it would appear that beween G_7 and G_6 there is a critical shift in the mode of attack by malt amylase preparations. Similar results and interpretations have been reported recently by Greenwood *et al.* (3).

In investigating the reasons for such a shift, we obtained evidence for the presence of a transferase type of activity, similar to that of the D-enzyme previously reported from potatoes (4,5). As yet we have not been able to separate the transferase activity from the alpha-amylase, but it should not be inferred that the two activities belong to the same protein.

Experimental

Preparation of Enzyme. Barley malt (Trophy)[3] was finely ground and stirred with 1% $Ca(OAc)_2$ at room temperature for 170 hr. The supernatant was decanted and clarified by filtration. It was then brought to 70°C. and held at this temperature for 20 min. (6). For purification the enzyme was handled by the method of Loyter and Schramm (7), generally considered to be specific for alpha-type

[1]Manuscript received January 6, 1966. Journal Paper No. J-5281 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Proj. 1116. Supported in part by grants from the Corn Industries Research Foundation and the USPHS (GM-08822).

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[3]Kindly supplied by The Fleischmann Malting Co., Chicago.

amylases. The heated enzyme solution was brought to room temperature and centrifuged at 10,000 × *g* at 5°C. for 10 min.; the supernatant was filtered through Whatman No. 1 paper. Ethanol was added to a concentration of 70% at 0°C. The solution was then centrifuged at 10,000 × *g* for 10 min. at 0°C. The precipitates were removed and dissolved in $Ca(OAc)_2$, and alcohol was added to a final concentration of 40% at 0°C. To this solution was added a 2% solution of shellfish glycogen (Sigma Chemical Co., St. Louis, Mo.), and the ethanol concentration was brought up to 40% again. Stirring was continued for 1 hr. and then the solution and enzyme-glycogen complex were centrifuged at 10,000 × *g* at 0°C. for 20 min. The insoluble complex was dissolved in 48 ml. of 0.025*M* $Ca(OAc)_2$ and 0.0025*M* $CaCl_2$. The solution containing enzyme and glycogen was stored overnight at 5°C. and was then kept at 35° for 3 hr. to digest the glycogen. The enzyme was removed from the glycogen fragments by passage through a column of Sephadex G25 (Pharmacia Fine Chemicals, Inc., New Market, N.J.). The enzyme preparation was assayed by the method of Robyt and Whelan (8), and contained 73 I.C.E. units (9) of alpha-amylase per ml. This stock solution of enzyme was used in the experiments a and c described below. A second amylase preparation, having apparently the same action pattern and similar activity, was prepared from Malt Diastase, Analytical Enzyme,[4] and used in experiment b. Attempts to crystallize the enzyme by the method of Schwimmer and Balls (10) were not successful, although in one trial experiment when the enzyme was purified on a DEAE cellulose column a small amount of microcrystalline material appeared.

Substrates. Radioactive ^{14}C D-glucose hydrate, nonspecifically labeled, was purchased from International Chemical and Nuclear Corp., City of Industry, Calif., and purified by paper chromatography. G_6 was obtained by charcoal chromatography (11–13) of a mixture of linear oligosaccharides obtained by action of crystalline Taka-amylase A (14) on commercial amylose ("Superlose").[5] Paper chromatography (15) indicated that the G_6 was substantially free from other oligosaccharides (see Fig. 1). G_6 was converted to maltohexaitol by treating 10.1 mg. in 1 ml. of H_2O with 20 mg. of sodium borohydride. After overnight standing at room temperature, the excess borohydride was destroyed with acetic acid and the solution was deionized by treatment with mixed-bed, carbonated ion-exchange resins. The deionized solution was taken to dryness and evaporated with methanol several times to remove boric acid. Paper chromatography of the maltohexaitol gave

[4] Kindly supplied by Wallerstein Co., Staten Island, N.Y.
[5] Kindly supplied by Stein-Hall and Co., New York.

a single spot sufficiently characteristic in appearance that it could be distinguished from G_6, even though the sugar alcohol has approximately the same R_F as the parent oligosaccharide. Similarly, other reduced oligosaccharides could be distinguished from the parent oligosaccharides by their appearance on paper chromatograms. Radioactive reducing end-labeled oligosaccharides were prepared as by French and co-workers (16).

Enzyme Digests. (a) *Reaction of* G_6. G_6 (10 mg. in 0.2 ml. of pH 5.8 acetate buffer) was treated with 0.1 ml. of stock enzyme solution. The mixture was incubated at 37°C. under toluene. Periodic samples were withdrawn, deionized, and submitted to paper chromatography. The results are presented in Fig. 1.

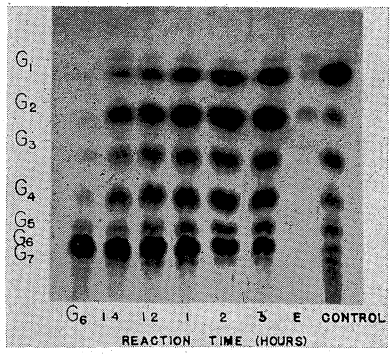


Fig. 1. Action of malt enzyme on maltohexaose. G_6 indicates the original substrate. E is the enzyme blank; the control contains D-glucose and the linear series of starch oligosaccharides G_2, G_3, etc.

(b) *Reaction of maltohexaitol.* Maltohexaitol (10 mg. in 0.2 ml.) was incubated at 37°C. with 0.1 ml. of stock enzyme in pH 5.8 acetate buffer. At intervals samples were withdrawn and submitted to paper chromatography (Fig. 2).

(c) *Reaction of* G_6 *with radioactive glucose.* G_6 (6.2 mg. in 0.3 ml.) was incubated with radioactive glucose (1.0 mg. in 0.1 ml. in pH 5.8 acetate buffer) with stock enzyme (0.1 ml.). The digest was incubated at 37°C. under toluene and samples were withdrawn after various time intervals. The samples were deionized and analyzed by paper chromatography (Fig. 3).

(d) *Reaction of reducing end-labeled oligosaccharides.* A mixture of radioactive oligosaccharides were separated by paper chromatography. The separated constituents were sprayed with stock enzyme directly on the paper, allowed to react for 6 hr. at 40°C., dried, and subjected to chromatography in a direction perpendicular to the first direction

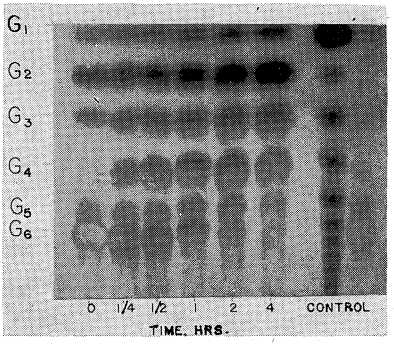


Fig. 2. Action of malt enzyme on maltohexaitol. Note the characteristic appearance of the oligosaccharide alcohols which have approximately the same R_F values as the parent reducing oligosaccharides (perhaps slightly slower). At equal concentration levels, the reducing oligosaccharides give an intense-black spot, whereas the oligosaccharide sugar alcohols give a light-gray spot with a slightly intensified border.

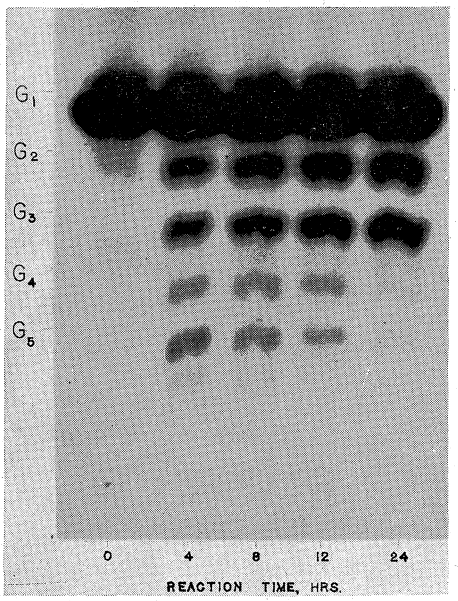


Fig. 3. Radioautograph showing action of malt enzyme on a mixture of maltohexaose and radioactive D-glucose. Samples were taken at 0, 4, 8, 12, and 24 hr. The chromatogram was held in contact with X-ray film for 48 hr.

(17). The dried chromatogram was subjected to radioautography (Fig. 4).

(e) *Reaction of reducing end-labeled* G_6. End-labeled G_6 obtained from the mixture used in d was mixed with inactive G_6 and enzyme so that the composition of the enzyme digest was similar to that in a.

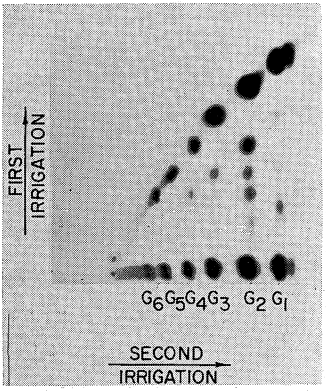


Fig. 4. Radioautograph of two-dimensional chromatogram showing action of malt enzyme on reducing end-labeled oligosaccharides. After separation on paper in the vertical direction, the oligosaccharides were sprayed with stock enzyme, allowed to react on the paper, and subjected to chromatography in the horizontal direction (radioautography as in Fig. 3).

Samples of digest were withdrawn after 0, 0.25, 0.5, 1, 2, 3, and 4 hr., and subjected to chromatography and radioautography (Fig. 5).

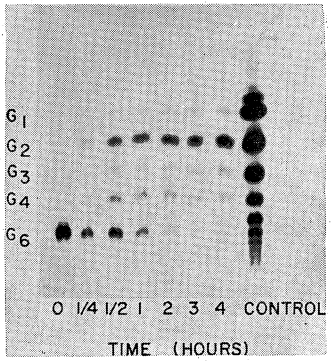
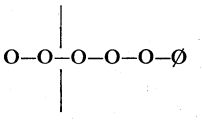


Fig. 5. Action of malt enzyme on reducing end-labeled maltohexaose.

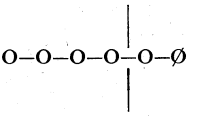
Results and Discussion

The results, as illustrated in Figs. 1 to 5, show that transferase activity is present in the malt enzyme preparation. With G_6 as substrate, the results (Fig. 1) are more or less in agreement with previous reports. Note, however, the formation of G_7 which can not be explained on the basis of a purely hydrolytic activity. The experiment with maltohexaitol as substrate was set up to test primarily for the presence of traces of beta-amylase in the enzyme preparation. If present, beta-amylase might cleave G_6 to G_4 plus G_2, as observed (Fig. 1). Single

cleavages of G_6 by beta-amylase or alpha-amylase may be envisioned as follows:

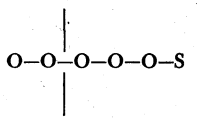


Expected attack of G_6 by beta-amylase

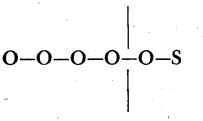


Specific attack of G_6 as suggested by Bird and Hopkins

In either case, the products are G_2 and G_4, and without using a labeled substrate it is impossible to distinguish between the two cases. On the other hand, with maltohexaitol as substrate, the products would be easily distinguished (S stands for a sorbitol unit):



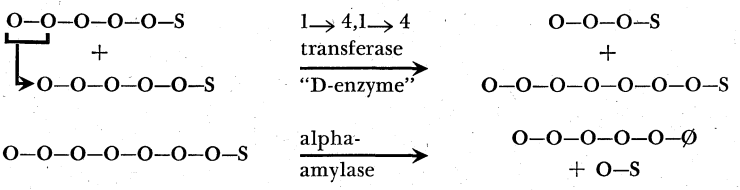
Expected attack by beta-amylase



Asymmetric attack by an alpha-type amylase

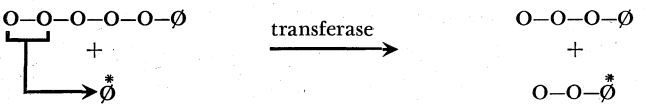
The actual products observed (Fig. 2) included G_6 as well as numerous lower oligosaccharides and sugar alcohols. Production of G_6 can be accounted for only if there is a transferase in addition to the amylase.

Reaction sequences such as the following are reasonable and would lead to the observed results:

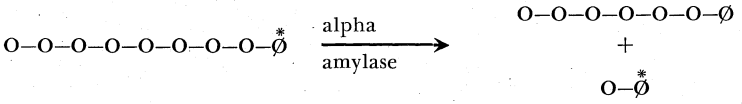


It would be expected that through repeated transferase and hydrolytic reaction, all higher oligosaccharides and sugar alcohols would eventually be degraded to low-molecular-weight products.

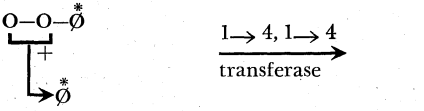
With G_6 and radioactive D-glucose as substrates, the results (Fig. 3) indicate clearly that a transferase activity is involved. In this particular case, the distribution of radioactivity suggests that the transferase selectively transfers a maltosyl unit, probably repetitively, to give initially G_3 and G_5, G_7, etc., with the reducing end labeled:



With increase in the chain size, the radioactive oligosaccharide eventually becomes a good substrate for alpha-amylase:



Reactions such as the foregoing lead to radioactive G_2 and higher oligosaccharides, which are only very slowly attacked by malt alpha-amylase, and which either cannot act as donors or substrates for the transferase or are regenerated in a dynamic equilibrium; for example:



Inasmuch as all evidence indicated the presence of transferase in malt alpha-amylase preparations, it was desirable to test the action of these preparations on reducing end-labeled oligosaccharides. The results (Figs. 4 and 5) show that action on G_3 gives D-glucose, together with a small amount of G_5. The absence of even-membered oligosaccharides is in harmony with a preferential transfer of maltosyl units followed by rapid alpha-amylase action on G_7, giving radioactive D-glucose and inactive G_6. With end-labeled G_4, transfer of maltose units gives radioactive G_6 and G_8, which in turn are rapidly cleaved by alpha-amylase to radioactive G_2 and inactive G_6. With end-labeled G_5, transferase activity gives radioactive G_3 or G_2 (from the donor) and G_7, G_8, and G_9. These in turn, by action of alpha-amylase, give radioactive D-glucose, G_2, and G_3, and inactive G_6 and G_7. With end-labeled G_6 (see also Fig. 5), it is clear that some of the radioactive G_4 must originate by transfer of a maltose unit from one G_6 to another. Subsequent rapid alpha-amylase action on the G_8 so formed gives radioactive G_2 together with inactive G_6. A small amount of radioactive G_3 is formed, possibly as a consequence of maltotriosyl transfer.

It is known from other work (3), and we have confirmed, that malt alpha-amylase acts readily on G_7 to give $G_6 + G_1$, and on G_8 to give $G_6 + G_2$. However, only by using specifically labeled substrate is it possible to decide whether the G_6 comes from the reducing or nonreducing end of the substrate molecule. It is seen in Fig. 4 that action of alpha-amylase on end-labeled G_7 gives radioactive D-glucose and inactive G_6, and G_8 gives radioactive G_2 and inactive G_6. G_7 and G_8 are obviously good substrates for the alpha-amylase and react rapidly;

there is no evidence for transferase action on them.

Evidence for preferential transfer of a maltosyl unit is incomplete at present, and some of the results (as with G_5 and G_6) can be explained most readily by assuming that a maltotriosyl unit can also be transferred. Moreover, it is not excluded that the transferase activity observed may be a dual activity of malt alpha-amylase itself. It is hoped that preparation of the transferase and amylase in pure form will enable us to resolve these questions.

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