[Chem. Pharm. Bull. 22(7)1593—1599(1599)]

UDC 547.466.1.09 : 577.15.07.08

Studies on Dextranase. III.[1] Action Patterns of Dextranase from *Penicillium funiculosum* on Substrate and Inhibition on Hydrolysis Reaction by Substrate Analogues

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(Received December 26, 1973)

Action patterns of dextranase from *P. funiculosum* on the substrate were investigated. Isomaltodextrins and their reduced derivatives (isomaltodextrinols) were used as the substrate. Comparison of the enzymatic digestion products did lead the conclusion that the attack points of the dextranase on isomaltodextrin were primarily at second and third glucosidic linkage from the non-reducing end of the substrate. On the increase of polymerization degree of substrate, the enzyme hydrolyzed also at fourth or fifth glucosidic linkage from the non-reducing end.

The inhibition of dextranase hydrolysis reaction by some mono- and disaccharides was also investigated. It was found that C-2 and C-6 positions of glucosidic residue were primary positions to effect the binding to active center of the enzyme; C-2 position was likely to effect the binding affinity and C-6 position was to exert the binding site.

In a previous paper,[3] we have reported the purification and some enzymatic properties of extracellular dextranase (EC 3.2.1.11) from *P. funiculosum*. Dextranase from *P. funiculosum* was devided into two parts, dextranase I (pI 3.98) and dextranase II (pI 4.19), by isoelectric focusing, but other different enzymatic properties were not found among them.

In general, dextranases from a variety of molds and a few bacteria are endo-types, whereas exo-types are found predominantly in mammalian tissue.[4] There are a few reports on action patterns of purified dextranases.[5–7] The authers have investigated the action patterns of purified dextranase II on various isomaltodextrins and their reduced derivatives.

Inhibition of amylase activity by some oligosaccharides was reported[8] and from the phenomena, inhibition mechanisms and binding pattern to active site of the enzyme were investigated.[9,10] These inhibitions, however, have not been reported on dextranases. In order to investigate the inhibition mechanisms of substrate analogues, the inhibition types of them on the dextranase were examined.

Material and Method

Preparation and Assay of Dextranase——Dextranase II from *P. funiculosum* was purified as described previously.[3] The enzyme activity was also assayed by the method described in the previous paper, and one unit of the activity was defined as that activity of the enzyme which produced reducing sugar in the amount equivalent to 1 μmole of glucose per min.

1) Part II: M. Sugiura, A. Ito, and I. Yamaguchi, *Biochim. Biophys. Acta.*, **350**, 61 (1974).
2) Location: *Ueno-sakuragi, 1-Chome, Taito-ku, Tokyo, 110, Japan.*
3) M. Sugiura, A. Ito, T. Ogiso, K. Kato, and H. Asano, *Biochim. Biophys. Acta*, **309**, 357 (1973).
4) E.H. Fisher and E.A. Stein, "The Enzymes," Vol. 4, P.D. Boyer, H. Lardy, and K. Myrback, ed., Academic Press, New York, 1960, pp. 304—307.
5) J. Fukumoto, H. Tsuji, and D. Tsuru, *J. Biochem. Tokyo*, **69**, 1113 (1971).
6) D. Tsuru, N. Hiraoka, and J. Fukumoto, *J. Biochem. Tokyo*, **71**, 653 (1972).
7) G.N. Richards and M. Streamer, *Carbohydr. Res.*, **25**, 323 (1972).
8) S. Ono, *J. Biochem. Tokyo*, **55**, 315 (1954).
9) M. Ohonishi, *J. Biochem. Tokyo*, **69**, 181 (1971).
10) N. Suetsugu, K. Hiromi, M. Takagi, and S. Ono, *J. Biochem. Tokyo*, **64**, 619 (1968).

Identification of Enzymatic Digestion Products——Enzymatic digestion products were identified by paper chromatography. The digests, about 50 μg as total sugars, were spotted periodically on a Toyo filter paper No. 50 (40×40 cm) and developed with ascending system for 6 hr at 20°. The solvent used for development was nitromethane–abs. ethanol–water (35: 40: 25 v/v). Localization of sugars were detected by silver nitrate–sodium hydroxide[11] or aniline hydrogen phthalate reagent.[12]

Preparation of Isomaltodextrins——Isomaltodextrins were prepared by partial digestion of dextran with dextranase II. To 300 ml of 8% dextran solution (in 100 mM phosphate buffer, pH 6.0) 160 units of dextranase II was added and incubated at 37° for 120 min. At which time, the rate of hydrolysis was 10%. After incubation, 2 volume of cold ethanol was poured into the mixture and stand in a cold over-night. The supernatant was collected by centrifugation and concentrated under reduced pressure. Then the supernatant concentrated was loaded on an active charcoal column (3×90 cm) which had been prepared as described by French.[13] The sugars adsorbed were eluted stepwise by aqueous ethanol, and sugar eluted was determined by phenol–sulfuric acid method.[14] Each sugar prepared was examined for its homogeneity by paper chromatography as shown in Fig. 1. Polymerization degree of the sugar was determined by the method of French and Wild,[15] and from the ratio of the reducing sugar to the total sugar content.

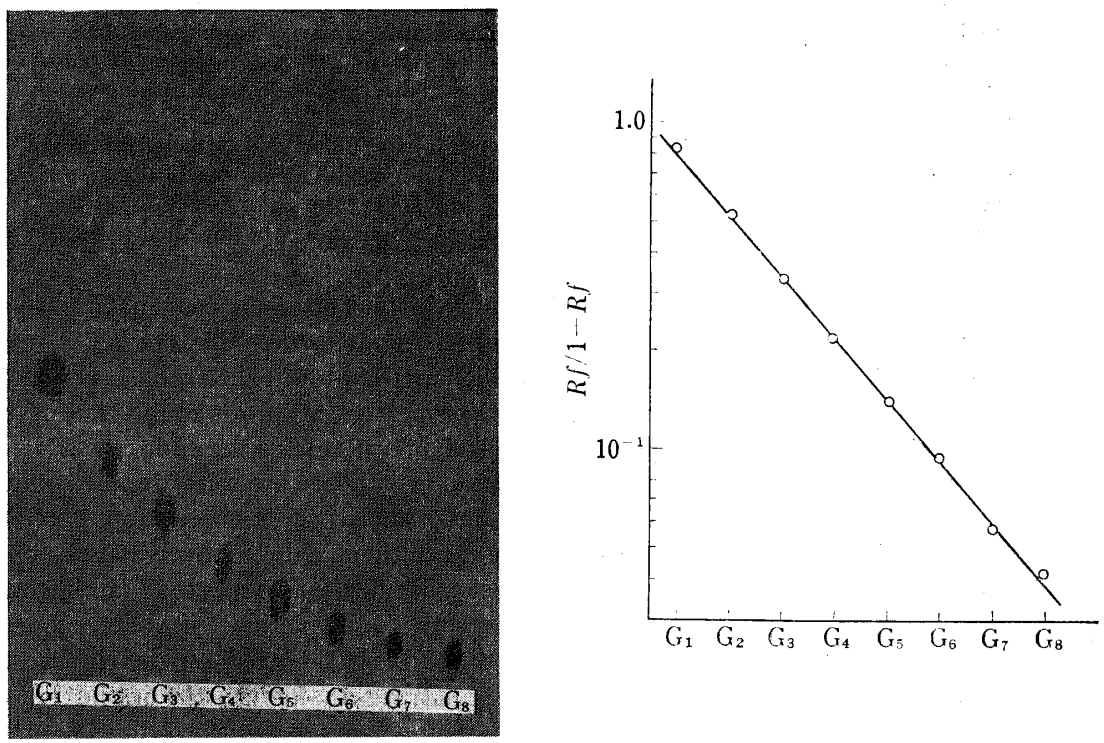

Fig. 1. Paper Chromatograms and Polymerization Degree of Isomaltodextrins Prepared from Dextran

G_1, G_2------G_8: glucose, isomaltose,------isomaltooctaose

Reduction of Isomaltodextrins——Isomaltodextrins were reduced according to the method of Frush and Isbell.[16] Aqueous solution of isomaltodextrin (1%, w/v) was mixed with the same volume of sodium borohydride solution (0.5%, w/v) and allowed to stand over-night at room temperature. To decompose the excess sodium borohydride, the mixture was treated with an appropriate amount of Dowex 50 (H^+-form) and then Dowex 50 was removed by filtration. The filtrate was evaporated to dryness under reduced pressure at 40°, and the residue were suspended in abs. methanol and dried up as above. This procedure was repeated several times to remove borate as the methyl ester, the residue were used as the reduced isomaltodextrins.

11) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).
12) S.M. Partridge, *Nature*, **164**, 443 (1949).
13) D. French, *J. Am. Chem. Soc.*, **77**, 1024 (1955).
14) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Roberts, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
15) D. French and G.M. Wild, *J. Am. Chem. Soc.*, **75**, 2612 (1953).
16) H.L. Frush and H.S. Isbell, "Methods in Carbohydrate Chemistry," Vol. 1, R.L. Whistler and M.L. Wolfrom, ed., Academic Press, New York, 1962, pp. 127—131.

Each isomaltodextrinol obtained did not react to the reducing sugar test (Somogyi-Nelson method[17]) and gave only one spot on a paperchromatogram.

Analysis for Inhibition Type of Several Oligosaccharides——Two ml of substrate solution which contained 0.45% of a saccharide and 0.35—0.75% dextran (in 100 mM phosphate buffer, pH 6.0) was preincubated at 37°. After 5 min, 1 ml of the enzyme solution was added and incubated for 30 min, and the reducing sugars liberated in 1 ml of the mixture were determined by Sumner's reagent.[18] Inhibition constant of each saccharide was analyzed by Lineweaver-Burk plots.

Substrate and Reagents——Dextran of *Leuconostoc mesenteroides* N-4 was a gift from Meito Sangyo Co. Ltd. and a partially hydrolyzed product, average MW 5×10^4, was used in the experiments. Active charcoal for chromatography was made by Wako Pure Chemical Industries Ltd. Dowex 50 was a product of Dow Chemical Company and sodium borohydride was of E. Merk, A.G. Other chemicals used were the special reagent grade.

Results

Action of Dextranase II on Dextran and Isomaltodextrins

Time course of hydrolysis and digestion products of dextran by dextranase II are presented in Fig. 2 and Fig. 3, respectively. Dextranase II digested dextran rapidly and at 120 min, maximum degree of hydrolysis attained to about 40%. According to the simultaneous paper chromatograms (Fig. 3), isomaltose, isomaltoriose, -tetraose, -pentaose, -hexaose and the other large isomaltodextrins were found as the digestion products at the biginning of the reaction.

The hydrolysis activity of dextranase II on each isomaltodextrin was observed by determining a liberated reducing sugar. Results are presented in Fig. 4. Isomaltotriose was not digested under the condition, and this may have proved that binding affinity between the enzyme and substrate would be weak. The enzyme, however, digested isomaltodextrins rapidly which contained more α-1,6 glucosidic linkages than isomaltoriose. On the increase of polymerization degree of substrate, the rate of hydrolysis was similar to that of dextran. Identification of digestion products from each isomaltodextrin was carried out simultaneously

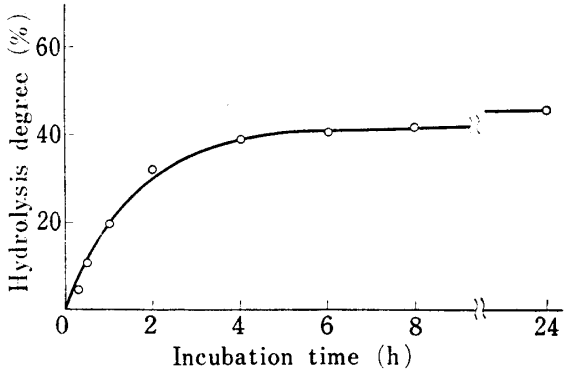

Fig. 2. Time Course of Dextran Hydrolysis by *P. funiculosum* Dextranase II

The reaction mixture contained 200 mg of dextran and 5 units of the enzyme in 10 ml of 100 mM phosphate buffer (pH 6.0), and was incubated at 37°. Hydrolysis degree was calculated by periodical determination of reducing sugars in aliquot of the reaction mixture. Reducing sugar was measured by Sumner's reagent.[18]

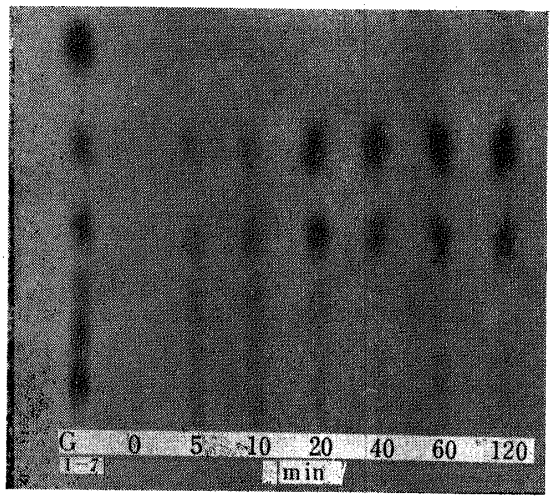

Fig. 3. Paper Chromatograms of Hydrolysis Products from Dextran by Dextranase II

The reaction conditions were the same as described in Fig. 2. 5 μl of the reaction mixture was periodically sampled and spotted on the filter paper. Locator was silver nitrate-sodium hydroxide.

17) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
18) J.B. Sumner, *J. Biol. Chem.*, **65**, 393 (1925).

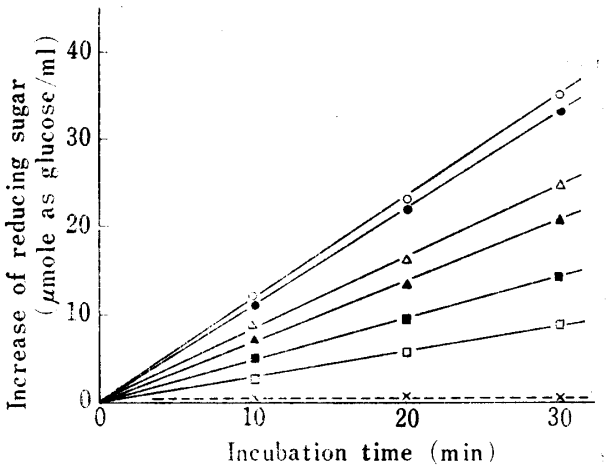

Fig. 4. Hydrolysis Curves of Several Isomaltodextrins by Dextranase II

The reaction mixture contained 20 mg substrate and 1 unit of the enzyme in 1 ml of 100 mM phosphate buffer (pH 6.0), and was incubated at 37°. Aliquots of the mixture were periodically sampled and liberated reducing sugars were determined by Summer's reagent.

×: isomaltotriose
□: isomaltotetraose
▲: isomaltopentaose
■: isomaltohexaose
△: isomaltoheptaose
●: isomaltooctaose
○: dextran

by paper chromatography as shown in Fig. 5 and Fig. 6. Isomaltose was not hydrolyzed at all by dextranase II even if use the 100-fold enzyme activity of this experiment. Isomaltose was regarded as one of the final digestion products from dextran. Isomaltotriose was hardly digested by dextranase II, but it was finally digested using large amount of the enzyme into glucose and isomaltose. Dextranase II digested isomaltotetraose much easier than isomaltotriose into isomaltose and partially into glucose and isomaltotriose as shown in Fig. 5. From isomaltopentaose, isomaltose and isomaltotriose were principally observed at first and afterwards glucose was also found. Paper chromatograms of digestion products from isomaltohexaose, isomaltoheptaose and isomaltooctaose are presented in Fig. 6. Isomaltohexaose was hydrolyzed into isomaltose, isomaltotriose and -tetraose. Isomaltoheptaose was also hydrolyzed to produce isomaltose, isomaltotriose, -tetraose and -pentaose. Isomaltose, isomaltotriose, -tetraose, -pentaose and -hexaose were produced from isomaltooctaose.

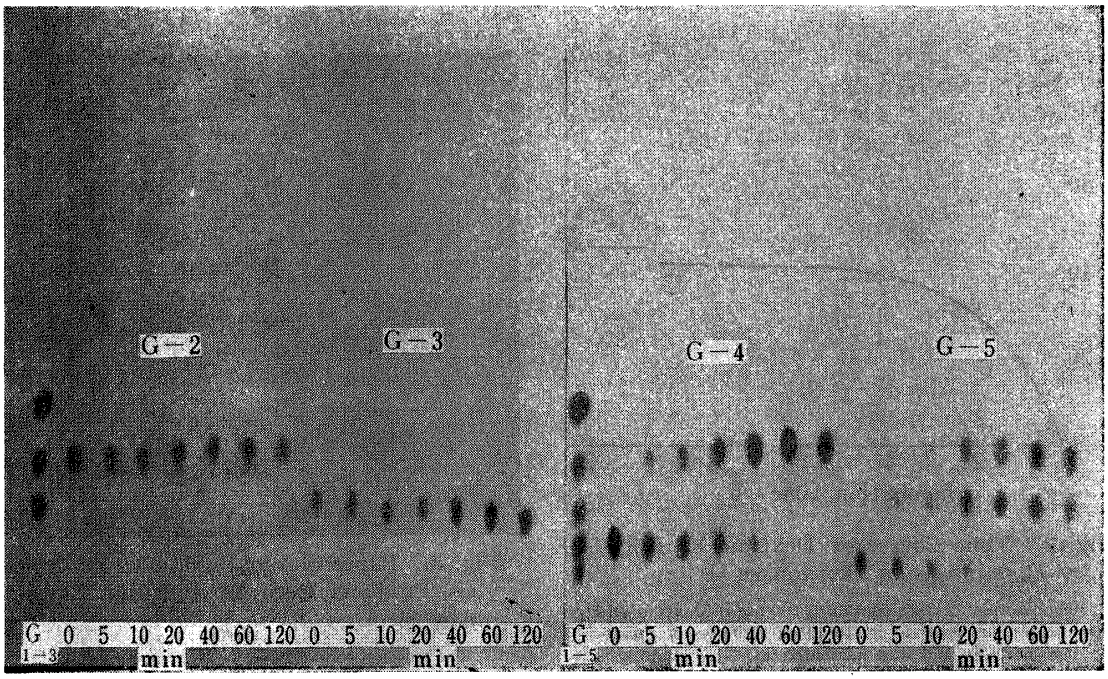

Fig. 5. Paper Chromatograms of Hydrolysis Products from Isomaltose, Isomaltotriose, Isomaltotetraose and Isomaltopentaose

Incubation conditions were the same as described in Fig. 4. 2 or 3 μl of the mixture was sampled periodically and spotted on the filter paper. After development, localization of sugars were identified by silver nitrate-sodium hydroxide.

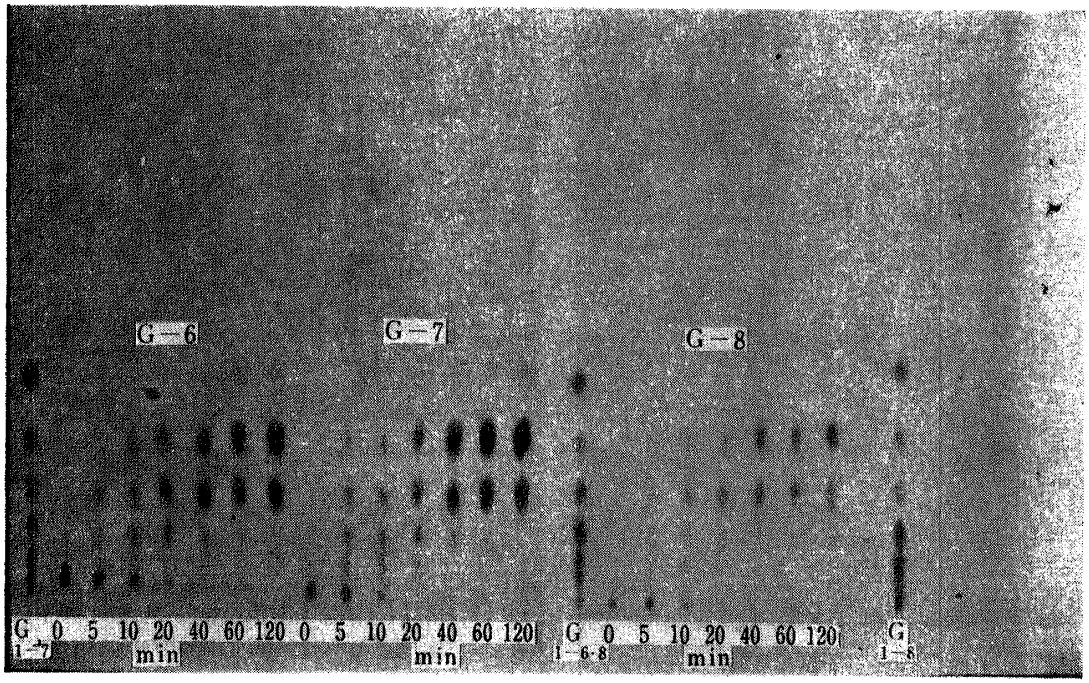

Fig. 6. Paper Chromatograms of Hydrolysis Products from Isomaltohexaose, Isomaltoheptaose and Isomaltooctaose

The procedures were the same as described in Fig. 5.

Action of Dextranase II on Isomaltodextrinols

To investigate the attack points of dextranase II on isomaltodextrins; from the reducing end or non-reducing end, isomaltodextrinol was digested by the enzyme and digestion products

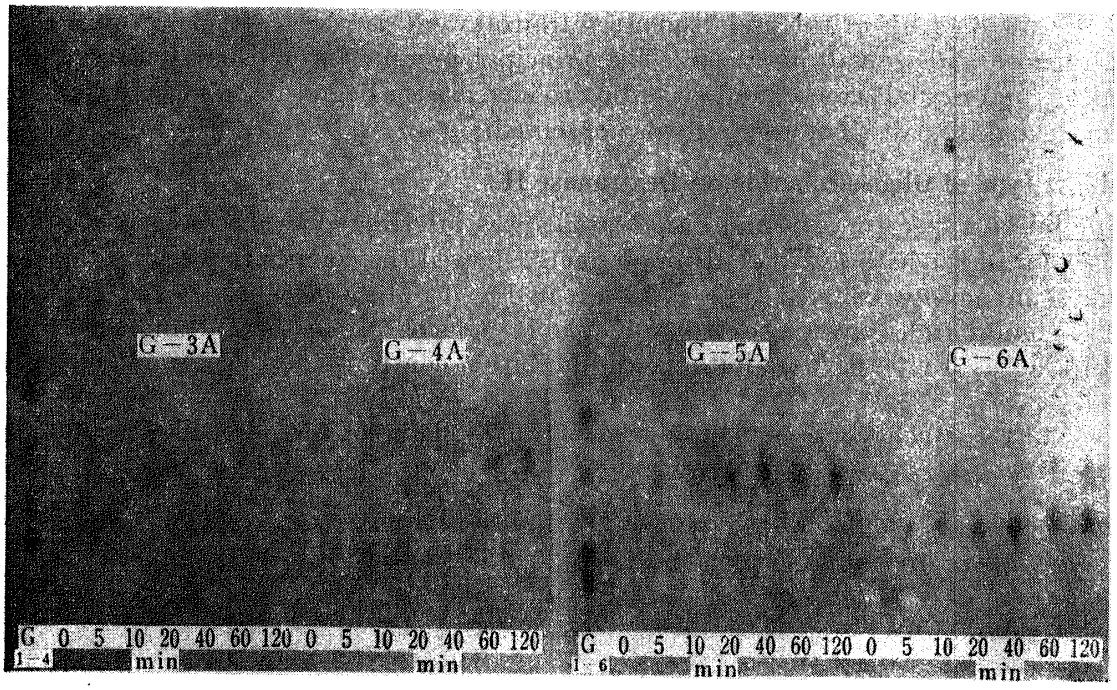

Fig. 7. Paper Chromatograms of Hydrolysis Products from Isomaltodextrinols

In these experiments, isomaltodextrinol was used as substrate instead of isomaltodextrin. Other conditions were the same as in Fig. 5. After development, only reducing sugars were detected by aniline hydrogen phthalate. G-3A: isomaltotritol, G-4A: isomaltotetraitol, G-5A: isomaltopentaitol G-6A: isomaltohexaitol

Fig. 8. Paper Chromatograms of Hydrolysis Products from Isomaltodextrinols

All the procedures were the same as described in Fig. 7. G-7A: isomaltoheptaitol, G-8A: isomaltooctaitol

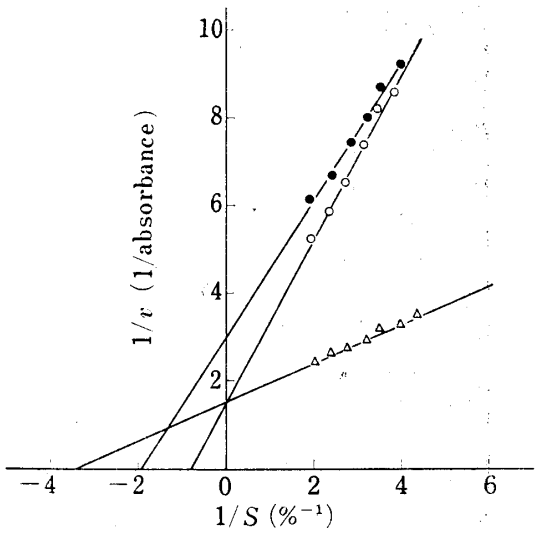

Fig. 9. Lineweaver–Burk Plots for Inhibitions of Dextranase II Catalyzed Hydrolysis by Glucose and Glucuronic Acid

Experimental conditions were described in the text.
○: with 2.5×10^{-2}M D-glucose
●: with 2.3×10^{-2}M D-glucuronic acid
△: without inhibitor

were identified by paper chromatography. Results are presented in Fig. 7 and Fig. 8. Isomaltodextrin and the corresponding isomaltodextrinol gave an identical *Rf* value under the solvent system used in the present experiments. However isomaltodextrin was distinguished from isomaltodextrinol by aniline hydrogen phthalate, since isomaltodextrinol did not react with the reagent. Isomaltotritol and isomaltotetraitol were far less attacked compared with isomaltotriose and isomaltotetraose. The principal products from isomaltodextrinols were isomaltose and isomaltotriose, and according to increase the degree of polymerization, isomaltotetraose and isomaltopentaose were also observed.

Inhibition Type of Oligosaccharide on Dextranase II

Inhibition type of mono- and disaccharides was interpreted by Lineweaver–Burk plots. Glucose, one of the hydrolyzed dextran products, showed the competitive inhibition and that of glucuronic acid was mixed type as shown in Fig. 9. Inhibition types and constants of other oligosaccharides are presented in Table I. Glucose, glucosamine and glactose were found to act as competitive inhibitors, and inhibition constants of glucosamine (5.6×10^{-2}M).

Table I. Inhibition of Dextranase II Catalyzed Hydrolysis by Several Oligosaccharides

Oligosaccharide	Type of inhibition	Inhibition constant $Ki(\times10^{-2}$M)	Oligosaccharide	Type of inhibition	Inhibition constant $Ki(\times10^{-2}$M)
D-Glucose	C	3.5	L-Rhamnose	N	32.0
D-Glucosamine	C	5.6	D-Xylose	C	3.9
D-Glucuronic acid	M	6.2	Maltose	M	1.6
D-Galactose	C	4.9	Lactose	M	4.0
D-Galacturonic acid	M	5.7	Cellobiose	M	3.1
D-Fructose	C	3.4			

C: competitive inhibition N: non-competitive inhibition M: mixed type inhibition

and galactose (4.9×10^{-2}M) were larger than that of glucose (3.5×10^{-2}M). Fructose and xylose showed mixed type inhibition and rhamnose was found to act as a non-competitive inhibitor. Lactose and cellobiose were also found to act as mixed type inhibitors. Such other saccharides as raffinose, salicine, trehalose, sucrose, sorbitol and mannitol did not show significant inhibition.

Discussion

Action patterns of *P. funiculosum* dextranase has been reported by Bourne, *et al.*[19] but the purified enzyme was not subjected to the experiments. This paper in the first description concerning that of the purified dextranase.

At the beginning of incubation, dextranase II from *P. funiculosum* produced almost equal amount of various isomaltodextrins (Fig. 3). These observations prove that this enzyme is endo-dextranase. Dextranase II, however, acted regularly on the isomaltodextrins. From the results of action on isomaltodextrinols, the attack points of the enzyme on isomaltodextrin are primarily at second and third glucosidic linkage from non-reducing end of the substrate, and according to the increase of polymerization degree of substrate, the enzyme attacks also at fourth or fifth glucosidic linkage. This is one of the proofs that this enzyme is the endo-dextranase. These results gave the proposed action patterns of dextranase II for isomaltodextrins as shown in Fig. 10.

Isomaltose was one of the digested products from dextran and not decomposed into more short parts by dextranase II. From these observations, it is presumed that distance between catalytic site and binding site for a substrate on the enzyme was longer than two glucosidic linkages, so isomaltose was not able to be catalyze by the enzyme.

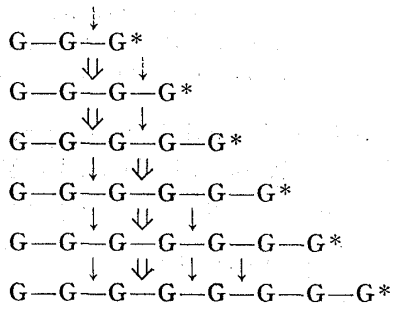

Fig. 10. Action Patterns of *P. funiculosum* Dextranase II on Isomaltodextrins

G: glucose, G*: reducing end, —: α-1,6 glucosidic linkage, ⇓: hydrolyzed very rapidly, ↓: hydrolyzed rapidly, ↓: slightly hydrolyzed when the reducing end was substituted with an alcohol group

Nitta and co-workers reported that partial alternation of substrate analogues changed the inhibition types of them in Taka-amylase A (EC 3.2.1.1).[20] Inhibition types of glucose and glucosamine were competitive and the inhibition constant of the latter was larger than that of the former. These were due to the difference of substituent at C-2, and therefore the substituent at C-2 was likely to effect on the degree of inhibition; *i.e.* strength of binding to active center of the enzyme. Since inhibition constant of galactose was larger than that of glucose, hydroxyl-group in β-form at C-4 was better than that in α-form for binding to active center of dextranase II. It was found that glucuronic acid acted as a mixed type inhibitor and rhamnose which had methyl group at C-6 acted as a non-competitive inhibitor. From these results, it is presumed that C-6 position of glucose is found to be the primary position to effect on the type of inhibition; *i.e.* binding part of the enzyme. This is supported by the difference of inhibition type between galactose and galactouronic acid. With dextranase II, it was assumed that C-6 position of glucosidic residue was the position which effected the enzyme activity. Disaccharides used in these experiments were found to act as mixed type inhibitors. It was no wonder that these disaccharides with α-1, 4 glucosidic linkage did not act as competitive inhibitors, since dextranase hydrolyzed only α-1, 6 glucosidic likage.

19) E.J. Bourne, D.H. Hutson, and H. Weigel, *Biochem. J.*, **85**, 158 (1962).
20) Y. Nitta, K. Hiromi, and S. Ono, *J. Biochem. Tokyo*, **69**, 577 (1971).