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STUDIES ON THE ACTION OF AMYLO-1,6-GLUCOSIDASE

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SUMMARY

The synthetic and hydrolytic properties of purified rabbit muscle amylo-1,6-glucosidase (dextrin 6-glucohydrolase, EC 3.2.1.33) were further investigated to more clearly define the mechanism of action.

The enzyme was found to retain anomeric configuration in the degradative direction and, in the reverse direction, to synthesize branched products from glucose and maltotetraose or α-Schardinger dextrin. These products were enzymatically analyzed by two-dimensional chromatography. The enzyme was also found to utilize glucose as a glucosyl acceptor with the formation of isomaltose.

The results indicate that the enzyme proceeds in both the synthetic and degradative directions with retention of anomeric configuration *via* the formation of a glucosyl–enzyme intermediate.

INTRODUCTION

Amylo-1,6-glucosidase (dextrin 6-glucohydrolase, EC 3.2.1.33) and oligo-1,4 → 1,4-transferase α-1,4-glucan:α-1,4-glucan 4-oligoglucantransferase, EC 2.4.1.24) comprise the mammalian debranching system of glycogen phosphorylase limit dextrin[1]. The two enzymatic activities are associated with each other throughout purification and even in the most highly purified preparations appear as a homogeneous protein entity[1,2].

The enzymatic activities have been classically assayed by their combined action on glycogen phosphorylase limit dextrin[1–4]. They can also be assayed independently. The glucosidase has been assayed by using specific substrates for its degradative action such as 6[3]-α-glucosyl-maltotetraose, "fast B_5" (ref. 1) or α-glucosyl Schardinger dextrin[5], or by utilizing the ability of the glucosidase to reincorporate

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glucosyl moieties into glycogen[6]. The transferase activity has been principally assayed by utilizing its transferase action on amylodextrins[1,7].

Recent investigations into the action of a highly purified glucosidase–transferase preparation have indicated that the properties of the glucosidase portion, such as the pH optimum, and the shift in the pH optimum and inhibition in the presence of Tris, are similar in the degradative (action on limit dextrin) as well as the synthetic direction (incorporation of glucosyl moieties) and suggest that the synthetic action of the glucosidase is due to micro-reversibility of the normal degradative reaction[2,8].

Although it has been known for some time that the glucosidase can reform α-(1→6)-glucosidic linkages with glucan acceptors[4,9], it was not known in the degradative direction whether the enzyme retained or inverted anomeric configuration in the products. Since retention or inversion of anomeric configuration in conjunction with the ability to catalyze synthesis reactions provides information concerning mechanism of action, it was of interest to further investigate the synthetic and degradative action of the glucosidase.

EXPERIMENTAL

Methods

Determination of enzymatic activity. The units of enzyme were determined by the glucosidase–transferase assay method described previously which measures glucose production from glycogen phosphorylase limit dextrin[2].

Preparation of the enzyme. The enzyme used in these experiments was the same as that described previously[2]. It is a highly purified preparation (specific activity 7.7 units/mg) of amylo-1,6-glucosidase–oligo-1,4 → 1,4-glucantransferase obtained from rabbit muscle.

Miscellaneous analytical methods. Protein was determined by the modified Folin–Lowry method described previously[2]. Carbohydrate concentration was determined by the phenol–H_2SO_4 method[1].

Anomeric configuration of the product. The enzyme incubation mixture consisted of 0.5% glycogen phosphorylase limit dextrin in 5 mM sodium maleate + 0.25 mM EDTA + 5 mM 2-mercaptoethanol (pH 6.6) at 25°. A quantity (18 ml) was prepared to fill a 0.5-dm cell with a slight excess (approx. 2.5 ml). The cell was fitted with a special aperture to allow rapid filling and emptying without bubble entrapment. The cell was used in a Bendix-Ericsson automatic polarimeter, model ETL-NPL, equipped with a type 143A optical unit. The optical unit was used in the vertical position with the sodium D line. The instrument was zeroed against water and checked with a solution of known rotation before use. Before addition of enzyme, 0.5 ml of incubation solution was removed and set aside as a zero time control, 0.175 ml of enzyme solution was added, with mixing, to the remaining solution (equilibrated to instrument temperature). A 1.0-ml aliquot was removed and placed inside the cell housing in a small tube. The remaining solution was poured into the polarimeter cell and the observed rotation followed for 15 min. At this point, 20 μl of concentrated NH_4OH were added and the solution mixed inside the cell by introduction of an air bubble and swirling (resultant pH approx. 10), and the rotational shift observed. At the point of introduction of the alkali, the aliquot placed inside the cell housing was removed and placed in a boiling-water bath for 2 min. The production of glucose was determined

using the spectrophotometric method described previously[2]. When the polarimetry experiment was repeated using the same conditions as above but with heat-denatured enzyme, no detectable shift in rotation was observed upon addition of alkali.

Synthesis of branched pentasaccharide and glucosyl schardinger dextrin. Branched pentasaccharide was prepared by incubating the glucosidase with glucose and maltotetraose. The synthetic incubation solution (1.0 ml) consisted of 50 mM sodium maleate + 5 mM EDTA + 10 mM 2-mercaptoethanol (pH 6.6) at 25°, containing 45% glucose + 5% maltotetraose and 1.8 units of glucosidase having a specific activity of 7.7 units/mg. The solution was incubated for 18 h at 30°. An aliquot of the incubation mixture was spotted directly on the paper for analysis. The product was then isolated preparatively by paper chromatography, using 1-propanol–ethyl acetate–water (7:1:2, by vol.) as the irrigating solvent.

Glucosyl Schardinger dextrin was prepared in the same manner as the branched pentasaccharide by substituting α-Schardinger dextrin for maltotetraose.

Synthesis of isomaltose. Isomaltose was synthesized by the glucosidase as described for maltotetraose using only 45% (w/v) uniformly labeled [^{14}C]glucose, specific activity 0.04 μC/μmole. The incubation solution (2 ml) contained 3.6 units of enzyme. The [^{14}C]glucose was subjected to chromatography before use and found to contain no radioactive compound corresponding to isomaltose. The unlabeled glucose used to dilute the labeled glucose to the above specific activity was also free of isomaltose (paper chromatography). The radioactive chromatograms were monitored *via* a Packard Instrument Co. radiochromatogram strip scanner before development of the spots. After formation of isomaltose the synthetic incubation solution was preparatively chromatographed on paper using 1-propanol–ethyl acetate–water (7:1:2, by vol.) as the irrigating solvent, and the portion corresponding to isomaltose cut from the chromatogram and eluted with water. The yield was approx. 0.2%. Due to the small quantity of radioactive isomaltose produced (approx. 2 mg), 100 mg of unlabeled isomaltose was added as a carrier. The isomaltose was concentrated to dryness under vacuum and converted to the β-octaacetate[11]. The octaacetate was recrystallized to constant specific activity from abs. ethanol, m.p. and mixed m.p. 143–145°; 5800 counts/min per mg.

Two-dimensional paper chromatography. The enzyme solution used to spray the paper consisted of 1.8 units of enzyme/ml (specific activity 7.7 units/mg) in 5 mM sodium citrate + 0.5 mM EDTA + 1 mM 2-mercaptoethanol (pH 6.0) at 25°. The paper was incubated in a moist atmosphere for 1 h and air-dried. It was then resubjected to descending chromatography at 90° to the first direction. The irrigating solvent was 1-propanol–ethyl acetate–water (7:1:2, by vol.)[12] in both directions. Reducing sugars and oligosaccharides were developed by the Tollen's method as modified by TREVELYAN *et al.*[13]. The chromatograms containing both reducing sugars and Schardinger dextrins were developed by first dipping them in 0.1% iodine + 0.02% potassium iodide in anhydrous methanol. The Schardinger dextrin spots were marked with pencil since the subsequent procedure eradicates the iodine color. After the paper was dry it was treated by the procedure of TREVELYAN *et al.*[13], referred to above.

Additional paper chromatographic solvent systems used in analysis of the synthetic products of glucosidase action were isoamyl alcohol–pyridine–water (1:1:1, by vol.), ethyl acetate–pyridine–water (10:4:3, by vol.), and 1-butanol–pyridine–water (6:4:3, by vol.)[14].

Materials

Glycogen phosphorylase limit dextrin was available in this laboratory, prepared as described previously[15]. Glucosyl Schardinger dextrin was a gift of Professor W. J. Whelan, University of Miami School of Medicine, Miami, Fla. α-Schardinger dextrin was a gift of Professor Dexter French, Iowa State University, Ames, Iowa. Isomaltose was obtained from the Miles Chemical Co., Elkhart, Ind. and purified by descending paper chromatography using 1-propanol–ethyl acetate–water (7:1:2, by vol.) before use. Authentic isomaltose β-octaacetate was a gift of Dr. B. A. Lewis, Cornell University, Ithaca, N.Y. Uniformly labeled [^{14}C]glucose was obtained from New England Nuclear Corp., Boston, Mass. and purified by descending paper chromatography using 1-propanol–ethyl acetate–water (7:1:2, by vol.) before use. Maltose, 3 times recrystallized was available in this laboratory and was purified by descending paper chromatography using 1-propanol–ethyl acetate–water (7:1:2, by vol.) before use. Maltotriose, maltotetraose, maltopentaose and maltohexaose were prepared from a partial acid hydrolysate of defatted corn starch using a column of acid-treated cocoanut charcoal (20–50 mesh, Fisher Scientific Co., Pittsburgh, Penn.) and an aq. ethanol gradient for preliminary separation. The column fractions were then chromatographed by descending paper chromatography using 1-propanol–ethyl acetate–water (7:1:2, by vol.) as the irrigating solvent and Whatman No. 31ET paper. The major maltodextrin fraction was cut from the paper, eluted with water and rechromatographed using the same solvent system. The maltodextrins were then deionized by passage through columns containing successive bands of acidic ion-exchange resin (Amberlite IR-120, Mallinckrodt Chemical Works, St. Louis, Mo.), weakly basic resin (Amberlite IR-45) and acidic resin. The solutions were concentrated (acidity checked) and lyophilized. Branched pentasaccharide was prepared from a glycogen α-amylase limit dextrin mixture by a variation of the usual enzymatic method[1] (T. E. NELSON AND J. LARNER, 1967; unpublished data). The product was purified by paper chromatography as described above for the maltodextrins and after incubation with purified amylo-1,6-glucosidase shown to yield maltotetraose and glucose upon paper chromatography. The pentasaccharide was tentatively identified as "fast B_5" (ref. 1) 6^3-α-glucosyl-maltotetraose (nomenclature of WHELAN[16]).

All paper chromatography was done using Whatman No. 1 paper (Reeve Angel, Newark, N.J.) for analytical purposes or Whatman No. 3MM for preparative purposes, unless otherwise stated.

Quantitative determination of radioactivity was made using the filter paper–liquid scintillation method described previously without prior washing of the papers[8].

All other chemicals or materials were of commercially available reagent or analytical grade.

RESULTS

Anomeric configuration

The anomeric configuration of the glucose released by the glucosidase was determined by following the course of the hydrolysis of limit dextrin in a polarimeter and observing the shift in rotation upon addition of alkali. The results are shown in Fig. 1. The mutarotational shift in the negative direction indicates qualitatively that the α-anomer of glucose was produced by the enzyme. The magnitude of the shift

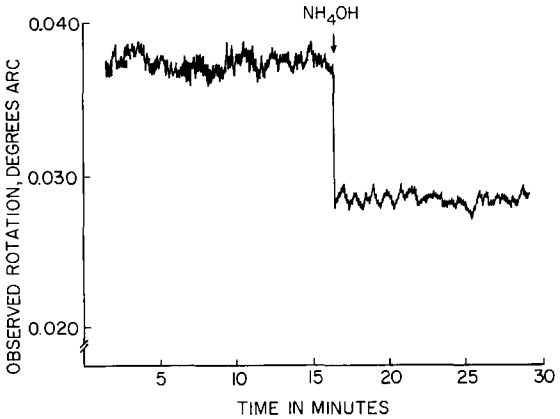

Fig. 1. Observed optical rotation during glucosidase–transferase hydrolysis of glycogen phosphorylase limit dextrin. The experiment was performed as described in the text using an automatic recording polarimeter. The recorder trace is shown. The polarimeter cell contained 0.02 unit of enzyme per ml having a specific activity of 7.7 units/mg. The arrow indicates the introduction of a small amount of concentrated NH_4OH into the cell.

approximated that calculated for the amount of glucose produced. The results indicate that the glucosidase proceeds with retention of configuration.

Enzymatic synthesis of low-molecular-weight substrates

The synthetic action of the glucosidase was investigated by allowing the enzyme to act on normal degradative products (glucose and maltodextrin) under conditions where the reverse reaction was favored—high product concentrations and lowered water concentration. Maltotetraose and α-Schardinger dextrin were chosen as potential glucosyl acceptors since the anticipated products could be readily identified chromatographically.

The incubation solutions were spotted on paper and examined by one-dimensional descending chromatography. Glucose (45%, w/v) and acceptor (5%, w/v) concentrations were determined experimentally by observing chromatographically which combination gave maximal synthesis.

In the case of maltotetraose, the appearance of a compound, having the same R_F in three solvent systems as a branched pentasaccharide (tentatively identified as 6^3-α-glucosyl-maltotetraose), increased with increasing glucose concentration. In the case of α-Schardinger dextrin the formation of a compound having the same R_F as α-glucosyl Schardinger dextrin was observed. Neither the spot corresponding to the branched pentasaccharide nor that corresponding to the branched Schardinger dextrin were observed in the zero-time controls (*minus* enzyme and enzyme alone). Of interest was the observation that in all cases (except the controls) a small amount of a product corresponding to isomaltose (6-*O*-α-glucosyl-glucose) was also formed.

The synthetic products were further identified by using them as substrates for the glucosidase. The products were isolated by paper chromatography and subjected to degradative action by the glucosidase. The resultant solution was concentrated and analyzed by descending paper chromatography. In the case of the pentasaccharide,

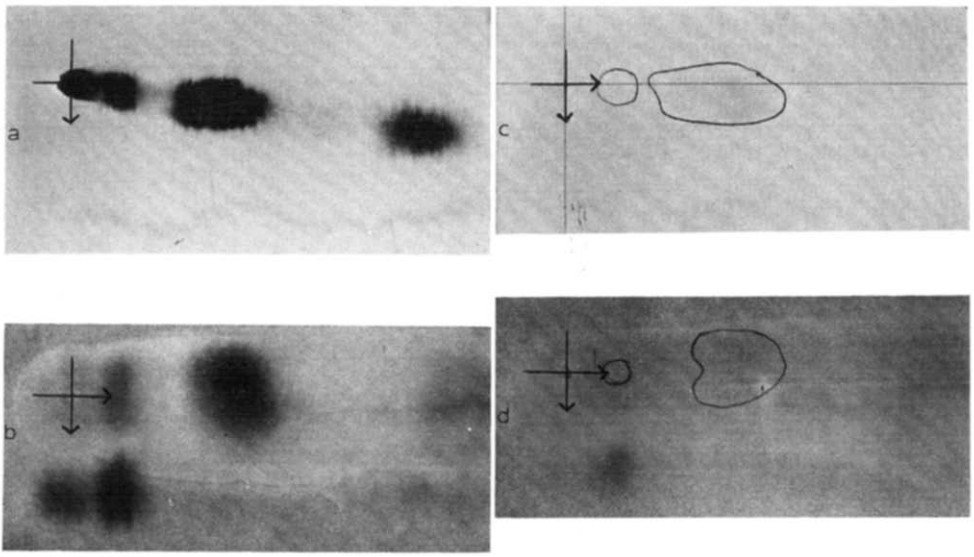

Fig. 2. Two-dimensional analysis of the synthetic products formed by the action of the glucosidase. The synthetic products formed by the action of the glucosidase in the presence of glucose and maltotetraose or α-Schardinger dextrin were chromatographed in the horizontal direction, as shown, for 72 h. The paper was sprayed with glucosidase solution, maintained in a moist atmosphere for 1 h and then chromatographed in the vertical direction, as shown, for 6 h. Details of the procedure are described in the text. The origin is at the left as shown. a. Two-dimensional chromatography control; maltotetraose incubation mixture without intermediate enzymatic spray treatment. The largest spot is maltotetraose, the spot between this and the origin is the branched pentasaccharide. The origin material was unidentified. The spot furthest from the origin is maltotriose, a trace component of the maltotetraose preparation. b. Two-dimensional chromatography of maltotetraose incubation mixture after intermediate enzymatic spray. The spots at the bottom of the chromatogram had an R_F corresponding to glucose. c. Two-dimensional chromatography control; α-Schardinger incubation mixture without intermediate enzymatic spray treatment. The largest spot is α-Schardinger dextrin, the spot between this and the origin is the glucosyl Schardinger dextrin. d. Two-dimensional chromatography of α-Schardinger dextrin incubation mixture after intermediate enzymatic spray. The spot at the bottom of the chromatogram had an R_F corresponding to glucose.

maltotetraose and glucose appeared as products. In the case of the glucosyl Schardinger dextrin, glucose and α-Schardinger dextrin appeared. The origin of the glucose was further substantiated by subjecting the original synthesis mixture to two-dimensional chromatographic analysis. This was done by running a descending chromatogram of the synthetic products without prior degradation by the glucosidase. The chromatogram was then allowed to dry and the portion containing the origin and the pentasaccharide or glucosyl Schardinger dextrin was removed, sprayed with glucosidase solution till damp, and allowed to incubate in a moist chamber at ambient temperature. The chromatogram was allowed to dry again, rotated 90°, and resubjected to descending chromatography. The results are shown in Fig. 2. As can be seen, glucosidase action released glucose from both the branched pentasaccharide and the glucosyl Schardinger dextrin. This type of analysis has recently been employed in analyzing complex oligosaccharides[17]. The maltotriose spot which appears in Fig. 2a was present in the zero-time control of the incubation mixture (*minus* enzyme) and is due to a trace in the maltotetraose preparation which was not completely removed

upon subsequent chromatography. The material yielding a trace of glucose at the origin in Fig. 2b is more than likely due to higher homologs of maltotetraose, which were also not completely removed, and apparently served as glucosyl acceptors. Although the maltotetraose was purified by the usual methods (*cf.* EXPERIMENTAL), it is difficult to remove traces of higher and lower homologs from such preparations, even upon repeated chromatography. The alkaline $AgNO_3$ detection method used, although highly sensitive, visually accentuates these trace quantities.

The synthesis of the branched pentasaccharide and the glucosyl Schardinger dextrin as well as the appearance of isomaltose could have been due to the synthetic action of a small amount of α-glucosidase (maltase) or glucoamylase (amyloglucosidase) present in the enzyme preparation. Although maltase activity had not been detected previously in the present purified preparation[2], the determination was not done under the conditions employed in the present experiments. In order to test for this possibility, a 5% (w/v) solution of maltose was incubated with the enzyme under the same conditions employed in the present experiments. The results showed (paper chromatography) no detectable production of glucose, thus indicating the absence of maltase and mammalian γ-amylase (glucoamylase) which readily degrade maltose to glucose[18].

The appearance of isomaltose in the synthetic incubation solutions indicated that the enzyme could use glucose itself as a glucosyl acceptor. This possibility was investigated by incubating the enzyme with uniformly labeled [^{14}C]glucose alone (45%, w/v). Examination of the incubation solution by paper chromatography indicated the production of isomaltose. The incubation solution was chromatographed preparatively on paper. The yield of radioactive isomaltose (calculated on a weight basis after chromatography) was approx. 0.2%. The radioactive isomaltose was characterized by recrystallization of the β-octaacetate to constant specific activity.

DISCUSSION AND CONCLUSIONS

The synthesis, using highly purified enzyme, of a branched pentasaccharide from glucose and maltotetraose and α-glucosyl Schardinger dextrin from α-Schardinger dextrin and glucose substantiates that amylo-1,6-glucosidase is capable of reforming an α-(1→6) linkage. The formation of isomaltose from glucose alone is of importance since it indicates that the enzyme does not require a polysaccharide or oligosaccharide acceptor and clearly demonstrates that the glucosidase transfers a glucosyl moiety by micro-reversibility of the degradative reaction. This indicates that hydrolysis proceeds *via* a glucosyl–enzyme intermediate. This is corroborated by previous evidence that the pH optimum of the glucosidase is the same in both the degradative and the synthetic directions and that the optimum is shifted and inhibited by Tris in the same manner in both directions[2,8].

The results with this highly purified glucosidase–transferase preparation substantiate previous reports that the glucosidase in tissue homogenates is able to synthesize branched maltodextrins from [^{14}C]glucose and linear maltodextrins[19] and that partially purified preparations of the glucosidase could synthesize a branched Schardinger dextrin from [^{3}H]glucose and α-Schardinger dextrin[20].

The fact that the glucosidase reforms the same linkage that it degrades and proceeds *via* a glucosyl intermediate does not indicate the hydrolytic mechanism by which this occurs. However, the fact that the glucosidase retains anomeric con-

figuration in the degradative direction suggests that hydrolysis may occur by one of the mechanisms proposed for enzymes that retain configuration[21,22].

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