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EFFECT OF VARIOUS ACCEPTORS ON THE RATES OF THE CYCLIZATION AND CHAIN-SHORTENING OF AMYLOSE CATALYZED BY THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Klebsiella pneumoniae* M 5 al. IMPROVEMENT OF NEW PHOTOMETRIC ASSAY METHODS.

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ABSTRACT

The effect of various acceptors on the cyclization and chain-shortening reaction of amylose catalyzed by cyclodextrin glycosyltransferase {(1→4)-α-D-glucan: [(1→4)-α-D-glycopyranosyl]transferase (cyclizing) EC 2.4.1.19} from *Klebsiella pneumoniae* M 5 al was studied by use of photometric-assay methods. The requirements for the acceptor were the same for both cyclization and chain-shortening, indicating the close relationship between both reactions. Maltose proved to be the most effective (2.48- and 5-fold acceleration of the cyclization and chain-shortening, respectively, in the presence of 584 μM maltose). The dependence of the chain-shortening reaction on the conformational state of the amylose molecules is discussed.

INTRODUCTION

Long-chain amyloses have been shown to be poor substrates for the reaction of cyclization catalyzed by cyclodextrin glycosyltransferase {(1→4)-α-D-glucan: [(1→4)-α-D-glucopyranosyl]transferase (cyclizing) EC 2.4.1.19; CGT} from *Klebsiella pneumoniae* M 5 al. Cyclohexaamylose is formed at maximum rate only after an initial degradation of the amylose into chains having lengths $< Glc_{100}$. The chain-shortening (evidenced by rapid retrogradation) is enhanced markedly by the addition of malto-oligosaccharides[1].

A photometric assay method for the determination of the initial cyclization-rate has been elaborated recently, based on the marked increase in u.v. absorption of iodine by complex-formation with cyclohexaamylose[2]. The values for enzyme activity obtained by the photometric assay were consistent with those found by the conventional methods[1,3]. It was obvious, therefore, to re-investigate the kinetics of the (1→4)-α-D-glucopyranosyl transfer-reactions catalyzed by CGT with amylose, using the new assay method. This paper describes the effect of various acceptors on both the cyclization and the chain-shortening reaction.

EXPERIMENTAL

Materials. — CGT was isolated from the culture filtrate of continuously grown *Klebsiella pneumoniae* M 5 al, and purified to electrophoretic homogeneity as previously described[4,5]. The specific activity of the enzyme preparation with (1→4)-α-D-glucopyranosyl chains (av. d.p. 20) was found to be 2.6×10^5 units/g of protein[2,3]. Maltooligosaccharides were prepared with the CGT from *Klebsiella pneumoniae* M 5 al according to Bender[1,3]. Amylose from potato starch (95% pure, mol. wt. 136,000 ±5%)[1] was from Serva (Heidelberg, F.R.G.). All other substances were purchased from commercial sources in the highest grade of purity available.

Methods. — The total carbohydrate content was determined with anthrone[6,7], and reducing aldehyde groups with the Nelson reagent[8]. Chain lengths were calculated according to Gunja-Smith *et al.*[9]. Protein content was determined by the biuret method[10].

Enzyme assays. — (*a*) *Cyclization reaction.* To a 4% solution (15 mL) of amylose in dimethyl sulfoxide was added 0.1M iodide-free iodine stock solution in dimethyl sulfoxide[11] to reach a mM concentration of iodine. The solution was completed to 100 mL with 30mM tris(hydroxymethyl) aminomethane hydrochloride (Tris) buffer (pH 7.2) containing 5mM calcium chloride. The substrate-iodine solution (initial substrate-concentration 44μM) was preincubated for 15 min at 30°, when its absorbance remained unchanged. The initial acceptor-concentrations were in the range of 5.84–584μM.

The transfer reactions were started by the addition of CGT. The increase in absorbance (corresponding to the rate of cyclohexaamylose formation) was followed for 6 min at 290 nm, at 30° for digests in quartz cuvettes (3 mL, 1-cm light-path), with a Shimadzu UV 100-02 spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan), and recorded with a W + W Recorder 1100 (W + W Electronic AG, Basle, Switzerland). The number of μmol of cyclohexaamylose that were produced at maximum rate by 1 mL of the enzyme solution was calculated according to the formula Δ_E digest · 3/3.45 · Δt · v, where Δ_E is the extrapolated maximum cyclization rate, the value of 3.45 is E of the cyclohexaamylose–iodine standard (corresponding to the increase in absorbance at 290 nm caused by complex formation of 1 μmol of iodine · mL^{-1} with 1 μmol of cyclohexaamylose · mL^{-1}), Δt is the incubation time, and v is the test volume.

A unit of enzyme activity is expressed as the amount of enzyme causing the formation of 1 μmol of cyclohexaamylose (at the maximum rate for an initial substrate-concentration of 44μM)/min at pH 7.2 and 30°. The specific catalytic activity is expressed both as unit/g of protein and as catal/kg of protein.

(*b*) *Chain-shortening reaction.* A hot, 6% solution of amylose in dimethyl sulfoxide (5 mL) was mixed with the iodine stock solution (0.5 mL), and poured into water (50 mL). The insoluble amylose–iodine complex (95% recovery) was removed by centrifugation (30 min, 12 000*g*) and dissolved in dimethyl sulfoxide (7 mL), and the solution was diluted to 100 mL with 30mM Tris · HCl buffer (pH 7.2)

containing 10mM calcium chloride. The bulk of iodine was removed by heating the solution for 5 min to 70°. The solution (21 μM initial substrate concentration) was stable for 2 h. The initial acceptor concentrations were in the range of 5.84–584μM.

The transfer reactions were started by addition of CGT (217 μg of protein · L^{-1}). In order to avoid any interference with the cyclization reaction, the increase in turbidity (relative determination of the chain-shortening reaction) was followed at 320 nm for 10 min at 30° (3 mL of digest and 1-cm light-path). The maximum rates of increase in turbidity, in the presence of acceptors, were related to the maximum rate of increase found without acceptor. The acceptor effect is expressed as "acceleration factor", corresponding to an x-fold increase in the reaction rate. For control, the changes of the average chain-lengths in the course of incubation were followed for a number of digests. The substrate residues were precipitated (by addition of 1.2 vol. of methanol), and the reducing power of the centrifuged-off and resuspended precipitates was determined (see ref. 1).

RESULTS AND DISCUSSION

Kinetics of the cyclization reaction. — The spectra of the amylose–iodine solution before and after incubation with CGT (21.7 mg of protein · L^{-1}, 30°, 8 min)

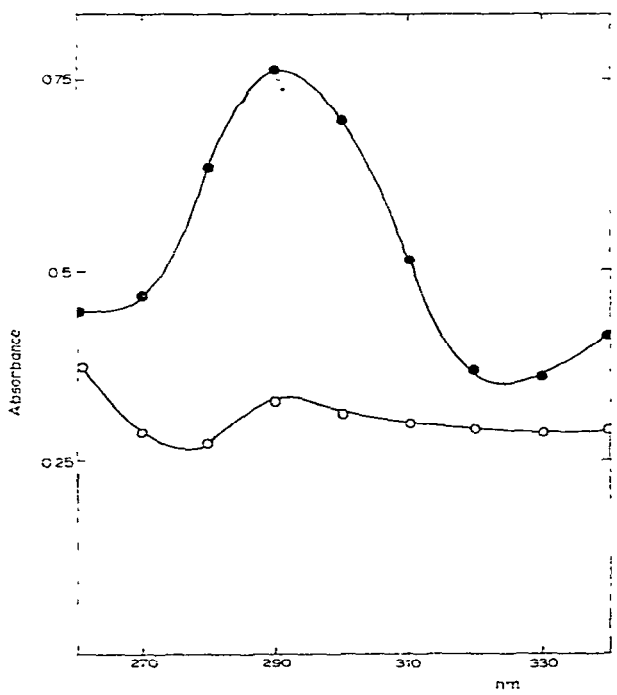

Fig. 1. u.v. absorbance of the amylose-iodine complex before (○——○) and after (●——●) incubation with CGT at 30°: The substrate-iodine solution (3 mL; initial substrate concentration, 44μM) was incubated with CGT (65.1μg) for 8 min.

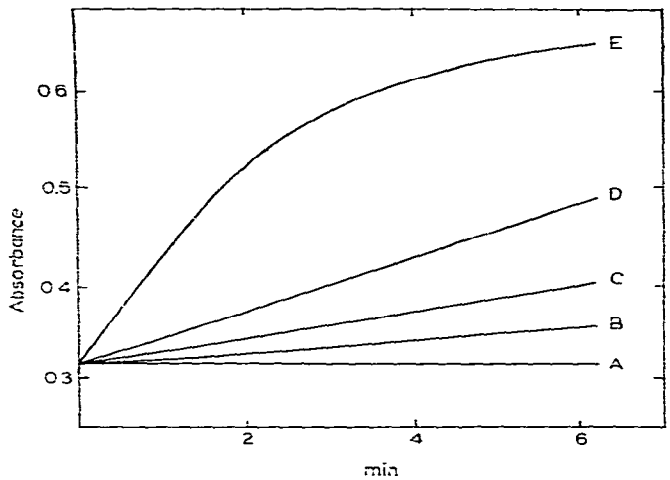

Fig. 2. Increase in absorbance at 290 nm due to the cyclization reaction catalyzed by various concentrations of CGT with 44μM amylose-iodine: (A) 0, (B) 1.085, (C) 2.17, (D) 4.34, and (E) 21.7 mg of CGT · L^{-1}.

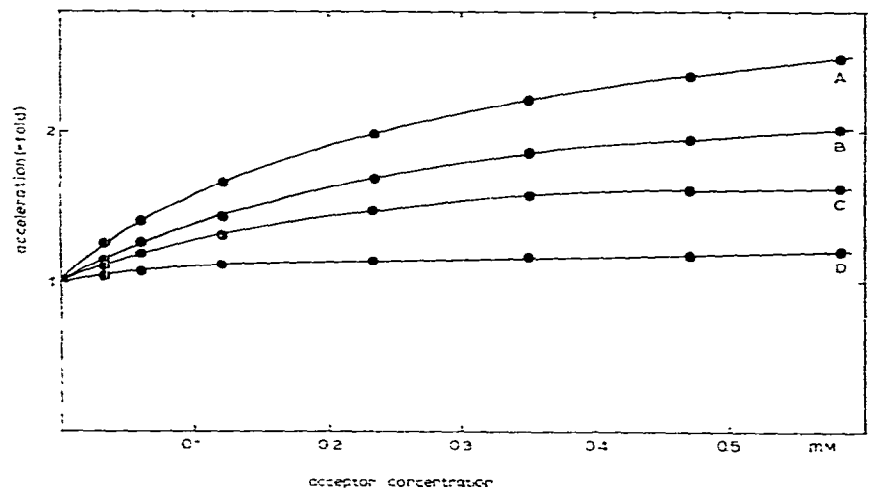

Fig. 3. "Acceleration effect" of various acceptors on the cyclization by CGT (1.085 mg · L^{-1}) on 44μM amylose-iodine at 30° relative to the acceptor concentration: (A) maltose, (B) maltotriose, (C) maltooligosaccharides (av. d.p. 7.8), and (D) D-glucose.

are shown in Fig. 1. The absorbance of the digest is characteristic of the cyclohexa-amylose–iodine clathrate[2]. Thus, the cyclization reaction of amylose–iodine catalyzed by CGT may be determined by the photometric assay as well (see Fig. 2). As shown by extrapolation for maximum velocity, the rates of cyclization are, with the exception of the highest, approximately proportional to the enzyme concentrations employed[2]. At lower enzyme concentrations, a slight increase in the reaction rate may be observed in the initial phase of incubation. From these data, an activity of 2.15 units · mg^{-1} of

protein (0.036 kat · kg^{-1} of protein) was calculated. By use of conventional methods, an initial cyclization-rate of 0.036 kat · kg^{-1} of protein was found[1] for an initial substrate concentration of 180μM.

As shown in Fig. 3, an "acceleration effect" of various acceptors on the cyclization reaction is still measurable for concentrations as low as 5.84μM. Maltose proved to be most effective (Fig. 3, A, see ref. 12). In the presence of 584μM disaccharide, the reaction rate was enhanced 2.48–fold. Maltotriose and maltooligosaccharides (av. d.p. 7.8) were less effective (Fig. 3, B and C). Apparently the acceptor properties decrease when the acceptor is also a substrate for CGT; maltose has been shown to be a very poor substrate for the enzyme[5]. D-Glucose was found to be a poor acceptor (Fig. 3, D), because of its presumably low affinity for the acceptor binding-sites of the enzyme.

Kinetics of the chain-shortening reaction. — At an initial substrate concentration of 180μM, maximum cyclization rates of 0.6 kat · kg^{-1} of protein (amylose without added acceptor) and of 1.8 kat · kg^{-1} of protein (amylose plus maltooligosaccharides) were observed. The maximum cyclohexaamylose formation coincided with a period of rapid retrogradation of the shortened amylose-chains[1]. Surprisingly, the digests used for the photometric assay remained completely clear for at least 30 min. This phenomenon may be explained by the low initial substrate concentration used, as the rate of amylose retrogradation depends upon various factors, such as concentration, chain length and conformation, pH, and the presence of mono- or poly-valent ions[13,14]. One possible explanation is that the chain-shortening reaction, which determines the cyclization rate, depends upon the conformation state of the amylose molecules, and is retarded under the conditions of assay; thus, the rate of chain-

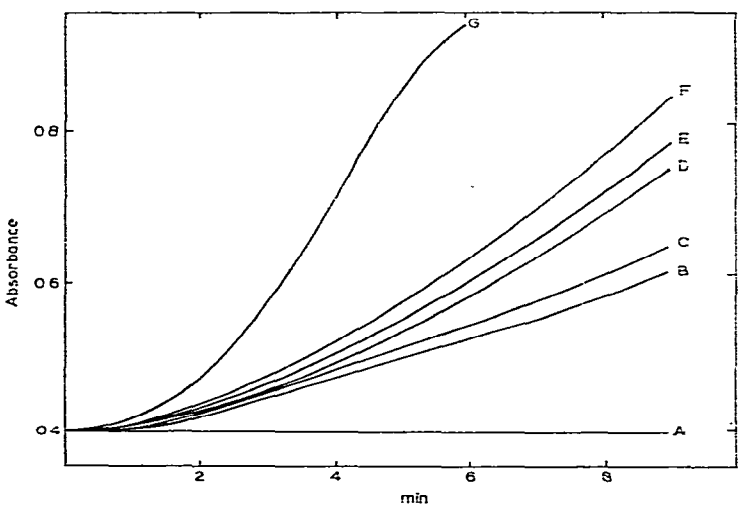

Fig. 4. Effect of maltose concentration on the chain-shortening reaction by CGT (0.217 mg · L^{-1}) with 21μM amylose at 30°, as shown by the increase in turbidity at 320nm: (A) no amylose, (B) 0, (C) 5.84, (D) 29.2, (E) 58.4, (F) 117, and (G) 584μM maltose.

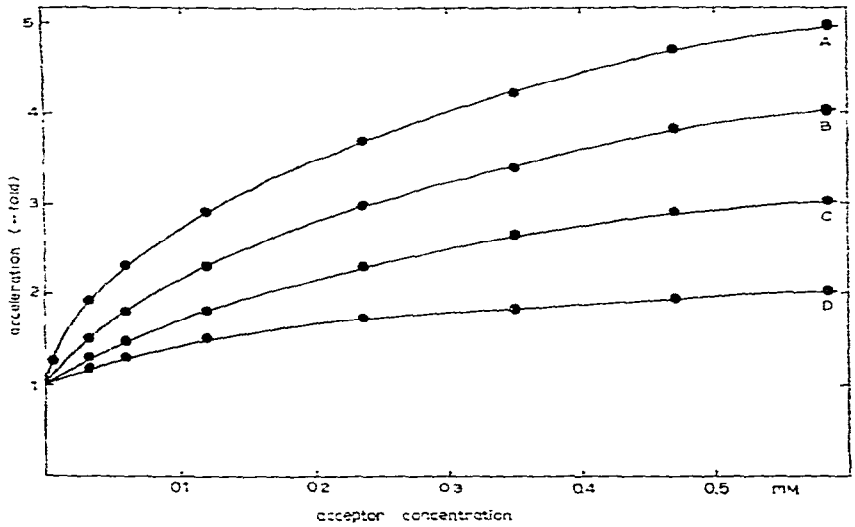

Fig. 5. "Acceleration effect" of various acceptors on the chain-shortening reaction by CGT (217ng·L^{-1}) with 21μM amylose at 30° relative to the acceptor concentration: (A) maltose, (B) maltotriose, (C) malto-oligosaccharides (av. d.p. 7.8), and (D) D-glucose.

shortening would be determined by an increase in turbidity (caused by retrogradation of the chains $<Glc_{100}$) at very low substrate concentrations. Indeed, a marked increase in turbidity was observed for digests of amylose prepared from the amylose–iodine complex.

In the study of the increase in turbidity (qualitative measure for chain-shortening) without acceptor (Fig. 4, B), and in the presence of various concentrations of maltose (Fig. 4, C–G), the effect of acceptor was still measurable at concentrations as low as 5.84μM ("acceleration factor" 1.19). In the presence of 584μM maltose, the retrogradation rate was enhanced 5-fold. The effects of the different acceptors were nearly identical to those found for the cyclization reaction, indicating the close relationship between both reactions (Fig. 5). As shown by the reducing-power of the substrate residues (precipitated by 1.2 vol. of methanol) of the digests, the average chain-lengths decreased permanently in the course of incubation[1], the decreases paralleling the rates of increase in turbidity.

These results clearly show the dependence of the chain-shortening reaction (apparently a special aspect of the disproportionation reaction resembling an endo attack) on the conformational state of the amylose. A marked increase in turbidity could be measured only with amylose freshly prepared from the insoluble amylose-iodine complex (molar ratio of amylose to iodine, 1:22.7), but not with the soluble amylose–iodine complex used for the assay of the cyclization reaction. Evidently the molar ratio of amylose to iodine (1:3.4) is insufficient to produce the conformational change necessary for the chain-shortening reaction at the low, initial substrate-concentration employed. Therefore, neither an increase in turbidity (measure for

chain-shortening) nor an increase in the cyclization rate in the course of prolonged incubation could be observed with this system.

Iodine-complex formation is known[15] "to lead to an appreciable shrinking of the amylose molecules, and an increase in rigidity, owing to dehydration of the polysaccharide (through hydrophobic interaction with iodine), and concomitant interturn hydrogen-bond formation". Evidently the conformation state of amylose favoring the chain-shortening reaction is retained for some time after removal of the bulk of iodine, and the more helical conformation of the substrate is responsible for the reaction. Accordingly, both the cyclization- and the chain-shortening reaction catalyzed by the CGT seem to depend upon helical regions[16].

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