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Isozymes of β-Glucosidase in *Dictyostelium discoideum*

M. BRUCE COSTON AND W. F. LOOMIS, JR.
Department of Biology, University of California, San Diego, La Jolla, California 92037

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The activity of β-glucosidase (EC 3.2.1.21) in extracts of *Dictyostelium discoideum* was investigated. The specific activity increased early in development, declined during pseudoplasmodium formation, and increased again during sorocarp formation. The β-glucosidase which was present in growing amoebae and during the first stages of multicellular development was electrophoretically distinct from the enzyme which accumulated during the final stages of morphogenesis. Ribonucleic acid synthesis and protein synthesis during development were required for the accumulation of the later isozyme. Analysis of β-glucosidase activity in a number of morphological mutants suggests that the enzyme which accumulates late in morphogenesis is developmentally controlled.

Depletion or removal of the supply of nutrients to vegetative myxamoebae of the cellular slime mold *Dictyostelium discoideum* initiates aggregation of the amoebae. The aggregates then proceed through a series of morphological stages resulting in the production of mature fruiting bodies composed mainly of differentiated stalk cells and spores (2). The specific activity of a number of enzymes has been shown to vary significantly during this development. Experiments with inhibitors of either ribonucleic acid (RNA) or protein synthesis imply that the increase in specific activity of four of these enzymes requires concomitant protein synthesis and prior RNA synthesis (1, 8, 13-15, 20, 21). Therefore, these changes in specific activity probably represent alteration in specific gene activity.

This study concerns the increase in the specific activity of β-glucosidase (EC 3.2.1.21) which occurs at two distinct stages of morphogenesis: immediately after initiation of development and again during the culmination phase of fruiting body formation. Variations in the specific activity of β-glucosidase during development in *D. discoideum* were first reported by Rosness (12). Our results indicate that β-glucosidase present during the early stages of development is electrophoretically distinct from the enzyme which accumulates later. Both RNA synthesis and protein synthesis are required for the accumulation of the second isozyme, whereas the increase in specific activity early in development is insensitive to inhibitors of RNA and protein synthesis. Investigation of the specific activity of the separated β-glucosidase isozymes in a number of morphogenetically aberrant mutant strains suggests that the late isozyme is developmentally controlled.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-(*N*-acetyl) glucosaminide, and *p*-nitrophenyl glucuronide were purchased from Calbiochem, Los Angeles, Calif. *p*-Nitrophenyl-β-D-lactoside was obtained from Cyclo Chemical Corp. Nitrophenyl-β-D-galactoside was obtained from K & K Laboratories, Jamaica, N.Y. Cellobiose and gentiobiose were obtained from Sigma Chemical Co., St. Louis, Mo. Cycloheximide (brand name Acti-Dione) was purchased from The Upjohn Co., Kalamazoo, Mich., and actinomycin D was the generous gift of Merck & Co., Inc., Rahway, N.J.

Black filters (AABP 047) and absorbant pads were purchased from the Millipore Corp., Bedford, Mass.

Glucose oxidase (Glucostat), from Worthington Biochemical Corp., Freehold, N.J., was used for the determination of glucose. Catalase was also obtained from Worthington.

Organisms. *D. discoideum* strain NC-4 (haploid wild type) and the mutant strains derived from it were grown in association with *Aerobacter aerogenes* (18). Morphological mutants VA-4, Fr-17, *min*-2, KY-19, and KY-3 have been described (1, 7, 8, 16, 26).

Morphogenesis was initiated by removal of nutrients from vegetative amoebae. About 10^8 amoebae washed free from bacteria were placed on membrane filters (Millipore Corp.) supported on absorbant pads saturated with streptomycin-salt solution (18). Mature fruiting bodies formed after 24 to 26 hr at

22 C. Development under these conditions was highly synchronous.

Supplements, in the following final concentrations, were administered by shifting the membrane filters (Millipore Corp.) to new support pads containing the desired agent: actinomycin D at 100 μg/ml, cycloheximide at 400 μg/ml, and ethylenediaminetetraacetic acid (EDTA) at 10^{-2} M.

Preparation of extracts. The cells were harvested from the membrane filters (Millipore Corp.) with 3 ml of distilled water and frozen. Crude extracts were prepared by sonic disruption of the thawed samples for 40 sec with a Branson Sonifier.

Extracts for gel electrophoresis were centrifuged for 75 min in a Spinco Ti-50 head at 135,000 × *g*, and the supernatant fluid was concentrated by vacuum dialysis.

The protein content of the extracts was determined by the method of Lowry et al. (10). Crystalline bovine serum albumin was used as a standard.

Assay of β-glucosidases. The assay of β-glucosidase activity was carried out in 0.5 ml which was 5×10^{-2} M in acetate buffer (*p*H 5.0) and 10^{-2} M in *p*-nitrophenyl-β-D-glucopyranoside. The reaction mixture and a blank without substrate were incubated for 50 min at 35 C, and the reaction was stopped with 1.0 ml of 1 M Na_2CO_3. The difference in optical density at 420 nm was measured with a Zeiss spectrophotometer. The reaction was linear with time and amount of extract over the ranges routinely employed. One unit of activity is defined as that amount of enzyme which produces 1 nmole of *p*-nitrophenol per min under these conditions. Specific activity is expressed in units of activity per milligram of protein.

Purification of β-glucosidases. Slime mold cells were collected in water, subjected to sonic disruption, and centrifuged for 75 min at 135,000 × *g* in a Spinco Ti-50 rotor. Streptomycin sulfate was added to the supernatant fluid to 0.5%. The precipitate was removed by centrifugation, and the activity was precipitated from the supernatant fluid at 60% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in water and extensively dialyzed against 10^{-4} M sodium phosphate, *p*H 7.0. The enzyme was applied to a diethylaminoethyl (DEAE)-Sephadex column (1 by 12 cm) and eluted with a 0.1 to 0.5 M NaCl gradient in 10^{-4} M sodium phosphate, *p*H 7.0. In some preparations, the 135,000 × *g* supernatant fluid was applied directly to the DEAE-Sephadex column. β-Glucosidase activity in extracts made from cells in early stages of morphogenesis elutes from the column in a single peak at 0.25 to 0.26 M NaCl; most of the activity in extracts of fully differentiated cells elutes at 0.13 to 0.14 M NaCl, but some activity still elutes at the higher salt concentrations at which the early enzyme elutes. The overall yield of purified enzyme from either aggregating or fully differentiated cells was typically 50 to 60%.

Acrylamide gel electrophoresis. Centrifuged samples in 15% sucrose at low ionic strength were loaded on continuous 5% acrylamide gels (4) containing 0.05 M sodium phosphate buffer, *p*H 7.2. Polymerization of the gels was catalyzed by final concentrations of 0.025% N, N, N′, N′-tetramethylethylenediamine and 0.07% ammonium persulfate. The tray buffer was 0.10 M sodium phosphate, *p*H 7.2.

The sample was "focused" (4) at 0.5 ma per gel for 30 min, and then electrophoresed at 5 to 6 ma per gel for about 165 min at 4 C. The gels were extruded and sliced into 2-mm fractions; each fraction was assayed for β-glucosidase activity. The acetate concentration was 0.15 M in the assay of gel slices to bring the *p*H to 5.0.

Sucrose gradient centrifugation. To estimate the sedimentation velocity (*S* value) of the β-glucosidase activities, clarified extracts were layered onto 17-ml, 5 to 20% sucrose gradients (10^{-4} M sodium phosphate, *p*H 7.0) and centrifuged for 24 hr in the Spinco SW-27 rotor at 90,000 × *g*. Catalase (11.2*S*) was added as an internal marker. After centrifugation, the gradient was pumped through a continuous flow cell in a Beckman DU spectrophotometer, and the optical density at 405 nm was recorded. Fractions were subsequently assayed for β-glucosidase activity.

Chromatography. Reaction products of the β-glucosidase assay were co-chromatographed with authentic compounds on Eastman 6065 cellulose thin layers in a solvent of *t*-butyl alcohol-methyl ethyl ketone-formic acid-water (8:6:3:3; reference 5). Compounds containing aryl groups could be located under ultraviolet light by using the fluorescent indicator contained in the thin layers. Reducing sugars were located with a silver nitrate stain (22).

RESULTS

The specific activity of β-glucosidase at various stages of morphogenesis of *D. discoideum* is shown in Fig. 1. The specific activity increased during the first 4 hr after removal of the food source and then decreased until culmination began about 14 hr later. At this time, a second period of increase occurred, reaching a maximum after culmination. Electrophoresis of cell-free extracts on 5% polyacrylamide gels showed that β-glucosidase present early in development is distinct from the enzyme which accumulates during culmination (Fig. 2). From the beginning of development until the formation of pseudoplasmodia, the activity was found in a single peak in the gels; at later times, this peak of activity decreased and a new, slower moving peak appeared and became the major component. Occasionally, a third component which migrates faster than either of the other major peaks was seen in both early and late samples, but when present it was always a small fraction of the total activity (less than 5%).

Both major components of β-glucosidase activity had been partially purified by column chromatography on DEAE-Sephadex. The purified enzymes migrated on acrylamide gels as single peaks at the same rate as the major components of crude preparations.

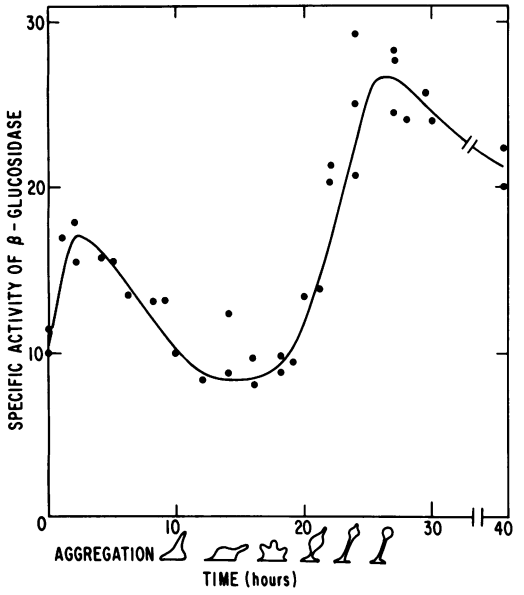

FIG. 1. *Specific activity of β-glucosidase (units/mg of protein) during development of D. discoideum. At the times indicated after the initiation of development, the cells were washed off one membrane filter (Millipore Corp.) into 3 ml of water, frozen, and subsequently assayed for β-glucosidase and protein content as described.*

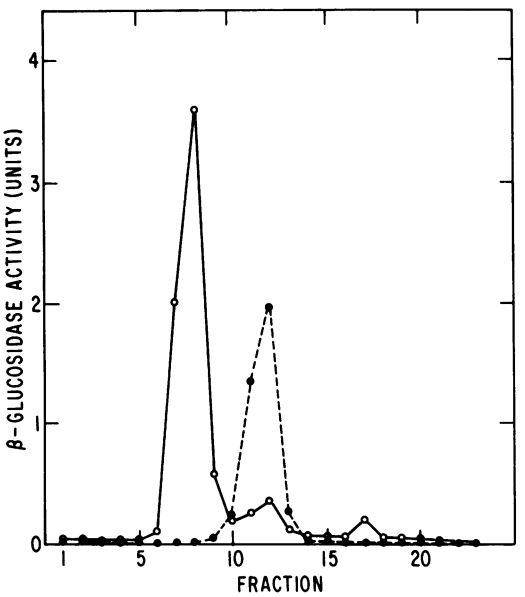

FIG. 2. *Polyacrylamide gel electrophoresis of β-glucosidase activities. Extracts were prepared from cells which had developed for 2 hr (●) or for 28 hr (○) and electrophoresed as described. The enzymes migrate toward the anode at the right.*

The two isozymes were similar in several respects (Table 1) but differed with respect to the Michaelis-Menten constant (K_m) for *p*-nitrophenyl-β-D-glucoside and the sedimentation velocity (*S*). We refer to the enzyme which is present early in development and which migrates rapidly toward the anode on gel electrophoresis as β-glucosidase-1. The enzyme which accumulates during culmination and migrates more slowly toward the anode is referred to as β-glucosidase-2.

β-Glucosidase activity in morphological mutants of D. discoideum. The specific activity of β-glucosidase during development was analyzed in several mutant strains unable to accomplish various steps in morphogenesis (Fig. 3 and Table 2).

The specific activity in strain VA-4, a mutant which does not aggregate (8), increased during the first 4 hr after removal of the food source, decreased during the next 14 hr, but did not increase thereafter as it does in wild-type cells. Similar results were found with five other mutants which fail to aggregate.

Strain KY-3, a mutant which does not develop beyond the pseudoplasmodial stage under our conditions (26), and strain KY-19, which forms spores but very few stalk cells (1), both showed typical increases in the specific activity of β-glucosidase during the first few hours of development. However, the amount of enzyme activity which accumulated during the second period, which began 14 hr later, was less than half that found in wild-type cells.

Strain *min*-2 proceeds through the first stages of morphogenesis normally and culminates to form mature fruiting bodies, but the cells in the sorus do not encapsulate to form spores (7). The pattern of specific activity during morphogenesis of strain *min*-2 was very similar to that of the wild-type strain (Table 2).

Strain Fr-17 completes development in about two-thirds of the time required by the wild-type strain; spores and stalk cells are both present by 16 hr (16). This mutant accumulated β-glucosidase-2 correspondingly early, beginning at about 12 hr (Fig. 3).

In the case of each mutant strain, a significant increase in specific activity between 18 and 30 hr (earlier in strain Fr-17) corresponded to the appearance of β-glucosidase activity which migrates on gel electrophoresis at the rate of β-glucosidase-2. Table 2 summarizes our studies on the mutants with respect to their morphological aberrations and the amount of β-glucosidase-2 accumulated. The results indicate that the initial increase in the specific activity of β-glucosidase

TABLE 1. *Comparison of some of the properties of the two forms of β-glucosidase found in D. discoideum at various times*[a]

Determination	β-Glucosidase-1	β-Glucosidase-2
K_m (*p*-nitrophenyl β-D-glucoside)[b]	$(1.27 \pm 0.27) \times 10^{-3}$ M	$(0.83 \pm 0.04) \times 10^{-3}$ M
K_i (for glucose)[b]	$(3.5 \pm 1.1) \times 10^{-3}$ M	$(2.3 \pm 0.3) \times 10^{-3}$ M
*p*H optimum	4.0–5.5	4.0–5.5
S value	8*S*	10*S*
Substrate specificities[c]		
p-Nitrophenyl β-D-glucoside	1.0	1.0
p-Nitrophenyl β-D-glucoside[d]	0.4	0.5
Cellobiose[d]	0.8	1.2
Gentiobiose[d]	0.5	0.6
Cellulose[d]	<0.01	<0.01
p-Nitrophenyl α-D-glucoside	0.009	0.05
o-Nitrophenyl β-D-galactoside	0.002	0.04
p-Nitrophenyl-glucuronide	<0.001	0.01
p-Nitrophenyl-(*N*-acetyl)-β-D-glucosaminide	0.0025	<0.001

[a] Partially purified enzymes were used (β-glucosidase-1 was purified 35 ×; β-glucosidase-2 was purified 15 ×), except in the determination of the sedimentation velocity (*S* value) where crude extracts were used. About 2 to 5 units was used, and the reaction was stopped when less than 4% of the substrates had been hydrolyzed.

[b] The values include the standard deviation.

[c] Relative rate of hydrolysis as compared with β-D-glucoside.

[d] The rate of hydrolysis of these compounds was estimated by measuring glucose production with Glucostat reagents compared on a molar basis with the amount of nitrophenol released from *p*-nitrophenyl-β-D-glucoside. Note that the hydrolysis of 1 mole of cellobiose or gentiobiose may release 2 moles of glucose.

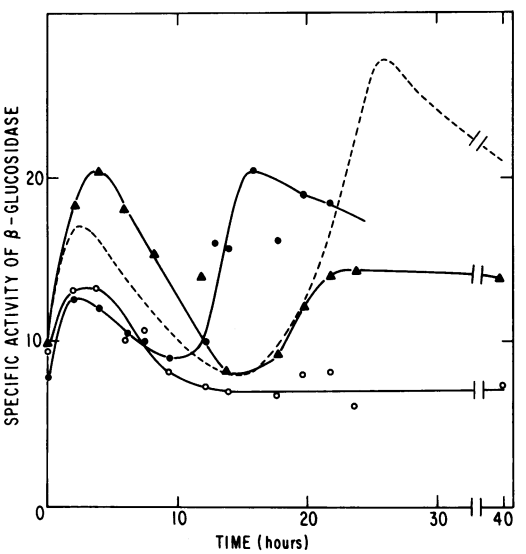

FIG. 3. *Specific activity of β-glucosidase in various morphological mutants of D. discoideum. At the times indicated after removal of the food source, cells were collected and the specific activity of β-glucosidase was subsequently determined: strain Fr-17 (●), strain VA-4 (○), and strain KY-19 (▲). The specific activity in wild-type cells (broken line) is included for comparison (from Fig. 1).*

TABLE 2. *β-Glucosidase-2 accumulation in morphological mutants*

Mutant	Morphogenesis			β-Glucosidase-2 accumulated[a]
	Aggregation	Culmination	Sporulation	
				%
VA-4	−	−	−	0
KY-3	+	−	−	15
KY-19	+	−	+	40
Min-2	+	+	−	100
Fr-17[b]	+	+	+	100

[a] Cells of the different strains were collected at the peak of β-glucosidase accumulation at least 16 hr after removal of the food source. Extracts were electrophoresed on acrylamide gels. The amount of activity migrating at the rate of β-glucosidase-2 was determined and is expressed as a per cent of the maximal amount found in wild-type cells.

[b] In Fr-17, morphogenesis occurs earlier and faster.

can occur in mutant strains unable to aggregate but that normal accumulation of β-glucosidase-2 takes place only in those mutant strains which are able to accomplish the morphogenetic steps resulting in culmination. Moreover, β-glucosidase-2

accumulated precociously in mutant strain Fr-17, in which the morphological differentiations are also completed more rapidly. We conclude that, although the initial increase in the specific activity of β-glucosidase-1 is not dependent on morphogenesis, the accumulation of β-glucosidase-2 is developmentally controlled.

Effect of cycloheximide on the accumulation of β-glucosidase activity. Cycloheximide preferentially depresses the rate of protein synthesis in *D. discoideum* by at least 85% for at least 8 hr under our conditions (1, 17). When the drug was added at the beginning of development, the initial increase in specific activity of β-glucosidase occurred normally, but the second increase was absent (Fig. 4). When cycloheximide was added at either 14 or 20 hr after the initiation of development, subsequent accumulation of β-glucosidase activity was inhibited (Fig. 4).

Electrophoresis of extracts of cycloheximide-treated and control cells (Fig. 5) showed that cycloheximide quickly inhibits the accumulation of β-glucosidase-2. The results indicate that concomitant protein synthesis is required for the accumulation of β-glucosidase-2. On the other hand, cycloheximide had no apparent effect on the increase in specific activity of β-glucosidase-1.

Effect of actinomycin D on the accumulation of β-glucosidase activity. To determine the requirement for RNA synthesis for the accumulation of β-glucosidase-2, actinomycin D was used. Actinomycin D has been shown to preferentially inhibit RNA synthesis in slime molds by 95% within 30 min under our conditions (19, 21). Actinomycin was added at various times, and the specific activity was determined at intervals after the addition of the drug (Fig. 6).

Inhibition of accumulation of β-glucosidase activity late in development did not immediately follow addition of the drug; rather, the level of specific activity which accumulated in the absence of RNA synthesis was a function of the time of addition of actinomycin. The limitation of the late accumulation resulted from inhibition of the production of β-glucosidase-2, as can be demon-

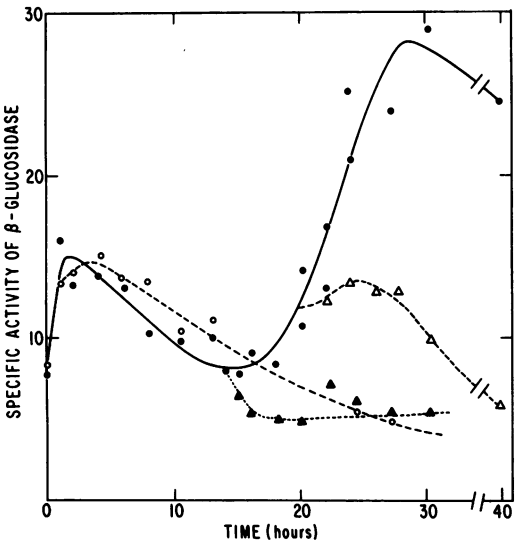

FIG. 4. *Effect of cycloheximide on the developmental kinetics of β-glucosidase activity. Developing cells on membrane filters (Millipore Corp.) were shifted to fresh support pads supplemented with cycloheximide at 0 (○), 14 (▲), or 20 hr (△) after the initiation of development. Control cells (●) were not transferred. Samples were taken at intervals, frozen, and subsequently assayed for the specific activity of β-glucosidase.*

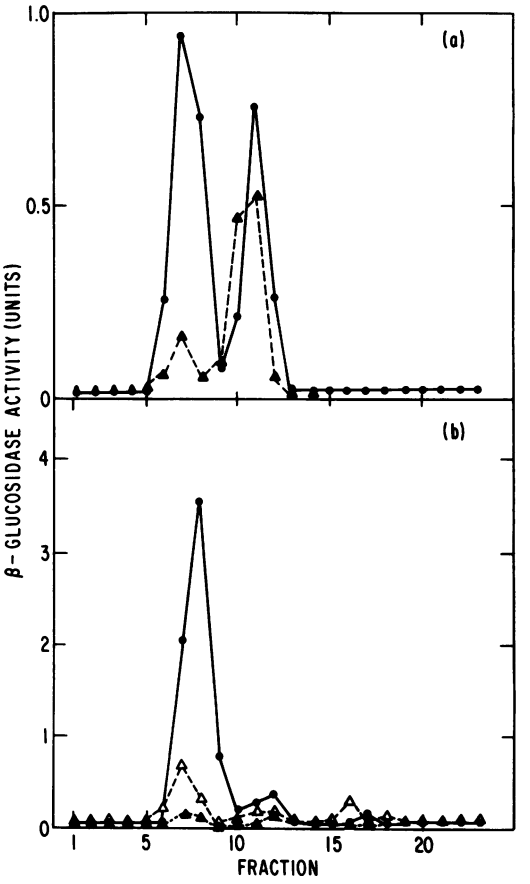

FIG. 5. *Inhibition of accumulation of β-glucosidase-2 by cycloheximide. β-Glucosidase activity was determined in gel fractions after electrophoresis of extracts made at (a) 19 hr and (b) 28 hr from control cells (●), cells that had received cycloheximide from 16 hr (▲), and cells that had received cycloheximide from 20 hr (△).*

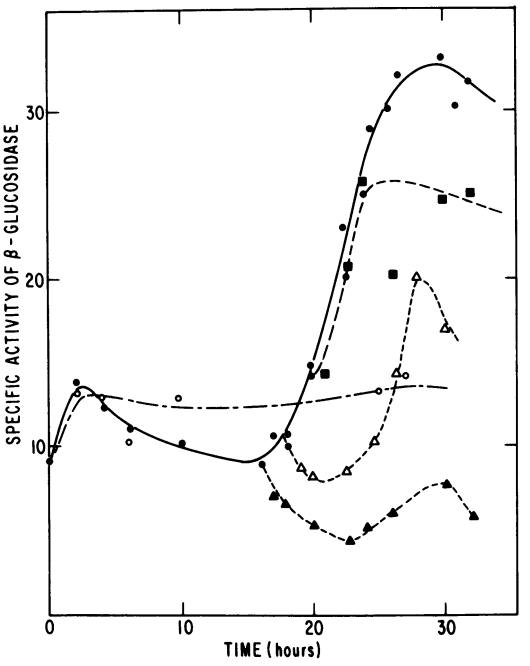

FIG. 6. *Effect of actinomycin D on the developmental kinetics of β-glucosidase activity. Developing cells on membrane filters (Millipore Corp.) were transferred to fresh support pads supplemented with actinomycin D at 0 (○), 16 (▲), 18 (△), or 20 hr (■) after the initiation of development. Control cells (●) were not transferred. Samples were taken at intervals, frozen, and subsequently assayed for β-glucosidase specific activity.*

strated by electrophoretic analysis of cell extracts (Fig. 7). Thus, RNA synthesis during the period of culmination seems to be required for the accumulation of β-glucosidase-2 activity.

The addition of actinomycin D immediately after the initiation of development was unable to prevent the increase in specific activity of β-glucosidase-1 (Fig. 6). It appears that the increase in specific activity of β-glucosidase-1 does not require RNA synthesis during development.

The results from numerous experiments on the effects of actinomycin on the accumulation of β-glucosidase-2 are summarized in Fig. 8. The development of the capacity to accumulate β-glucosidase-2 in the presence of actinomycin D preceded the accumulation itself by about 3 to 4 hr.

Effect of dissociation on β-glucosidase-2 accumulation. The relationship between morphogenesis and accumulation of β-glucosidase-2 during culmination was investigated by physically disrupting the pseudoplasmodia. It has been shown (6) that if the pseudoplasmodia are triturated extensively they can be dissociated to single cells and clumps of several cells. When these cells are redeposited on membrane filter (Millipore Corp.) supports, they re-aggregate within 4 hr and proceed rapidly through morphogenesis to form mature fruiting bodies. If, however, they are redeposited on membrane filters (Millipore Corp.) containing 10^{-2} M EDTA in the saturating buffer, the cells do not reaggregate and do not differentiate (6).

Pseudoplasmodia were collected 18 hr after the initiation of development, triturated, and redeposited on membrane filter (Millipore Corp.) supports saturated with the normal buffer or with normal buffer containing 10^{-2} M EDTA. The subsequent changes in the specific activity of β-glucosidase can be seen in Fig. 9.

When the cells were redeposited on normal buffer, mature fruiting bodies were formed within 9 hr. The specific activity of β-glucosidase first declined but then increased to a peak about 10 to 12 hr after dissociation. Electrophoretic analysis (Fig. 10) showed that more than 90% of the activity in these mature fruiting bodies migrated at the rate characteristic of β-glucosidase-2. When the cells were redeposited on buffer containing 10^{-2} M EDTA, the cells did not reform normal aggregates. The specific activity of β-glucosidase declined rapidly and remained at a low value. EDTA at 10^{-3} M had no inhibitory effect on the

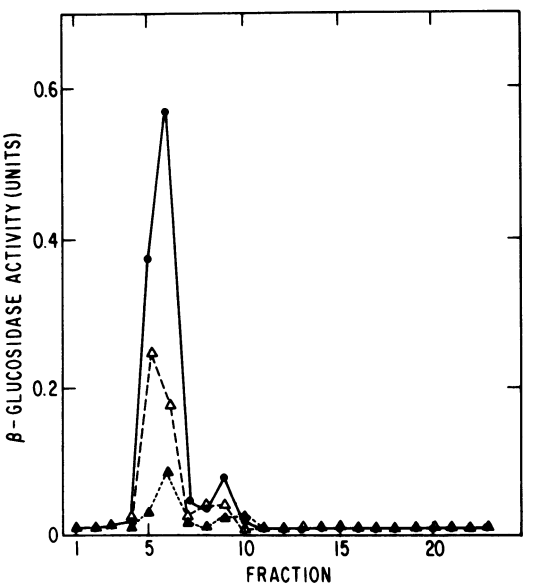

FIG. 7. *Inhibition of accumulation of β-glucosidase-2 by actinomycin D. β-Glucosidase activity in gel fractions was determined after electrophoresis of extracts made at 26 hr from control cells (●) and from cells that had received actinomycin D from 14 (▲) or from 18.5 hr (△).*

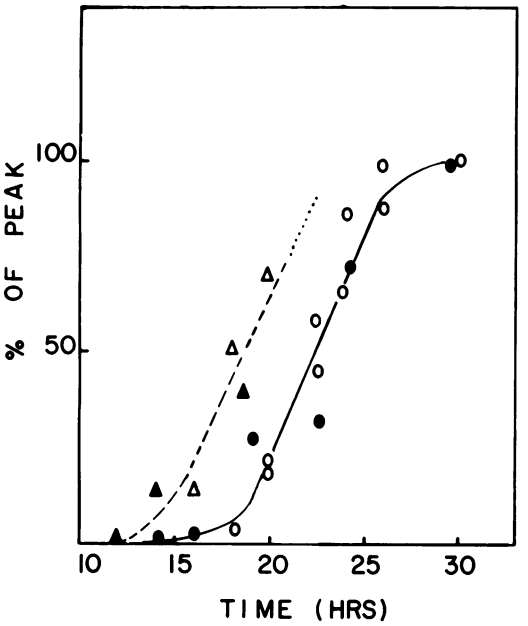

FIG. 8. *Capacity to accumulate β-glucosidase-2 in the presence of actinomycin D. The triangles indicate the maximum amount of β-glucosidase-2 ultimately accumulated after the addition of actinomycin D at the indicated times. β-Glucosidase-2 was estimated either as activity in the slower electrophoretic peak (▲) or as the net increase in β-glucosidase specific activity after 16 hr (△). These data are plotted as the percentage of the peak activity accumulated by control cells (no actinomycin D). The circles indicate the activity of β-glucosidase-2 expressed as the percentage of the peak activity achieved in control cells. Again, the isozyme was estimated either as activity in the slower electrophoretic peak (●) or as the net increase in specific activity after 16 hr of development (○).*

in vitro activity of β-glucosidase. It thus appears that specific multicellular organization is required for the accumulation of β-glucosidase-2.

Transferase activity of β-glucosidase enzymes. The data given in Table 1 indicate that the catalytic activities of both β-glucosidase-1 and β-glucosidase-2 are specific for β-glucosyl linkages and that glucose and *p*-nitrophenol are the main hydrolysis products when *p*-nitrophenyl-β-D-glucoside is used as substrate. However, only about 40% as much glucose as *p*-nitrophenol is produced initially with partially purified preparations of the isozymes.

To determine what other compounds are formed, the reaction mixture was chromatographed after the hydrolysis of *p*-nitrophenyl-β-D-glucoside was either 10 to 20% or 60 to 80% complete as judged by nitrophenol release. Intense spots corresponding to the expected compounds glucose, nitrophenol, and substrate were found, but two lighter spots were also found when the reaction was 60 to 80% complete. One of these lighter spots co-chromatographed with cellobiose, whereas the other had an R_F of 0.49 (Fig. 11). When the reaction was 10 to 20% complete, only the light spot at an R_F of 0.49 could be detected in addition to the intense spots; the light spot co-chromatographing with cellobiose was absent. In the solvent system used, *p*-nitrophenyl-β-lactose had an R_F of approximately 0.51. Thus, it appears that, in the early stages of the reaction, a *p*-nitrophenyl disaccharide is made by transfer of the hydrolyzed glucosyl moiety to another molecule of substrate. As the reaction progresses toward completion, there is an accumulation of glucose which can act as an acceptor and a disaccharide is formed. At all times during the reaction, water (present in much higher molar concentration) can act as an acceptor liberating free glucose. Both isozymes, β-glucosidase-1 and β-glucosidase-2, catalyzed these transferase reactions to essentially the same extent (Table 1).

Since transfer to an acceptor other than water occurred to such an extent (about 60% of the time initially, Table 1), one would expect the

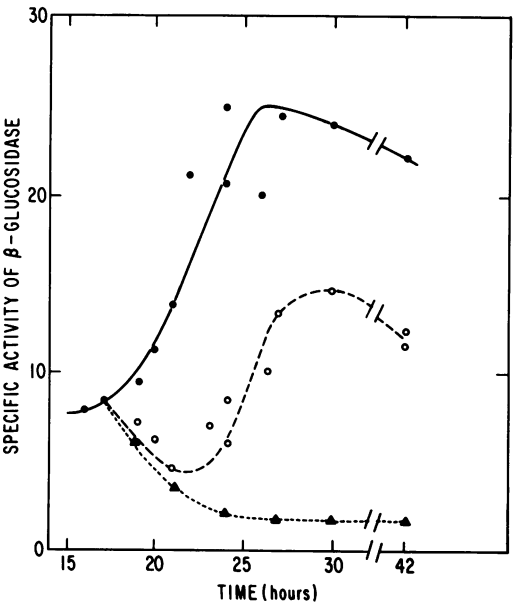

FIG. 9. *Effect of dissociation on the accumulation of β-glucosidase. At 18 hr after the initiation of development, cells were harvested from several membrane filters (Millipore Corp.), triturated, and redeposited at the same cell density on fresh membrane filters with (▲) or without (○) 10^{-2} M EDTA as a supplement to the buffer-salt solution in the supporting absorbant pad. Control cells (●) were undisturbed. At intervals thereafter, samples were collected for the determination of the specific activity of β-glucosidase.*

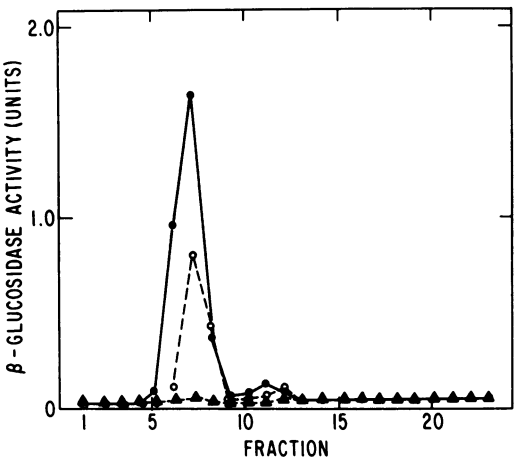

FIG. 10. *Electrophoretic analysis of β-glucosidase in dissociated cells. Pseudoplasmodia were dissociated 18 hr after the initiation of development and replaced on fresh membrane filters (Millipore Corp.). Twelve hours later, the cells were collected. Cell-free extracts were electrophoresed and gel fractions were assayed for β-glucosidase activity; extract of cells which re-aggregated on normal buffer (○); extract of cells which were redeposited on buffer containing 10^{-2} M EDTA (▲). An extract of undissociated cells collected at 30 hr was run as a marker (●).*

transferase products to accumulate. However, judging by the intensity of the pertinent spots on the chromatograms, this did not occur. It is probable that these products of the transfer reaction were hydrolyzed themselves by the β-glucosidases and thus must be β-glucosides.

DISCUSSION

The specific activity of β-glucosidase in *D. discoideum* increases during the early stages of morphogenesis, decreases during the pseudoplasmodial stages, and then increases a second time during culmination. Our results agree qualitatively with those reported by Rosness (12). Acrylamide gel electrophoretic analysis of cell-free extracts shows that these variations result from changes in the amounts of at least two electrophoretically distinguishable components having β-glucosidase activity. The component we have called β-glucosidase-1 moves rapidly toward the anode and is present early in development, decreasing to a minor fraction of the activity later. β-Glucosidase-2 moves more slowly toward the anode and is only found later in development, increasing from about 16 hr until after culmination is completed.

Experiments with cycloheximide and actinomycin D indicate that the increase in the specific activity of β-glucosidase during the first stages of morphogenesis is not dependent on RNA or protein synthesis. This increase in the specific activity of β-glucosidase-1 may result from preferential protection of the enzyme present in vegetative cells from the fairly extensive protein turnover which occurs during the first few hours after removal of the food source (25). Alternatively, the increase in specific activity may result from activation of the enzyme. In either case, synthesis of the enzyme apparently takes place during the growth phase and is independent of development.

Cycloheximide, an inhibitor of protein synthesis, prevents the accumulation of β-glucosidase-2 when added before accumulation begins and stops accumulation almost immediately when added during the period of increase in specific activity (Fig. 4 and 5). Actinomycin D added

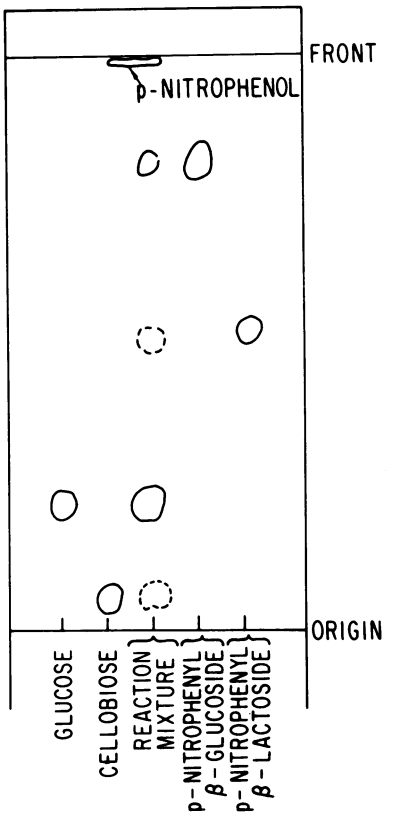

FIG. 11. *Diagram of a thin-layer chromatogram of the reaction products formed from p-nitrophenyl-β-D-glucoside. About 5 units of 400-fold-purified β-glucosidase-1 was incubated with substrate under standard assay conditions. When approximately 60% of the substrate had been hydrolyzed as judged by the release of nitrophenol, the reaction mix was applied to a cellulose thin-layer chromatogram along with authentic compounds. Chromatographic analysis was carried out as described.*

during development limits enzyme-accumulating ability (Fig. 6 and 7). The capacity to accumulate β-glucosidase-2 which can be expressed in the presence of actinomycin D precedes the accumulation itself by 3 to 4 hr (Fig. 8). Thus, concomitant protein synthesis and prior RNA synthesis seem to be required for the accumulation of β-glucosidase-2. A likely possibility is that β-glucosidase-2 is a new protein synthesized de novo between 16 and 28 hr on messenger RNA made between 12 and 24 hr. This hypothesis has not been tested more directly.

The accumulation of β-glucosidase-2 can be inhibited by disruption of the multicellular organization during culmination (Fig. 9 and 10). If the cells are allowed to reaggregate and form fruiting bodies, the accumulation begins again in about 4 hr, i.e., at about the same time that culmination begins again. If, on the other hand, reaggregation is inhibited by 10^{-2} EDTA, no more β-glucosidase-2 accumulates. EDTA does not inhibit the enzyme in vitro, nor does it appear to affect the overall rate of protein synthesis or accumulation of another developmentally controlled enzyme, uridine diphosphate-galactose polysaccharide transferase (9). Thus, it is possible that control of the accumulation of β-glucosidase-2 synthesis involves the development of multicellular organization.

The discovery that β-glucosidase from other organisms can transfer the β-D-glucosyl unit to acceptors other than water is credited to Rabaté (11), and transferase activity has since been shown in β-galactosidases (23, 24). Burstein et al. (3) showed that lactose does not itself induce the *lac* operon in *Escherichia coli*. Active β-galactosidase, however, is able to transfer the galactosyl part of lactose to any of a number of acceptor molecules, and some of these transfer products are inducers of the *lac* operon. Whether transfer products formed by β-glucosidase in *D. discoideum* have any regulatory significance is open to speculation.

Rosness (12) assumed that the function of β-glucosidase in slime mold is the hydrolysis of cellobiose and cellotriose. These oligosaccharides are apparently the products of the cellulase activity also studied by Rosness, and the combined action of the two activities hydrolyzes cellulose to glucose. Supposedly, the two activities are stored in spore cells, to be activated later, at germination, in order to open the spore coat and utilize the resulting saccharides as an energy source. Accumulation of β-glucosidase-2 in some form such that it becomes physiologically active only at germination could be accomplished by localization in some special cellular compartment, presumably distinct from the localization of β-glucosidase-1.

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