Acta Chemica Scandinavica B 33 (1979) 239—243

Enzyme Activities of the Mannitol Cycle and Some Connected Pathways in *Alternaria alternata*, with Comments on the Regulation of the Cycle

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The specific activities of the following enzymes have been measured: mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, mannitol dehydrogenase, hexokinase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, phosphofructokinase and aldolase. The maximal capacities of the enzymes were calculated and were compared with the metabolic fluxes in the mannitol cycle. No enzyme limitations for the fluxes in the cycle were found. It is proposed that the coenzyme availability governs the fluxes in the cycle.

No large differences in the specific enzyme activities between an alternariol producing strain and a nonproducing strain were found that could explain the production of the secondary metabolite.

The mannitol cycle was recently shown to be an important part of the metabolism of the fungus *Alternaria alternata*.[1] The following enzymes are involved in the cycle: mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, mannitol dehydrogenase and hexokinase.* The first enzyme catalyzes the reduction of fructose 6-phosphate to mannitol 1-phosphate with NADH as the coenzyme. Mannitol 1-phosphate is subsequently hydrolyzed to mannitol and inorganic phosphate. Mannitol is deposited in a large pool prior to oxidation to fructose with concomitant formation of NADPH from $NADP^+$. Fructose is then phosphorylated to fructose 6-phosphate. 10 – 100 % of the glucose metabolized by the fungus enters the mannitol cycle.

In order to obtain some information on the regulation of the cycle, the enzyme activities in the cycle and some connected pathways were studied. Two strains of *A. alternata*, differing in polyketide production and mannitol oxidation,[1] were investigated to detect differences in the enzymic compositions.

MATERIAL AND METHODS

Cultural conditions. The alternariol producing strain *Alternaria alternata* (CMI 89 343) was obtained from Commonwealth Mycological Institute (Kew, Surrey, England). The non-producing strain was a spontaneous mutant isolated from the producing strain. Conical flasks of 500 ml capacity containing 150 ml modified Czapek-Dox medium [2] were inoculated from agar slants and incubated on a rotary shaking table at 25 °C.

Preparations of cell free extracts. For preparation of cell free extracts the mycelia from three flasks were used from two days old cultures. For older cultures one flask was used. The mycelium was harvested by filtration, washed twice with deionized water and once with the buffer used for the homogenate preparation.

Buffer used for the preparation of homogenates was 20 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], 5 mM $MgCl_2$, 5 mM mercaptoethanol, 1 mM EDTA (ethylenediaminetetraacetate) and 0.1 mM phenylmethylsulfonyl fluoride. The pH value was adjusted to 7.5 with KOH. The cells

* *Enzymes.* Mannitol 1-phosphate dehydrogenase (EC 1.1.1.17); glucose 6-phosphate dehydrogenase (EC 1.1.1.49); mannitol dehydrogenase (EC 1.1.1.138); hexokinase (EC 2.7.1.1); phosphofructokinase (EC 2.7.1.11); mannitol 1-phosphatase (EC 3.1.3.22); aldolase (EC 4.1.2.13); glucose phosphate isomerase (EC 5.3.1.9).

0302-4369/79/040239-05$02.50
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were disintegrated by grinding in a mortar with sand in the double amount of buffer compared to cell wet weight. Polyvinylpolypyrrolidone 1 g was added during grinding.

The homogenate was centrifuged at 48 000 *g*, 4 °C, 20 min. The supernatant was desalted on Sephadex G-25 (Pharmacia PD-10 columns were used). The desalted supernatants were stored frozen and used for analysis of enzyme activities on the following day.

Assay of enzyme activities. All enzyme assays were performed at 25 °C in the following buffer: 20 mM HEPES, 5 mM $MgCl_2$, pH 7.5 (adjusted with KOH). Assays were checked for linearity with time and amount of enzyme added. Blanks without enzyme extracts added were run in all assays containing auxiliary enzymes.

Mannitol dehydrogenase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase and hexokinase were assayed by following the increase of fluorescence in a Turner Model 430 spectrofluorometer. Wavelengths used were 340 nm for excitation and 470 nm for emission. Total volume was 2 ml including 5 – 50 µl enzyme extract.

Mannitol dehydrogenase was assayed in a medium containing, in addition to the buffer, 0.1 mM $NADP^+$ and 50 mM mannitol. Controls were run without mannitol.

The assay medium for glucose 6-phosphate dehydrogenase consisted of glucose 6-phosphate and fructose 6-phosphate with a total concentration of 1.39 mM. The sugar phosphates were preequilibrated with glucose phosphate isomeras (0.05 U/ml assay mixture) to give a final concentration of 1 mM glucose 6-phosphate ($K_{eq} = 0.27$ [3]). The preequilibration was done because the high glucose phosphate isomerase activities in the cell extracts would otherwise have changed the concentration of glucose 6-phosphate in the incubation during analysis.[4] In addition to the sugar phosphates the assay mixture contained 0.1 mM $NADP^+$. Control lacked sugar phosphates.

Glucose phosphate isomerase was assayed in a medium containing 0.1 mM $NADP^+$, 1 mM fructose 6-phosphate and 0.07 U glucose 6-phosphate dehydrogenase/ml. Controls were run without fructose 6-phosphate.

The assay medium for hexokinase consisted of 0.1 mM $NADP^+$, 1 mM glucose, 1 mM ATP and 0.07 U glucose 6-phosphate dehydrogenase/ml. Controls were run without glucose.

Mannitol 1-phosphate dehydrogenase was assayed by following the change in A_{340} in a Perkin-Elmer 124 doublebeam spectrophotometer. Total volume was 2 ml including 50 – 100 µl enzyme extract. Fructose 6-phosphate and glucose 6-phosphate were equilibrated in the assay medium in the same manner as for the glucose 6-phosphate dehydrogenase assay. The total sugar phosphate concentration was 4.7 mM giving 1 mM fructose 6-phosphate. NADH was used with a concentration of 0.13 mM. Controls were run without sugar phosphates.

Mannitol 1-phosphatase was not assayed continuously but incubated in 0.5 ml buffer containing 2 mM mannitol 1-phosphate for 5 min before termination of the reaction with 1.5 ml phosphate reagent.[5] Separate controls were run without enzyme extract and mannitol 1-phosphate respectively. A_{820} was measured with sodium dihydrogen phosphate as standard.

For the assay of aldolase 10 µl enzyme extract was preincubated 15 min with 115 µl buffer supplemented with 224 mM hydrazine sulfate and 1 mM $ZnCl_2$. The reaction was started by addition of 15 µl fructose 1,6-diphosphate to a final concentration of 1 mM. After 15 min incubation the reaction was terminated by addition of 40 µl 20 % (w/v) trichloroacetic acid. Controls were without fructose 1,6-diphosphate.

Phosphofructokinase was assayed in 125 µl medium containing buffer and 224 mM hydrazine sulfate, 0.15 U aldolase, 0.15 U glucose phosphate isomerase, 1 mM fructose 6-phosphate (glucose 6-phosphate plus fructose 6-phosphate was 4.7 mM, see mannitol 1-phosphate dehydrogenase and glucose 6-phosphate dehydrogenase), 1 mM ATP and 25 µl enzyme extract making a total volume of 150 µl. 40 µl 20 % (w/v) trichloroacetic acid was added after 30 min incubation. Controls without fructose 6-phosphate were run concurrently.

The products formed during aldolase and phosphofructokinase assays were quantitated with a method described by Lowry.[6] Samples with aldolase and known concentrations of fructose 1,6-diphosphate, but without enzyme extracts, were run in parallel with the analysis and were used as standards. The acidified incubations were added 200 µl 0.75 M NaOH and were left at room temperature for 10 min. Dinitrophenylhydrazine, 200 µl 0.1 % (w/v) in 2 M HCl, was added and the mixtures were incubated for 10 min at 38 °C. After addition of 1800 µl 3.33 M NaOH – ethylene glycol monomethylether (1:2) and 10 min at room temperature the absorbance at 570 nm was measured.

Protein in the cell extracts was measured with fluorescamine.[7] Total cell protein was measured with the biuret method.[8]

All substrates and auxiliary enzymes were obtained from Sigma.

RESULTS AND DISCUSSION

Two strains of *Alternaria alternata* were used in this investigation; one strain is a producer of the polyketide alternariol but the other, which is a spontaneous mutant of the producing strain, is a nonproducer. Further known differences between the two strains are that the nonproducing strain synthesizes much more fat and oxidizes mannitol in a greater extent than the producer.[1]

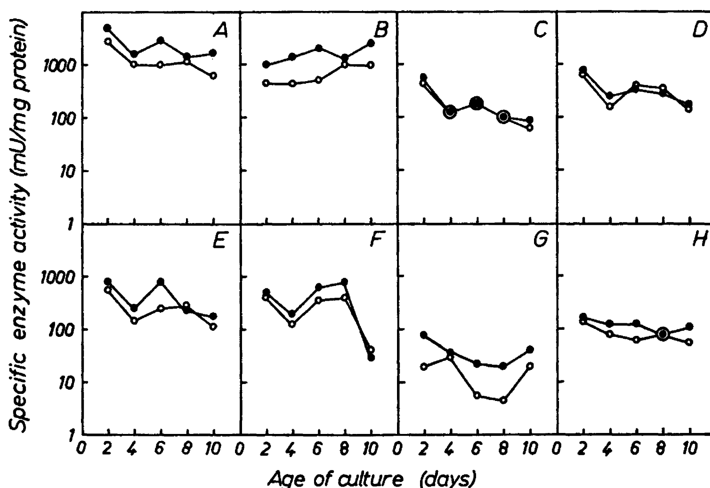

Fig. 1. Specific enzyme activities. (A) Glucose phosphate isomerase, (B) mannitol 1-phosphatase, (C) mannitol dehydrogenase, (D) glucose 6-phosphate dehydrogenase, (E) hexokinase, (F) mannitol 1-phosphate dehydrogenase, (G) phosphofructokinase and (H) aldolase. Specific activities were measured at 25 °C in 20 mM HEPES, 5 mM $MgCl_2$ buffer adjusted to pH 7.5 with KOH. (●) Nonproducing strain, (○) alternariol producing strain.

All enzyme activities were measured under conditions that gave the maximal activities of the enzymes at pH 7.5. The results are shown in Fig. 1 and it can be seen from the figure that there are no pronounced differences in specific enzyme activities between the non-producing and the producing strains. However, slightly lower activities are seen in the producing strain than in the nonproducing strain for the three enzymes glucose phosphate isomerase, mannitol 1-phosphatase and phosphofructokinase.

The specific activities of the enzymes fall into three different groups. Glucose phosphate isomerase and mannitol 1-phosphatase have both high specific activities, while mannitol dehydrogenase, mannitol 1-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase and hexokinase all have moderate specific activities and finally phosphofructokinase and aldolase have low specific activities.

The specific enzyme activities in *A. alternata* agree with the activities published for related fungi. Lee measured the specific activities in a mannitol accumulating *Aspergillus* species.[9] Aldolase was much more active and glucose 6-phosphate dehydrogenase more active in this *Aspergillus* species than in *A. alternata*. Mannitol dehydrogenase was not detected in the mannitol accumulating *Aspergillus* although it was demonstrated in *Aspergillus candidus*.[10] Mannitol-1-phosphatase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase and phosphofructokinase had all roughly the same activities while aldolase was more active in *Aspergillus nidulans* than in *A. alternata*.[11,12] Boonsaeng *et al.* reported on mannitol 1-phosphate dehydrogenase and phosphofructokinase activities, among others in a number of *Aspergillus* and *Penicillium* species,[4,13] and the activities in *A. alternata* agree with these data.

A. alternata differs from the other fungi mentioned, except for *Aspergillus candidus*, only by having an active mannitol dehydrogenase. This enzyme is very active in crude extracts from *A. alternata* without addition of mannitol to the assay medium, which is explained by the fact that the crude extracts contain large amounts of mannitol derived from the cells (10 – 15 % of the dry weight is mannitol [1]), and its efficient removal is needed to detect the mannitol dehydrogenase activity.

The metabolic fluxes in *A. alternata* are known for the four enzymes hexokinase, mannitol dehydrogenase, mannitol 1-phosphate dehydrogenase and mannitol 1-phosphatase.[1]

Table 1. Protein contents in *A. alternata*. Total cell protein was measured, after boiling the mycelium 5 min in 1 M NaOH, with the biuret method.[8] Values represent the mean of three determinations of each two cultures per strain, followed by the standard error of the mean.

Age, days	Nonproducing strain [a]	Producing strain [a]
4	0.41±0.02	0.31±0.03
6	0.41±0.02	0.37±0.05
8	0.31±0.03	0.39±0.04

[a] (mg protein)/(mg cell dry wt).

The fluxes can be compared with the maximal enzyme capacity if the fluxes are calculated on protein basis instead of mg cell dry weight. The amount of mg protein per mg cell dry weight are given in Table 1. Fluxes calculated

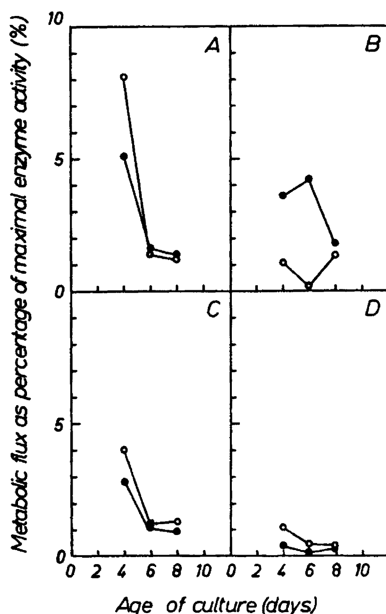

Fig. 2. Metabolic fluxes as percentage of maximal enzyme activities. (A) Hexokinase, (B) mannitol dehydrogenase, (C) mannitol 1-phosphate dehydrogenase and (D) mannitol 1-phosphatase. The metabolic fluxes were taken from Ref. 1 and were calculated on protein basis with the figures taken from Table 1. The maximal enzyme activities were taken from Fig. 1. (●) Nonproducing strain, (○) alternariol producing strain.

as percentages of maximal enzyme capacity are given in Fig. 2. The figure shows that hexokinase and mannitol 1-phosphate dehydrogenase are occupied to the same extent in the nonproducing and the producing strains to maintain the measured fluxes. On the other hand mannitol dehydrogenase and mannitol 1-phosphatase are used to significantly different extents in the two strains. The observed lower rate of mannitol oxidation in the producing strain[1] is evidently not caused by a limiting activity of mannitol dehydrogenase but must be induced by other factors.

All enzymes in the mannitol cycle are used to a low extent and hence none of the enzymes seems to be rate limiting. For the mannitol 1-phosphatase step, which is used to less than 1 % of its maximal capacity, the availability of mannitol 1-phosphate should be the rate limiting factor. It is likely that mannitol 1-phosphate is in equilibrium with fructose 6-phosphate and glucose 6-phosphate in the cells by the action of the enzymes mannitol 1-phosphate dehydrogenase and glucose phosphate isomerase, which are present with high activities. The concentration of mannitol 1-phosphate will consequently fluctuate with the $NADH/NAD^+$ ratio and with the concentrations of fructose 6-phosphate and glucose 6-phosphate in the cells. The levels of these sugar phosphates are rather constant in *A. alternata*[14] and hence the rate of mannitol synthesis should be governed by NADH availability and the synthesis can be looked upon as a sink for NADH during growth.

The situation for mannitol dehydrogenase is very similar to the situation for mannitol 1-phosphatase as mannitol concentration and enzyme capacity are both abundant. The rate of mannitol oxidation would then be limited by the $NADP^+$ availability. It is suggested that the fluxes in the mannitol cycle are dependent on the redox states of the two nicotinamide adenine nucleotide systems of the cells and that the involved reactions are of importance for maintaining the right redox states of these coenzyme pools.

Acknowledgement. This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Council for Forestry and Agricultural Research.

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Received December 21, 1978.