Alterations in Trehalase Solubility during Development in the Cellular Slime Mould *Dictyostelium discoideum*

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Previous studies have indicated that during development in the slime mould *Dictyostelium discoideum*, compartmentation of the isoenzymes of trehalase (α,α' -trehalose 1-D-glucosylhydrolase, (EC 3.2.1.28) occurs between the extracellular and intracellular environments. The compartmentation of trehalase between soluble and particulate cell fractions was examined in this work. The trehalase present in crude homogenates prepared during the first 12 h of development was completely soluble. Starting at about the pseudoplasmodial stage (i.e. the 14th hour of development), trehalase activity became associated with insoluble cellular material and this increased to a maximal value in homogenates from mature sorocarps, where 50% of the activity was insoluble. Spore cells accounted for only 2 to 3% of the trehalase associated with mature sorocarps, with the remaining 97% being localized in stalk cell material. Although trehalase recovered from spores was completely soluble, more than half of that from the stalk was recovered in the buffer-insoluble pellet fraction.

INTRODUCTION

The life cycle of the cellular slime mould *Dictyostelium discoideum* consists of growth and multicellular differentiation. In the absence of exogenous nutrients, development is initiated and the myxamoebae stream together to form aggregates, each of which is subsequently transformed over a 24 h period (at 22 °C) into a mature sorocarp, consisting of a spore mass supported by a cellulose-ensheathed stalk. During development, there is an accumulation of structural polysaccharides and the nonreducing disaccharide, α,α' -trehalose. The latter sugar serves as the major energy source during spore germination at which time its hydrolysis to glucose is catalysed by the enzyme trehalase (α,α' -trehalose 1-D-glucosylhydrolase, EC 3.2.1.28). Prior to germination, trehalase catabolism is absent in spore cells due to the *in vivo* latency of trehalase. In contrast to spores however, trehalase turnover is extensive in stalk cells (Jefferson & Rutherford, 1976).

Recently, evidence has been presented which indicates that extracts from mature fruits of *Dictyostelium* contain electrophoretically distinct multiple trehalase activities and that these isoenzymes are under developmental regulation (Killick, 1981*a, b*; 1983). Thus, although a single intracellular trehalase activity (isoenzyme I) was detectable prior to 14 h of development, during culmination a second isoenzyme (isoenzyme II) accumulated. Additional evidence has also suggested that selective channelling (i.e. compartmentation) of the multiple trehalase activities occurs between the extracellular and intracellular environments with which the enzyme is associated. To explore further the role that compartmentation may play in the regulation *in vivo* of trehalase activity, additional studies were undertaken to determine whether alterations in trehalase solubility occur *in vitro* such that a differential partitioning of enzyme activity between soluble and particulate cell fractions occurs as a function of developmental stage. The results of these investigations form the basis of the present report.

METHODS

Materials. Trehalose dihydrate, maleic acid, MES, glucose-6-phosphate dehydrogenase and ATP were purchased from Sigma; Tris (enzyme grade) was from Schwartz/Mann; Statzyme reagent was from Worthington; Percoll was from Pharmacia; NADP⁺ and yeast hexokinase were purchased from Boehringer-Mannheim. All other chemicals were of the highest analytical grade available.

Organism and culture conditions. *D. discoideum* strain NC-4 (ATCC 24697) was grown on nutrient agar with *Escherichia coli* as the bacterial associate (Liddel & Wright, 1961).

Initiation of differentiation. Upon depletion of the bacterial food source, the stationary phase myxamoebae were harvested from the nutrient agar surface and washed free of residual bacteria by repeated centrifugation in the presence of cold distilled water and the washed cells were spread on 2% non-nutrient agar and incubated at 22 °C until the desired stage of morphogenesis was reached.

Preparation of spores and stalks. Spores were isolated from 3-d-old mature sorocarps and purified with Percoll density gradient centrifugation as previously described (Killick, 1981*b*). Stalks were isolated from washed fruiting bodies (Killick, 1981*b*) which were homogenized into suspension and briefly vortexed to dislodge spores from the stalks. The cell suspension was then filtered under gravity through several sections of cheesecloth or nylon mesh (Killick, 1981*b*) and the retentate of stalks resuspended in 50 mM-MES/NaOH (pH 6.5), frozen in dry ice/acetone and stored at -20 °C.

Preparation of spore and stalk extracts. Harvested spore and stalk preparations were frozen in a dry ice/acetone bath and thawed at 23 °C. Crude homogenates were passed through a French Pressure cell (18000 lbf in⁻²; 1 lbf in⁻² $\approx$ 6.9 kPa) and the resulting extracts centrifuged at 33000 *g* (30 min) to obtain particulate and soluble cell-free fractions.

Preparation of cell-free extracts from developing cells. Cells were harvested at specified periods with 50 mM-MES/NaOH (pH 6.5) buffer, pelleted by low speed centrifugation (4 °C), washed twice and resuspended in the same buffer. After shell-freezing in dry ice/acetone, the pellets were stored at -12 °C. To effect cell breakage, the frozen pellets were thawed at 23 °C. The suspension was centrifuged at 33000 *g* (15 min) and the supernatant and pellet served as the sources of trehalase activity.

Assay of trehalase activity. Trehalase activity was assayed at 35 °C in an incubation mixture that contained 25 mM-trehalose and either 50 mM-potassium citrate buffer (pH 5.5) (discontinuous colorimetric and coupled spectrophotometric assays) or 50 mM-MOPS/NaOH buffer (pH 7.0) (coupled continuous spectrophotometric assay; Killick, 1979*b*). The initial rates of glucose production were measured either continuously over a 10 min interval, or discontinuously at 5 to 10 min intervals over a period of 20 to 60 min. One unit of enzyme activity is defined as that amount of enzyme that catalyses the synthesis of 1 μ mol product per 30 min at 35 °C. The specific activity is expressed as enzyme units per mg acid-precipitable protein.

Measurement of glucose. Glucose was assayed according to methods previously described (Killick, 1979*a*).

Trehalose-6-phosphate synthase assay. Activity was assayed at 23 °C in an incubation mixture that contained 25 mM-glucose 6-phosphate, 10 mM-UDP-glucose, 400 mM-KCl, 62.2 mM-MgCl₂, 1 mM-EDTA and 50 mM-MES/NaOH buffer (pH 6.5) according to previously described methods (Killick, 1979*a*). For this assay, cell-free extracts were prepared in 50 mM-MOPS/NaOH, 10 mM-potassium phosphate, 25 mM-trehalose and 5 mM-2-mercaptoethanol (pH 7.5).

Measurement of protein. This was done by the Lowry method.

RESULTS

Equivalence of trehalase assays

Prior to investigating compartmentation of trehalase activity, the values for enzyme activity obtained with a continuous coupled spectrophotometric assay were compared with those from discontinuous procedures that use either the Worthington Glucostat or Statzyme reagents. In both procedures, controls were included to account for product formation that occurred in the absence of exogenous trehalose. Both methods gave equivalent values for trehalase activity with soluble trehalase preparations (the 33000 *g* supernatant fraction).

Effect of method of cell rupture on the recovery of soluble trehalase activity

As shown in Table 1, maximal release of soluble enzyme activity from vegetative slime mould cells occurred after a single freeze-thaw cycle; rupture of mature sorocarps by this method solubilized half as much trehalase activity as did Yeda or French pressure cell treatment. When the sorocarp homogenate obtained by freeze-thaw treatment was subjected to a single passage through a Yeda pressure cell (2300 lbf in⁻²) or to two consecutive passages through a French

Table 1. *Effect of rupture method on the recovery of trehalase activity from vegetative cells and mature sorocarps*

Rupture method	Vegetative cells*		Sorocarps	
	Total act.†	Sp. act.‡	Total act.†	Sp. act.‡
Freeze-thaw	24	0.40	27	0.59
+ Yeda press	25	0.33	49	1.20
+ French press	23	0.26	42	0.38

* Vegetative cells were harvested during the stationary phase of growth and starved for 2 h on non-nutrient agar.

† Total units of enzyme activity recovered in the supernatant liquid following centrifugation (33000 g; 15 min) of the crude homogenate. One unit of activity is that amount of enzyme which catalyses the synthesis of 1 μ mol product in 30 min at 35 °C.

‡ Specific activity is expressed as enzyme units per mg acid-precipitable protein.

Table 2. *Distribution of trehalose-6-phosphate synthase and trehalase activities in sorocarp homogenates*

Fraction	Activity (% of total)	
	Trehalose-6-phosphate synthase	Trehalase
Crude homogenate*	100	100
33000g supernate	96	39
Pellet	2†	51‡

* Homogenates were prepared by subjecting the cells to a single freeze-thaw and French pressure cell treatment as described in Methods.

† The pellet was washed and resuspended in 50 mM-MOPS/NaOH (pH 7.5) buffer containing 10 mM-phosphate, 1 mM-EDTA, 5 mM-2-mercaptoethanol and 25 mM-trehalose.

‡ The pellet was washed and resuspended in 50 mM-MES/NaOH (pH 6.5) buffer.

pressure cell (7500 lbf in⁻²), the amount of recoverable soluble trehalase activity increased about 1.5- to 2-fold. Although the two pressure cell treatments released the same amount of enzyme activity, the specific activity of the enzyme prepared by Yeda pressure cell treatment was 2 to 3 times higher than that obtained for enzyme prepared with the French pressure cell.

Comparison of the distributions of trehalose-6-phosphate synthase and trehalase activities in sorocarp homogenates (Table 2) prepared by a freeze-thaw/French press treatment indicated that 96% and 39% of the synthase and trehalase activities, respectively, were solubilized by this procedure. About 57% of the trehalase recoverable from crude sorocarp homogenates was insoluble. When the latter fraction was analysed by differential centrifugation, about 50% of the trehalase activity sedimented at 1085 g (1 min), 27% by centrifugation of the previous supernate at 4340 g (20 min), 7.5% by centrifugation of the supernate from the previous step at 20000 g (20 min) and the remainder of trehalase activity was recovered in the 33000 g (60 min) pellet and supernate.

Compartmentation of trehalase during development

The trehalase profile was determined during differentiation in *D. discoideum* by measuring both soluble and pellet-associated enzyme activities. During the first few hours of development, the specific activity began to fall linearly, with a minimum value at the pseudoplasmodial/pre-culmination stage, corresponding to a net reduction of 7- to 14-fold. During culmination, the enzyme specific activity increased 10- to 15-fold reaching its maximal value at the mature sorocarp stage. This value then remained fairly constant, when the cells were incubated for an additional 2 to 3 d on non-nutrient agar.

Because previous studies had shown that about 50% of the trehalase activity in homogenates from 3-d-old sorocarps was associated with insoluble material (the 33000 g pellet fraction) the profile for pellet-associated trehalase activity was determined during slime mould development.

Table 3. *Partitioning of trehalase activity during development and subsequent cellular ageing in Dictyostelium*

Stage of development	Trehalase activity [(pellet/homogenate*) × 100]
2.5 h Amoeba	0
5.0 h Amoeba	0
8.0 h Amoeba	0
10.0 h Aggregate	0
12.5 h Late aggregate/early pseudoplasmodium	11.2
15.0 h Late pseudoplasmodium	17
18.0 h Preculmination	27.7
20.0 h Culmination	34.4
23.0 h Young sorocarp	45
40.0 h Mature sorocarp	50.4
72.0 h Mature sorocarp	50.4

* Homogenates were prepared by subjecting the cells to a single freeze-thaw cycle followed by passage of the extract through a Yeda pressure cell. The crude homogenate was centrifuged (33 000 *g*; 15 min), the supernate was decanted and the pellet was washed (twice) and re-isolated by the same centrifugation techniques.

Table 4. *Inter- and intracellular compartmentation of trehalase in sorocarp homogenates*

Cell type	Relative trehalase content*	Intracellular distribution of trehalase activity (% of total recovered)†	
		Soluble	Insoluble
Sorocarp	1.0	39	61
Spore	0.02	100	0
Stalk	0.98	31	69

* Trehalase content relative to that of the mature sorocarp expressed as units per cell sample.

† Fractions were prepared as described in Table 2 and were assayed for trehalase activity as described in Methods.

As shown in Table 3, starting at about the pseudoplasmodial stage, pellet-associated trehalase activity was detectable. Between the early pseudoplasmodial and mature sorocarp (i.e. 40 h) stages, the amount of insoluble enzyme activity increased linearly with time at least 50-fold.

Trehalase activity from spore and stalk tissue

Dormant spores were purified from 3-d mature sorocarps according to methods previously described (Killick, 1981*a*), and crude homogenates prepared by French pressure cell treatment were fractionated into soluble and particulate fractions by centrifugation at 33 000 *g* for 15 min, as described in Methods.

Spore cell trehalase was completely soluble, with no trehalase activity detectable in the insoluble pellet fraction. The specific activity of spore cell trehalase was maximally 0.03 to 0.05 units mg^{-1} . Comparison of the activity of spore cell trehalase with that from the mature sorocarp indicated that the spore enzyme comprised about 2 to 3% of the total activity of the whole sorocarp.

Comparable analysis of the enzyme prepared by French pressure cell treatment of isolated stalk tissue indicated that at least 55 to 63% of recovered trehalase activity was pellet-associated (i.e. insoluble). The content of stalk cell trehalase per cell sample was about 40 times greater than that associated with spore cells (Table 4) and it comprised about 97% of the total activity associated with the whole sorocarp.

DISCUSSION

Trehalase in crude homogenates of *D. discoideum* prepared during the first 12 h of development was completely soluble, as is the enzyme from *Saccharomyces cerevisiae* (Kelly & Catley, 1976; Panek, 1969; Souza & Panek, 1968), *Trichoderma reesei* (Vijayakumar *et al.*, 1978) and *Pullularia pullulans* (Meridinger *et al.*, 1971). In contrast, enzyme preparations from culminating *D. discoideum* exist as both soluble and insoluble forms, similar to the trehalases from the water mould, *Lagenidium* sp. (McInnis & Domnas, 1973) and the harvester termite, *Trinervitermes trinervoides* (Retief & Hewitt, 1973). On the other hand, most of the trehalase activity in crude homogenates from the honey bee (Lefebvre & Huber, 1970), rat intestine (Sacktor, 1968) and rabbit kidney (Sasajima *et al.*, 1975) is particulate, with little enzyme activity being soluble.

By using ultramicrochemical techniques and enzymic cycling, Jefferson & Rutherford (1976) demonstrated that the increase in trehalase activity occurring between the 14th and 24th hours of development in cell-free extracts from the whole *Dictyostelium* organism was due to a 30-fold increase in enzyme activity in the stalks. Spore trehalase activity was either very low or non-detectable. This conclusion is supported by the present work, which also shows that the insoluble form of the enzyme is restricted to the stalk cells.

Coincident with alterations in trehalase solubility is the appearance of multiple forms of the enzyme during terminal differentiation in *D. discoideum* (Killick, 1983). A single major enzyme activity, isoenzyme I, is detectable prior to the 12th hour of development, but during culmination a second electrophoretically distinct form of trehalase (isoenzyme II) accumulates, and at least 70% of the soluble trehalase activity prepared during culmination is this isoenzyme (Killick, 1981a).

The intra- and intercellular distribution of a particular enzyme may reflect its role in metabolism. Thus, the association of high trehalase activity with stalks, coupled with the fact that the rate of trehalose turnover in this tissue is maximal over the morphogenetic period when stalk cellulose accumulation occurs at its fastest rate, may suggest a role for trehalose metabolism in both cellulose accumulation and stalk cell differentiation.

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