

Procedure for preparing permeabilized hyphae for enzyme assays.

Permeabilization procedures have been developed for measuring many enzyme activities in dormant and germinating conidia (Basabe et al., 1979, *Anal. Biochem* 92: 356; Christensen and Schmit, 1980, *J. Bacteriol.*, 144: 983). We have modified this procedure in order to measure enzyme activities in permeabilized hyphae. The protocol used to prepare permeabilized hyphae for glutamic acid decarboxylase (GAD) assays is given below.

Vegetative hyphae and aerial hyphae were obtained from standing cultures (Table I). The vegetative hyphae formed a pad on the surface of the liquid medium and, after a few days, aerial hyphae grew from the mycelial pad. The aerial hyphae and then the mycelial pad were removed from the test tube with a sterile spatula. Immediately the material was agitated on a vortex mixer for 2 min. with 5.5 ml of the permeabilization solution. The permeabilization solution contained 0.5 ml of toluene:methanol (1:4 v/v) and 5 ml of 20 mM potassium phos-

TABLE I.
GAD^a activities in conidiating cultures^b

Days ^c	Specific Activity nmoles CO ₂ min ⁻¹ (mg lyophilized weight) ⁻¹	
	Vegetative hyphae	Aerial hyphae
2	0.323 ± 0.005 ^d	
4	0.885 ± 0.007	0.735 ± 0.02

^aGAD was assayed by measuring the amount of ¹⁴CO₂ released from DL[1-¹⁴C] glutamic acid (Christensen and Schmit, 1980 J. Bacteriol. 144: 983).

^bStanding conidiating cultures of nada (FGSC #2688) were grown in test tubes (18 x 150) on 5 ml of liquid complete medium containing 1% casamino acids, 0.5% yeast extract, 2% sucrose, 1% glycerol and Vogel salts (Vogel 1964, Am Nat. 98: 435) at 25°C.

^cDays after inoculation with 5 x 10⁵ conidia/tube.

^dThe assays were done in duplicate.

phate (pH 6.8), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzimidazole hydrochloride (BHCl). The permeabilized cells were collected by centrifugation, transferred to preweighed disposable micro test tubes (1.5 ml) and frozen with liquid nitrogen. The frozen cells were lyophilized in a Speed-vac Concentrator (SAVANT). The lyophilized cells were weighed and ground to a fine powder. Small samples, 5-15 mg of cells, were ground on a cold spot plate with a glass rod whereas larger samples were ground with a cold mortar and pestle. The ground cells were suspended in a buffer (20 mM potassium phosphate (pH 6.8), 10 mM EDTA, 1 mM PMSF, 1 mM BHCl) to give a final concentration of 20 mg/ml of lyophilized mycelia.

Aliquots of the crude cell suspension were transferred directly to the GAD assay mixture with an automatic pipetter. The pipet tips were cut off to ensure that a uniform sample was transferred. The specific activities of GAD in vegetative hyphae and aerial hyphae were determined (Table I). Each assay was done in duplicate and the deviation between duplicates was less than 3%. This experiment has been repeated several times and the specific activities of duplicate assay; always varied less than 10%

We have found that this procedure for measuring enzyme activities in hyphae has the following advantages, as compared to preparing cell free extracts.

1. The permeabilization of the hyphae assures that all the enzyme activity, intraorganellar as well as cytosolic, is accessible for measurement.
 2. Small molecules are removed rapidly during permeabilization; thus, dialysis is not necessary.
 3. The cell interior can be rapidly exposed to EDTA and protease inhibitors minimizing enzyme degradation.
 4. Lyophilized cells can be weighed to calculate specific activities.
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