Separation of Glutamate Dehydrogenases of *Coprinus cinereus* on Polyacrylamide Gels

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INTRODUCTION

It is now well established that two forms of glutamate dehydrogenase (GDH) exist in fungi (Holzer & Schneider, 1957; Sanwal, 1961; Sanwal & Lata, 1961; Kato *et al.*, 1962; Dennen & Niederpruem, 1965; Fawole & Casselton, 1972; Hynes, 1974) and in bacteria (LeJohn & McCrea, 1968; Kramer, 1970; Brown, Macdonald-Brown & Meers, 1974). Studies on fungal GDH have recently been reviewed by Brown *et al.* (1974). In some of these, two distinct enzyme proteins were separated. One form is linked to nicotinamide adenine dinucleotide (NAD-GDH; EC. 1.4.1.2) and the other to nicotinamide adenine dinucleotide phosphate (NADP-GDH; EC. 1.4.1.4).

The present study was designed to separate the two glutamate dehydrogenases of *Coprinus cinereus* using polyacrylamide gel electrophoresis.

METHODS

Organism and growth of cultures. The wild-type monokaryon, H9, of *Coprinus cinereus* (previously called *C. lagopus*) was grown as described by Fawole & Casselton (1972), using ammonium medium with 1 % (w/v) sucrose instead of 2 % (w/v) glucose, and vigorously aerating the cultures in the dark at 25 °C. Although glucose is better than sucrose as a carbon source for *Coprinus* (e.g. Moore, 1969), it strongly repressed NAD-GDH activity in the fungus (Fawole & Casselton, 1972; Stewart & Moore, 1974), and so sucrose was used to minimize this effect.

Preparation of cell-free extracts. Mycelial pellets were collected by filtration through four layers of muslin and washed with water. In a preliminary experiment it was found that there was a substantial loss of GDH activity in mycelia stored at -20 °C even for one week. Therefore, fresh mycelia were used in subsequent experiments. Mycelium was homogenized at full speed for two 90 s periods using a Virtis S45 homogenizer (Techmation, Edgware, Middlesex HA8 8JP) cooled in ice. The extractant was as used by Fawole & Casselton (1972). The crude extract was centrifuged at 30000 g for 20 min at 2 °C. The clear supernatant was dialysed for about 16 h at 2 °C against three changes of the extractant: before use, the dialysis tubing had been boiled for 20 min in 10 % (w/v) sodium carbonate and then rinsed in tap water followed by distilled water (Smith, 1968).

Enzyme assays. GDH was assayed at room temperature by measuring the oxidation of either NADH or NADPH at 340 nm in a Gilford 2000 automatic recording spectrophotometer using the reaction mixtures of Fawole & Casselton (1972). Proteins were precipitated with 10 % (w/v) trichloroacetic acid (TCA), washed once with 5 % TCA and then twice

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with ethanol. The precipitate was dissolved in 0.1 M-NaOH and the protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Specific activities of GDH were expressed as nmol NADH or NADPH oxidized min^{-1} (mg protein) $^{-1}$.

Polyacrylamide gel electrophoresis. This was carried out by the method of Davis (1964) with the following modifications. Experiments were done at room temperature, but the electrode solution container was immersed in a cold bath at 0 to 4 °C. The concentration of the main gel was reduced to 5 % acrylamide plus 0.17 % methylene bisacrylamide. The sample (200 μg in 0.2 ml) was mixed with 40 % (w/v) sucrose instead of using a sample gel, and then layered on the spacer gel. A constant current of 2 mA per gel was applied for the first 15 min and then increased to 5 mA per gel for 2 h, during which time the reference bromophenol blue dye migrated to about 6 mm from the end of the gel. Gels were 75 mm long.

The gels were either stained for protein in 1 % (w/v) Coomassie brilliant blue in 7 % (v/v) acetic acid, or stained for GDH activity. In the latter case, gels were first extruded into cold 0.1 M-Tris/HCl buffer, pH 9.5 for 30 min, and then stained in a freshly prepared incubation mixture for 15 min at 35 °C. The incubation mixture was modified from that of Laycock, Thurman & Boulter (1965) and contained: 0.1 M-Tris/HCl buffer, pH 9.5; sodium glutamate, 50 mg; NAD^+ or NADP^+ , 10 mg; *N*-methylphenazonium methosulphate, 1 mg; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg; in a total volume of 10 ml.

Partial purification of enzymes. All operations for the partial purification of GDH were carried out at 0 to 4 °C (Table 1). Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the crude enzyme extract to 35 % saturation. The precipitate which formed after 50 min stirring was removed by centrifuging and more solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 55 % saturation. The resulting 35 to 55 % $(\text{NH}_4)_2\text{SO}_4$ protein fraction, after centrifuging, was redissolved in a minimum volume of extractant. It was then dialysed for 16 h against three changes of the extractant and treated with alumina Cy gel at 20 mg gel (mg protein) $^{-1}$ (Yamasaki & Suzuki, 1969). The suspension was stirred for 15 min, and the gel was recovered by centrifuging at 10000 *g* for 10 min, and washed once with 0.05 M-phosphate buffer, pH 7.65, plus 1 mM- β -mercaptoethanol. The enzyme was eluted from the gel by stirring for 1 h with 5 ml 0.1 M-phosphate buffer, pH 7.65, containing 1 mM- β -mercaptoethanol. After centrifuging the suspension at 20000 *g* for 15 min, the supernatant was separated and the pellet was again stirred for two 1 h periods each with 5 ml 0.2 M-phosphate buffer, pH 7.65, containing 1 mM- β -mercaptoethanol, followed by centrifuging as before. The three eluates were pooled and then dialysed as above. Attempts to concentrate the enzyme using polyethylene glycol resulted in reduced enzyme activities, a result similar to that reported by Dennen & Niederpruem (1967) using *Schizophyllum commune*.

RESULTS AND DISCUSSION

Most of the NAD-GDH and NADP-GDH activities of *C. cinereus* were found in the same 35 to 55 % ammonium sulphate fraction (Table 1). Both enzyme activities showed a single band after electrophoresis, the NADP-GDH band being more distinct than the NAD-GDH band. The difference in the intensities of the bands could be due to an instability of NAD-GDH under the conditions of electrophoresis, as has been observed in *Neurospora crassa* (Kapoor & Grover, 1970). A faint band developed below the NAD-GDH band long after the normal incubation period and was greyish, in contrast to the normal purple GDH bands. When glutamate, NAD or NADP was omitted from the

Table 1. Summary of the procedure for the partial purification of GDH from *C. cinereus*

Procedure	Total protein (mg)	Specific activity		Recovery of activity (%)	
		NAD-GDH	NADP-GDH	NAD-GDH	NADP-GDH
Crude undialysed extract	250	0.425	0.224	100	100
Crude dialysed extract	216	0.496	0.247	101	95.2
(NH ₄) ₂ SO ₄ 35 to 55 % saturation	20	2.877	0.502	54.2	17.9
Pooled alumina Cy eluates	4.4	4.286	0.539	17.8	4.2

incubation mixture, no band appeared on the gel. The two stained enzyme bands coincided with two bands observed when gels were stained with Coomassie brilliant blue. The electrophoretic study of the partially purified enzymes confirms the existence of two distinct enzyme proteins. An indication of high molecular weights for the two enzymes was observed when the concentration of acrylamide was increased from 5 to 7 %, but with the same 0.17 % methylene bisacrylamide. After 2 h in 7 % acrylamide, NAD-GDH migrated only 1 mm and NADP-GDH only 1.6 mm from the top of the running gel. However, after 2 h in 5 % acrylamide, NAD-GDH and NADP-GDH migrated 18 mm and 34 mm respectively. Although no attempt has been made to estimate the molecular weight of GDH in *C. cinereus*, the enzyme is known to be a large molecule in *N. crassa* (Blumenthal & Smith, 1973), bacteria (Yarrison, Young & Choules, 1972), animals (Cassman & Schachman, 1971) and higher plants (Chou & Splittstoesser, 1972).

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