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A method of visualization of Neurospora nucleosidase in polyacrylamide gels.

In order to study nucleosidase and its isozymic pattern in N. crassa, it was of considerable interest to develop a suitable staining method to locate the enzyme activity in gels, since this would facilitate genetic and developmental studier. In this communication a method to visualize nucleosidase activity in polyacrylamide gel is described. The method involves the use of the D-ribose reductase of Eremothecium orhbyii (Mehta et al. 1972 Biochem. J. 130: 159) to reduce either ribose (or ribose phosphate, the product of nucleosidase), in the presence of reduced NADP; the $NADP^+$ liberated in this reaction is coupled to isocitrate dehydrogenare in the presence of phenazine methosulphate (PMS) and nitro-blue tetrazolium (NBT).

Cultures of N. crassa were obtained as previously described (Mattoo et al. 1973 Indian J. Exp. Biol. 11: 551). The methods for the preparation of cell-free extracts, estimation of protein and the array for nucleosidase activity have been described (Mattoo and Shah 1974 7. allge. Mikrobiol. 14: 581). The specific activity of nucleosidase in the cell-free extracts used was 12-14 μmoles ribose liberated/min/mg of protein.

For the preparation of D-ribose reductore, a 48-hr harvested culture of E. arhbyii was transferred to a fresh liquid medium (Mehto et al. 1972 Biochem. J. 130: 159)in which glucose was replaced with D-ribose (1%). The flasks were incubated at 25° C on a rotary shaker (150 r.p.m.) for 16 hr. Cells were harvested by filtration at 0-5° C, frozen immediately and cell-free extracts prepared. The following operations were carried out at 0-5° C, unless otherwise indicated. To the crude extract protamine sulphate (0.1 mg/mg protein, pH 7.0) was added after 10 min the precipitate was centrifuged down and discorded. The supernatant was added to calcium phosphate gel (18 mg/ml dryweight, pH 7.0) in the gel-to-protein ratio of 16. The suspension was centrifuged immediately and the residue discarded. To the supernatant liquid from the gel were added rephodex-G-15 beads (Pharmacia), 1 gm per 10 ml solution. The mixture was allowed to stand at 25° C for 1 to 2 hr prior to an overnight incubation at 5" C. The supernatant was decanted, dialysed for 4 hr against 200 volumes of glass distilled water and used as the partially purified preparation of D-ribose reductare. The specific activity of the enzyme in this preparation was 7 micromoles NADP formed/min/mg protein.

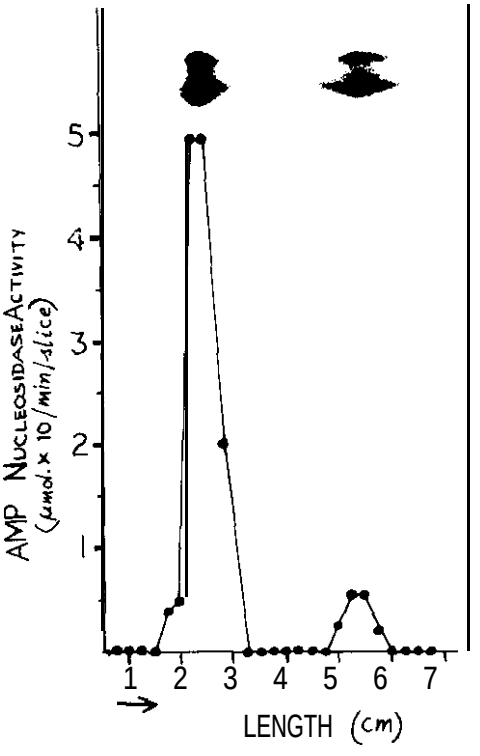

Figure 1. Gel electrophorerir of Neurospora crassa nucleosidase in 7% polyacrylamide gel. After electrophorerir the gel was sliced into 2.5 mm sections, suspended in 1 ml of Tris-HCl buffer (pH 7.0), frozen and thawed, and the enzyme activity determined. The top portion of the figure depicts a duplicate gel stained in the complete staining medium described in the text. 100 μg of the Neurospora cell extract was applied on the gel.

Polyacrylamide gels 7.5% without sample and spacer gels were prepared as described by Davis (1964 Ann. N.Y. Acad. Sci. 121:404) using Tris-glycine buffer at pH 8.3. Gels were mode in gloss cylinders (gel size, 0.5 x 7.0 cm) and a current of 4 mA/gel was maintained. The gels were loaded with Neurospora cell-free extract and overlayered with 0.5 ml of 1 M sucrose solution, followed by the buffer used for separation. The electrophoretic separation of the sample lasted for 75 min at 2° C. Rods of gel removed from the tuber were immersed in water (2 min) and then placed in the staining solution. Staining was accomplished in the dark at 25° C. After completion of the staining, the gels were washed once again in the water and then transferred to tuber containing 7.5% acetic acid. Duplicate gels were sliced into 2.5 mm sections, proteins eluted by overnight freezing in Tris-HCl buffer (pH 7.0) and then thawing, and the enzyme activity determined.

Sharp and well-defined bonds of enzyme activity in the gels were obtained (Fig.1) in the following standardized staining solution: AMP (sodium salt, Sigma), 3 mM; NADPH, 0.06 mM; $MnCl_2$, 5 mM; DL-irocitric acid, 1 .4 mM; pig heart isocitrate dehydrogenare (specific activity 3-10 μMolar units/min/mg, type IV, Sigma), 0.15 μMolar units/ml; partially purified Eremothecium D-ribore reductare, 7 units/ml; PMS, 0.3 mg/ml; NBT, 0.6 mg/ml; and citrate-NaOH buffer (pH 6.5), 50-100 mM. The intensity of the bands could be increased with higher concentrations of NADPH (up to 0.15 mM), isocitrate dehydrogenare (0.3 μMolar units/ml) and D-ribose reductare (21 units/ml). Staining war complete in 45-60 min. Staining for longer periods of time did not result in an increased intensity of staining or in the detection of additional bonds of the enzyme activity. It is evident that N. crassa grown under our conditions contains 2 isozymes of nucleosidase.

No band of enzyme activity developed in those gels which were stained in the absence of any of the following components of the staining solution: AMP, NADPH, isocitrate dehydrogenare, or D-ribose reductore, indicating the absolute requirement for these in the development of enzyme activity bonds.

Duplicate unstained gels were sliced, proteins eluted in buffer after freezing and thawing, and the enzyme activity determined. The peaks of enzyme activity correlated well with the stained bands, the intensely stained bond clearly exhibiting much higher enzyme activity than the less stained one (Fig. 1). The intensity of the bands increased with increasing concentrations of the applied enzyme protein showing enzyme-concentration-dependent development of enzyme activity bands. Further, GMP (3 mM) could replace AMP as the substrate for the development of both the enzyme activity bonds, although the bands were less intensely stained. On storage at 0-4° C or at -10° C the eluted fraction corresponding to the fast moving isozyme lost considerable activity in contrast to the slow moving isozyme fraction, indicating the unstable nature of the former. ---- The encouragement of Professor V.V. Modi and valued cooperation of Mr. A.M. Madia and Mr. A. Wali are gratefully acknowledged.

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