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Abstract
Phosphofructokinase determination

Kapoor, M. Phosphofructokinase of Neurospora crassa.

Neurospora crassa fluffyoid strain P628 (FGSC#553) was used as a source of phosphofructokinase (PFK). All cultures were prepared in Vogel's minimal medium with 2% sucrose as the carbon source. Conidial suspensions containing ca. 5 x 10^7 conidia/ml were prepared from agar slants grown for 6 doys at 28°C. Liquid cultures were started by inoculating 5 ml of the conidial suspension into a 2.8 l Fernbach flask containing 1.5 l of the medium and grown in an environmental rotatory shaker (New Brunswick) at 28°C for 30 h rs; the myoelia were then harvested, lyophilized and stored at -20°C.

Assay methods: PFK was assayed by two methods. Method 1, a slight modification of that of Uyeda and Racker (1965 J. Biol. Chem. 240: 4682), consisted in using a reaction system containing the following: Tris-H Cl, pH 8.0, 100 µmoles; ATP 1. 25 pmoles; fructose-6-phosphate 2.5 pmoles; $MgCl_2$ 5 pmoles; rabbit muscle aldolase (Sigma) 100 µg; a-glycerophosphate dehydrogenase-triose-phosphate isomerase (Sigma) 10 µg; reduced NAD 0. 14 µmole; and enzyme preparation in a total volume of 3 ml. The reaction rote was measured by following the initial decrease in OD at 340 mµ accompanying the disappearance of reduced NAD in a Giford model 2000 recording spectrophotometer at 25°C.

Method II consisted in following the appearance of ADP by coupling with PK-LDH system. The following reaction mixture was employed: Tris-HCl, pH 8.0, 100 µmoles; fructose-6-phosphate 2.5 µmoles; ATP 1 µmole; $MgCl_2$ 5 pmoles; phosphoenolpyruvate 0.3 µmole; PK/LDH (Sigma) in excess; reduced NAD 0. 14 µmole; and the enzyme preparation in a total volume of 3 ml. The reaction rate was determined by the decrease in OD at 340 mµ.

Preparation of the enzyme: Fifteen grams of the lyophilized mycelium powder was extracted with 100 ml of TPMSAF buffer (Tris-phosphate 0. 05 M-ß-mercaptoethanol 5 x 10^{-4}M-sorbitol 0. 1 M-ATP 2.5 x 10^{-4}M-FDP 5 x 10^{-4}M, pH 8.0) for 20 min, homogenized in a gloss homogenizer and centrifuged at 15,000 rpm for 15 min in a Sorvall RC-2 refrigerated centrifuge. The supernatant was used as the crude extract. To 75 ml of the supernatant solid ammonium sulfate, sufficient to give a saturation of 0.45, was added. The precipitate was centrifuged out and the supernatant was fractionated by adding solid ammonium sulfate successively to obtain protein fractions precipitating between saturations of 0.45 and 0.70, between 0.70 and 0.90 and between 0.90 and 1.0, respectively. Each of the precipitates was dissolved in TPMSAF buffer (fractions II, 111 and IV). The activities of PFK in all these fractions were determined by methods I and II, method I being used largely in assaying extracts during different steps of purification; method II was used in crude extracts to assay the ATPase activity simultaneously with that of PFK. Since both ATPase and NADH oxidase ore present in crude extracts, PFK values obtained in fraction II and subsequent fractions only were considered to be reliable. ATPase precipitated out in ammonium sulfate at 0.45 saturation and, although fraction II had some NADH oxidase activity, fraction 111 was completely free of it. The specific activities of PFK in these fractions are given in Table I.

As is evident from these data, a large proportion of PFK activity precipitates with proteins at 0.7-0.9 saturation of ammonium sulfate. Further purification con be achieved by ion exchange chromatography on DEAE cellulose columns. However, purification of this enzyme presents serious difficulties as PFK is a very unstable enzyme. In the absence of sorbitol, ATP and FDP, the enzyme activity in crude extracts and ammonium sulfate precipitates is not retained for more than a day. With ATP and FDP alone in the extraction medium, there is a slight stabilization of the enzyme, but if sorbitol is present in the buffer enzyme activity can be maintained for at least a period of 10 days at -20°C. A further problem that has been encountered is the extreme variation of PFK activity recorded in different batches of mycelia obtained from the same stock cultures grown under apparently identical conditions. In some botches, it is almost impossible to detect enzyme activity and others may show a low to a fairly high level of PFK. Attempts at reactivating the inactive enzyme in such extracts have proved unsuccessful. We are unable to explain this variation on genetic or environmental grounds. It therefore seems reasonable to conclude that some parameter, upon which PFK activity is entirely dependent, and that we ore completely unaware of, comer into operation at a critical phase in the growth of a given culture. It is also possible to speculate that perhaps Neurospora PFK undergoes drastic changes in conformation in response to slight differences in growth conditions and/or chemical substances present in the medium as impurities. On account of these difficulties it has not been possible for us to prepare extracts in bulk quantities or

Table 1.

Preparation	Activity/ml in OD units	Protein/ml	Sp. act.
Crude		13.5	-
Fraction II (0.45-O. 70)	1. 30	20.0	0.06
Fraction 111 (0.70-O. 90)	8.5	3.7	2.3
Fraction IV (0.90-I. 0)	1.50	0.8	1.9

to undertake purification on a large scale. I would appreciate hearing from other Neurosporologists if they hove any suggestions in this regard.

Preliminary kinetic experiments conducted with partially purified preparations of PFK indicate that the enzyme is inhibited both by fructose-6-phosphate and ATP at higher concentrations. At least in some respects the Neurospora PFK appears to be similar to that of other microorganisms. - - - Department of Biology, University of Calgary, Calgary, Alberta, Canada.