Agar gel electrophoresis of amylases

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Abstract
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\[
\text{Fructose-6-phosphate + ATP} \rightarrow \text{Fructose-1,6-diphosphate + ADP + H}^+ \\
\text{Fructose-1,6-diphosphate} \rightarrow \text{Dihydroxyacetone phosphate} \\
\text{Glyceroldehyde-3-phosphate} \rightarrow \text{Dihydroxyacetone phosphate}
\]

Preparation of crude extract: The washed mycelial mat is lyophilized and milled (Wiley) to pass 60 mesh sieve. One gram of powder is homogenized with 10 ml of 0.03 M KF, 0.001 M EDTA on an ice bath. 0.5 ml of 1 M MnCl₂ is added to precipitate the nucleic acids. The homogenate is centrifuged to 60,000 x g and the precipitate is discarded.

Assay Procedure: The assay mixture has a final volume of 1 ml and contains 50 mM Tris-HCl, pH 8.4, 25 mM fructose-6-phosphate, 5 mM ATP, 4 mM MgCl₂, 6.6 mM mercaptoethanol, 0.16 mM DPNH, and 0.05 ml auxiliary enzyme solution (0.2 mg/ml aldolase, 0.04 mg/ml triosephosphate isomerase, 0.04 mg/ml α-glycerophosphate dehydrogenase, and 0.2 mg/ml bovine serum albumin in 0.01 M Tris-HCl, pH 8.0). The reaction is initiated by the addition of 0.002 ml of extract and the OD change at 340 mμ is measured. After the addition of extract, the initial velocity will normally remain linear with time, and it is therefore important to use the initial velocity to determine PFK activity. Background DPNH oxidation is also occasionally encountered before the addition of extract. This must be subtracted from the DPNH oxidation rate after the addition of extract.

One unit of PFK activity is defined as that amount catalyzing the formation of 1 μmole of fructose-1,6-diphosphate per min. at 25°C under the conditions of the standard assay. Specific activity is expressed as units per mg of protein. The value for crude extracts is ca. 0.1. PFK from N. crassa is very labile and activity will be lost rapidly in crude extract delayed by adding an equal volume of glycerol to the extract and storing at -20°C.

Fass, D. N. Isolation of γ-amylase (gluc-amylase) from the culture filtrate of N. crassa. An amylase-super-producer strain, e.g., inox (89601) α, (FGSC #498) is grown 84-96 hours in 1% sucrose Vogel’s medium. Good yields may be expected from 1 liter cultures in 2.8 1 Fernbach flasks incubated at 25°C on a shaker. Adequate aeration is essential for production of the enzyme. At the end of this time, the mycelia are removed by filtration and the medium is chilled to 4°C and cold ethanol is added to 40%. The solution is allowed to stand overnight at 4°C and the resulting precipitate is removed by centrifugation at 25000 x g for 10 min. To the alcoholic supernatant is added a water solution of 2% α-glycerophosphate in the proportion of 25 ml/l of original medium. The white precipitate which forms immediately at 4000 X g for 10 min and redissoles in a small volume of Vogel’s salts. This mixed enzyme solution is incubated for 1 hr at 37°C and then dialyzed twice at 4°C against 50 vols of citrate buffer 0.01 M in NaOH, pH 5.0, for 4 hrs and 8 h. The sample is applied to a 2 x 15 cm column of Amberlite CG-50 equilibrated with the same buffer. Elution is carried out at 4°C with a 500 ml linear gradient from 0.01 to 1.1 N NaOH at approximately 40-50 ml/hr. Citrate is the counter-ion.

The amylolytic activity recovered at about 0.4 N NaOH shows an E-fold increase in specific activity, no invertase or α-amylase activity, and a single band in acrylamide gel electrophoresis. This work was supported in part by the NSF and NIH Training Grant in Genetics (T01-GM01316) to Florida State University. Genetics Laboratories, Department of Biological Science, Florida State University, Tallahassee, Florida 32306.

Fass, D. N. Agar gel electrophoresis of Neurospora amyloses. The procedure of Kikkawa (1963 Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ. 11:41) previously used for the production of zymograms of Drosophila amyloses has been modified for use with Neurospora crassa. A glass plate 10 x 10 cm is frosted on one side by grinding with household cleanser ( Comet). The electrophoretic medium consists of 2% Difco Purified Agar in 0.01 M citric acid buffered to pH 5.0 with NaOH. This some buffer is used in the electrode reservoirs. A layer 1 mm thick is pipetted onto the frosted side of the plate and is evenly with the rise of pipette. A layer 1 cm thick is pipetted onto the frosted side of the prewetted plate and is evenly with the rise of pipette. Whatman #2 filter paper strips 1 x 0.15 cm soaked with enzyme are applied too line 3 cm from a long edge of the plate. The enzyme is then applied for 10 min, after which the strips are removed. The plate is connected to the reservoirs by the thicknesses of Whatman #1 filter paper. A potential gradient of 40 V/cm is applied and maintained for 2 hours. The amylases will migrate to the plate.

The plate is then immersed in 4 mg/ml soluble starch solution for 15 min, followed by a brief water rinse. Digestion of the starch is allowed to occur for 20 min in a 37°C incubator, after which the agar is stained in a solution of 0.3% KI - 0.03% I₂. Two types of bonds will be visible; clear (against dork blue) and faint pink. The former are the Y-amylases (gluc-amylases) and the latter are the a-amylase.
Enzyme may be obtained by growing most strains for 8 days in Vogel's salts plus 2% sucrose with necessary supplements in stationary culture. The medium is decanted and the pads are washed for 1 hr in Vogel's salts. This is also decanted and Vogel's salts plus 1% maltose is added to the pad. After 24 hrs of shaking at 25°C, easily detectable quantities of enzyme will be present in the medium. This medium should be concentrated 20-50 fold by dialysis against air or sucrose before electrophoresis. Strain inos 89601a (FGSC#498), grown for 4 days with shaking in Vogel's salts plus 1% sucrose and inositol, will produce sufficient enzyme in the medium for electrophoresis without concentration. Strain inos 89601A (FGSC#497) does not produce elevated levels of amylose (H. G. Grotzner, personal commun.).

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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 \( 5 \times 10^7 \) conidia/ml were prepared from agar slants grown for 6 days at 28°C. Liquid cultures were started by inoculating 5 ml of the medium with some mycelia. The mycelia were then harvested, lyophilized and stored at -20°C.

Assay methods: PFK was assayed by two methods. Method I, a modification of that of Uyeda and Racker (1965 J. Biol. Chem. 240: 4682), consisted in using a reaction system containing the following: Tris-HCl, pH 8.0, 100 \( \mu \)moles; MgCl2, 5 \( \mu \)moles; fructose-6-phosphate, 2.5 \( \mu \)moles; rabbit muscle aldolase (Sigma) 100 \( \mu \)g; \( \alpha \)-glycerophosphate dehydrogenase-triose-phosphate isomerase (Sigma) 10 \( \mu \)g; NAD 0.05 \( \mu \)mole; and enzyme preparation in a total volume of 3 ml. The reaction rate was measured by following the initial decrease in OD at 340 \( \mu \)m accompanying the disappearance of reduced NAD in a Gilford 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 \( \mu \)moles; fructose-6-phosphate, 2.5 \( \mu \)moles; ATP 1 \( \mu \)mole; MgCl2, 5 \( \mu \)moles; phosphoenolpyruvate 0.3 \( \mu \)mole; PK/LDH (Sigma) in excess; reduced NAD 0.14 \( \mu \)mole; and enzyme preparation in a total volume of 3 ml. The reaction rate was determined by the decrease in OD at 340 \( \mu \)m.

Preparation of the enzyme: Fifteen grams of the lyophilized mycelium powder was extracted with 100 ml of TPMSAF buffer (Tris-phosphate 0.05 M - B-mercaptoethanol 5 \( \times 10^{-4} \) M - sorbitol 0.1 M - ATP 2.5 \( \times 10^{-4} \) M - FDP 5 \( \times 10^{-4} \) M - pH 8.0) for 20 min, homogenized in a glass 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, III, IV, and V) and fractionated by DEAE cellulose columns. The activities of PFK in these fractions were considered to be reliable. PFK precipitated out in ammonium sulfate at 0.45 saturation and, although fraction II had some NADH oxidase activity, fraction III 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 can 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 maintained 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 batches, 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 in genetic or environmental grounds. It therefore seems reasonable to conclude that perhaps Neurospora PFK undergoes drastic changes in conformation in response to slight differences in growth conditions or some chemical imperfections present in the medium. As a result of these difficulties it has not been possible for us to prepare extracts in bulk quantities or...