Arginosuccinate synthetase determination

R. W. Tateson

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Arginosuccinate synthetase determination

Abstract
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This enzyme methodology is available in Fungal Genetics Reports: http://newprairiepress.org/fgr/vol14/iss1/19
Tateson, R. W. A method for the determination of adenylsuccinate lyase.

The reported method for this enzyme (Adenylsuccinate AMP lyase 4.3.2.2) required a supply of adenylsuccinate (Giles et al. 1957 Proc. Natl. Acad. Sci. U.S. 43: 305). As adenylsuccinate is not available commercially, a method has been devised to measure the back reaction whose substrates, fumarate and AMP, are readily available.

The enzyme was prepared in the same way as for the ASA synthetase below. The reaction: Into a 1 cm. cuvette are added 0.1 ml of 0.01 M AMP, 0.05 ml of 0.05 M fumarate, 2.75 ml of 0.1 M phosphate/citrate buffer pH 7.5. and 0.1 ml enzyme extract. The increase in OD at 280 m for the back reaction whose substrates, fumarate and AMP, are readily available.

The following stock solutions are made up: CIT = 0.986 g L-citrulline in 25 ml water; ASP = 1.00 g L-aspatic acid in 100 ml water; FUM = 16.0 g Na fumarate in 100 ml water; Mg = 4.94 g MgSO 4·7H 2 O in 50 ml 1 M Tris; TRIS = 1.0 M Tris; PGA = 4.46 g Na D(-)-3-phosphoglyceric acid. 2H 2 O in 75 ml 1 M HCl (add 20 ml saturated K 2 SO 4 spin off ppt., make up to 100 ml); ATP = 0.031 g ATP (disodium salt) in 10 ml water. All solutions are adjusted to pH 7.4 before being made up to final volume. All solutions may be stored frozen.

The reaction mixture consists of:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Vol. ml.</th>
<th>μmoles in 0.1 ml rxn. mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIT</td>
<td>1.0</td>
<td>2.25</td>
</tr>
<tr>
<td>ASP</td>
<td>3.0</td>
<td>2.25</td>
</tr>
<tr>
<td>Mg</td>
<td>0.5</td>
<td>2.58</td>
</tr>
<tr>
<td>TRIS</td>
<td>0.5</td>
<td>10.0</td>
</tr>
<tr>
<td>FUM</td>
<td>1.0</td>
<td>15.0</td>
</tr>
<tr>
<td>PGA</td>
<td>4.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

to this is added:

- Creatine phosphate 0.127 g
- Creatine phophokinase 0.001 g

(Sigma London Ltd.)

Tateson, R.W. A method for the determination of argininosuccinate synthetase.

A method for determining argininosuccinate synthetase (L-citrulline: L-aspartate lyase (AMP) 6.3.4.5.) by measuring the production of argininosuccinic acid (ASA) has been developed in this laboratory. This has been found to be more reliable and accurate than methods measuring the rate of loss of citrulline in the presence of large amounts of citrulline. To prevent the destruction of ASA, by the enzyme ASAase, fumarate is included in the reaction mixture which keeps the reaction: ASA -> arginine + fumarate well over to the left by a mass action effect. As ATP is inhibiting in high concentrations, a low concentration is used, plus two ATP generating systems.

The enzyme is prepared by homogenizing 40 g of freeze-dried Neurospora powder in 1 ml of 0.05 M Tris pH 7.5 buffer, and centrifuging at 3000 rpm for 5 min. The supernatant is dialyzed against two changes of 0.05 M Tris pH 7.5 at 4°C.

0.1 ml of enzyme extract is added to 0.1 ml of reaction mixture in a small centrifuge tube and allowed to equilibrate at 35°C. The reaction is started by adding 0.1 ml of ATP solution. For each assay three tubes are made up and the reaction is stopped after 0, 30 and 90 min by adding 0.05 ml of 5% TCA. The tubes are boiled for 15 min and then spun at 3000 rpm for a short time.

0.05 ml of the supernatant are spotted onto 3MM chromatography paper which is subjected to electrophoresis in a pyridine-acetic acid buffer pH 3.7 for 60 min at 3000 volts, in an Anfinson-type tank. The papers are developed by the method of Bronk and Fisher (1956 Biochem. J. 64: 108). ASA runs as two spots, as it is converted into its onhydrides by boiling with acid. These spots are cut out, eluted with 2 ml methanol, and the OD at 504 μm is measured. The OD is proportional to time and protein concentration.

ENZYME METHODOLOGY


The reaction mixture contained 0.16 μmole pyridoxal phosphate, 3 μmole magnesium sulfate, 0.06 μmole kynurenine, 50 μmole Tris-(hydroxymethyl) aminomethane pH 8.0, and protein (50-500 μg) in a final volume of 1.2 ml. The reaction was conducted at 30°C in the cuvette of an Aminco Bowman spectrophotofluorimeter equipped with thermal spacer. Anthranilic acid which appeared during the reaction was detected by measuring the intensity of its fluorescence at 409 nm. Activation was at 315 nm.

A rapid method for assaying kynureninase has been developed. As in the procedure described by Jakoby and Bonnet (1953 J. Biol. Chem. 205: 699), the method involves detection of anthranilic acid produced by measuring the intensity of its fluorescence. Extracts prepared from tryptophan-induced wild type strain 74A were used as a source of kynureninase. The activity was purified approximately 4-fold by amanmonium sulphate precipitation and Sephadex chromatography.

The reaction mixture contained 0.16 μmole pyridoxal phosphate, 3 μmole magnesium sulfate, 0.06 μmole kynurenine, 50 μmole Tris-(hydroxymethyl) aminomethane pH 8.0, and protein (50-500 μg) in a final volume of 1.2 ml. The reaction was conducted at 30°C in the cuvette of an Aminco Bowman spectrophotofluorimeter equipped with thermal spacer. Anthranilic acid which appeared during the reaction was detected by measuring the intensity of its fluorescence at 409 nm. Activation was at 315 nm.

The amount of anthranilic acid produced is linear with time for about 5 minutes, after which the rate decreases. The velocity of the reaction is proportional to protein concentration from 0.045 to 0.54 mg/ml and extrapolates to zero at zero protein concentration. The velocity of the reaction approached a maximal value when the kynurenine concentration was 0.05 μmole/ml. Kynur-