Protocol for glyoxalase II extracts and kinetic assays

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1. Introduction

This is a general protocol of how to perform the purification of glyoxalase II (GlxII) as well as enzyme kinetic assays. The strain used for this protocol is MJF609, which contains one chromosomal copy of glxII gene under control of the lac promoter. Induction of gene expression is achieved by addition of IPTG to the growth medium.

The protocol follows four basic steps: growth of cells, induction of gene expression by IPTG, preparation of cell extracts and kinetic assays. It is possible to measure SLG concentration spectrophotometrically at 240 nm in 50mM potassium phosphate buffer pH 7.8.

GlxII is a metalloenzyme capable of accepting different ions such as Fe$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ [1]. Each metal form of the enzyme exhibit different kinetic properties and supplementation of growth medium with the ion of interest is of great importance for the analysis of those kinetics properties.

The growth medium is decided according to the purpose of the experiment, and this protocol will describe how to perform the experiments in $K_{0.2}$ medium, with 200μM ZnCl$_2$, for the analysis of the Zn$^{2+}$-enzyme.

2. Materials

2.1. Reagents

- Na$_2$HPO$_4$.12H$_2$O (Fischer Scientific, cat no S/4360/60)
- Na$_2$HPO$_4$.2H$_2$O (Fischer Scientific, cat no S/3760/60)
- (NH$_4$)$_2$SO$_4$ (BDH Laboratory Supplies Poole, cat no 100334C)
- KCl (VWR International Ltd Poole, cat no 101984L)
- MgSO$_4$.7H$_2$O (Fischer Scientific, cat no M/1050/53)
- (NH$_4$)$_2$SO$_4$.FeSO$_4$.6H$_2$O (BDH Laboratory Supplies Poole, cat no 10112)
- Thiamine (Sigma, cat no T-4625)
- HCl (VWR International Ltd Poole, cat no 103076P)
- D-glucose (VWR International Ltd Poole, cat no 101174Y)
2.2. Reagents setup

- **Kx** buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄.12H₂O</td>
<td>46</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>23</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>7.95</td>
</tr>
</tbody>
</table>

x is KCl concentration in mM

- 50 mM Potassium Phosphate buffer pH 7.8

The standard method for this buffer preparation is described in [2], however, due to impurities in the salt stocks, the pH is adjusted by changing slightly the proportions from the standard method with the help of a pH meter.

2.3. Supplements

In order to prepare Kx minimum media, the following supplements are added to the buffer:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄.12H₂O</td>
<td>40mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>(NH₄)₂SO₄.FeSO₄.6H₂O</td>
<td>6 mM</td>
<td>6 µM</td>
</tr>
<tr>
<td>Thiamine.HCl*</td>
<td>0.1 %</td>
<td>0.001 %</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>20 %</td>
<td>0.02 %</td>
</tr>
</tbody>
</table>

*The stock solution was prepared with 0.1 M HCl
x is the final concentration of KCl.
ZnCl₂ stock solutions of 80 mM were prepared.

3. Equipment

- RC-5 Superspeed Sorvall centrifuge
- Jouan centrifuge MR 22
- Optima ultracentrifuge
4. Protocol description

4.1. Cell growth

**Timing: 4 hours**

Prepare an overnight culture of MJF609 in K0.2 medium, at 37°C. Next morning, dilute the culture 10x into fresh pre-warmed K0.2 and incubate cells at 37°C. Grow cells until OD650 of 0.4, dilute them into fresh K0.2 in the presence of 200 µM ZnCl2 and 0.5 mM IPTG at 30°C. Induce the gene expression until OD650 of 0.4 is reached and harvest cells by centrifugation at 5000 rpm 15 min at 4°C.

4.2. Protein extracts

**Timing: 4 hours**

Discard supernatant and resuspend the pellet in 50 mM potassium phosphate buffer pH 7.8. In order to wash cells, spin them down in 4000 rpm for 15 min at 4°C. Discard supernatant, resuspend the pellet in 50 mM potassium phosphate buffer pH 7.8 to the desired cell density. French press cells twice at 1800 Psi. Lysed cells are ultra-centrifuged at 50000 rpm at 4°C for one hour and supernatant frozen in aliquots.

**PAUSE point: protein extracts are frozen**

It is necessary to do the Lowry assay [3] to quantify the total protein content obtained. The amount of protein extract used to be used in the enzyme kinetic assay will depend on the activity of the protein extract obtained. It is possible that dilutions of the protein extract should be performed before starting the assay.

4.3. Enzyme kinetic assay

**Timing: 3 hours**

This assay consists in obtaining the initial enzyme activities when subjected to different initial SLG concentrations, in a temperature controlled way (set to 37°C).

The assay is started by the addition of protein extract to a cuvette containing a solution of SLG in potassium phosphate buffer. SLG disappearance is then measured spectrophotometrically at 240 nm.

An example of the different SLG solutions prepared, from 100 mM stock solutions are listed in table 1. Note that those solutions will be diluted 10 times further while performing the assay (100 µl of protein extract, 100 µl of SLG solution, 800 ul of potassium phosphate buffer).

The actual concentrations of SLG solutions should be calculated according to the saturation level of the enzyme and the cuvette available for the assay. In the case described,
Table 1.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Volume of MG</th>
<th>Volume of distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM</td>
<td>150 µl of 100 mM solution</td>
<td>850 µl</td>
</tr>
<tr>
<td>12.5</td>
<td>420 µl of 15 mM solution</td>
<td>84 µl</td>
</tr>
<tr>
<td>10</td>
<td>130 µl of 100 mM solution</td>
<td>1170 µl</td>
</tr>
<tr>
<td>7.5</td>
<td>405 µl of 10 mM solution</td>
<td>135 µl</td>
</tr>
<tr>
<td>5.0</td>
<td>400 µl of 10 mM solution</td>
<td>400 µl</td>
</tr>
<tr>
<td>2.5</td>
<td>350 µl of 5 mM solution</td>
<td>350 µl</td>
</tr>
<tr>
<td>1</td>
<td>200 µl of 2.5 mM solution</td>
<td>300 µl</td>
</tr>
</tbody>
</table>

A 1 ml cuvette with a path length of 4 mm was used, and the maximum SLG concentration that could be measured in this case was 1.5 mM.

Initially, it is necessary to measure the absorbance of the desired SLG solution present in the cuvette, prior to the addition of the protein extract, as a control to SLG stability. For this, we add 100 µl of the desired SLG solution to 800 µl of pre-warmed buffer and measure SLG absorbance for 3 min. After addition of 100 µl of protein extract and mixing, the data acquisition of the decrease in SLG absorbance is started.

The absorbances obtained must be subtracted by the absorbance of the solution of extract and buffer only (100 µl of extract + 900 µl of buffer), as it is possible to have absorption of light by the protein extract itself.

Calculations should be performed in order to obtain the SLG concentration measured in the assay. From these concentrations, the initial activities (v) are be obtained for each starting SLG concentration ([S]₀). A graph of v × [S]₀ is plotted, as well as the Hanes-Woolf plot (\( \frac{v}{[S]₀} \times [S]₀ \)) to help the estimation of the kinetic parameters.

References