

**Department of Biology and Biochemistry**

**CELL BIOLOGY**

**(BIOL 244)**

**Experiment 6: Analysis of Subcellulr Fraction III**

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**Analysis of subcellular fraction III**

* **Objectives:**

Testing the presence of an phsphatase enzyme in lysosome and measuring its value

* **Introduction:**

Lysosomes are membrane-bound cytoplasmic organelles that play a role in protein breakdown within cells. Lysosomes ,Enzymatic and non-enzymatic deficits cause lysosomal dysfunction, which results in aberrant material buildup.

The non-proteinaceous, non-specific substrate p-Nitrophenyl Phosphate (PNPP) is used to test protein, alkaline, and acid phosphatases. Under any non-standard circumstances, the test may be used to quickly assess protein phosphatase activity.

In basic solutions, alkaline phosphatase (ALP) catalyzes the hydrolysis of different phosphate esters. ALP is present in the body in different forms, although the isoenzyme from the plasma membrane of hepatocytes contributes the most to ALP activity in blood serum.In alkaline conditions, ALP hydrolyzes p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate.NaCl is used as an activator in this solution. The amount of p-nitrophenol released is a measure of enzyme activity.activity. In an alkaline solution, its yellow hue is spectrophotometrically quantified.The kinetic approach or the so-called end-point method can both be used to quantify ALP activity. The enzymatic process is stopped by the inhibitor NaOH/EDTA solution.

* **Materials**
* 1M Sodium acetate buffer, pH 5.7
* Lysosome fraction
* 150 mM KCl in 10mM HEPES,PH7.4
* Water bath at 30c
* 0.1 M MgCl2
* 50 Mm p-Nitrophenyl phosphate
* 0.5 N KOH
* Spectrophotometer and tubes
* Biuret protein assay
* **Procedure**

The Mitochondrial and Nuclear was isolated from the rat liver by kiiled a rat and removed its liver and put it in the ice-cold, we was cut 1.2g from the liver,we added amount of HB-buffer to the liver(thats help to remove the free fatty and lipids) and chop it into small piceces with scalpels,then homogenized by Place the mixture in a homogenizer tube and homogenize with a motor homogenizer (up to 10-15 strokes) while keeping it chilled, then centrifuged the homogenate at 400xg for 5 minutes thats to remove the cell debris and unbroken cells, after that in the centrufuge tubes appeared pellet( un broken cell ) we got rid of it, and the supernatant(SN1) thats what we need this is the whole homogenate, we collected it volume (v\*) and talked 2ml and saved it on ice on tube H; to use it later, we repeat the centerfuge for the rest of the homogenate(SN1) for 15 minute at 4000xg, in the senterfuge tube we had a pellet (nuclei) we resupended it in the same volume of v\* of H-buffer and saved it on ice in tube N, and supernatant(SN2) we plased into a clean centerfuge tube to sentrifuge it at 12000xg for 15 minute the pellet which resulted is the mitochondrial fraction we was resuspend it in the same volume of v\* of H-buffer and saved it on ice in tube M, and the supernatant(SN3) recorded its volume, to (SN3) we added 80 mMCaCl2 to yield final concentration of 8mM of CaCl 2 in the sample 1 ml of CaCl per 9 ml of supernatant, stirred gently and centrifuged at 20000xg for 25 minute at 4 C , this give a microsomal fraction enriched in lysosomes we resuspend the pellet in the same volume of v\*of 150 mM KCL in 10 mM HEPES buffer, PH7 saved it on ice in tube L,we removed the supernatant, to assessed for the acid phosphatase anzyme we prepared a series of six tubes consists of 0.5 ml of 1M Sodium acetate buffer, 0.5 ml of 0.1 M MgCl2, and 0.5 M p-nitrophenyl phosphateand we added 3.3 ml of distilled H2O to each of the six tubes befor put it in a 30c water bath we mixed it well, prepared anathor serial dilution of lysosome fraction by added 200µl lysosome suspension to 1.8 ml of 150 mM KCl, 10 mM HEPES bufers mixed the solution well, we was do dilution for 5 time for that we added 200µl of the diluted suspension to a new tube contain 1.8 ml of buffer and mixed well.

* **Data & Results**

Table 1 Data table

|  |  |  |  |
| --- | --- | --- | --- |
| **(Tube)dilution** | **Absorbance (at 5 min )** | **[p-nitrophenol]**  **(µM)** | **Activity/min**  **(µM/min)** |
| (1) Blank | 0 | 0 | 0 |
| (2) 100 | 0.39 | 2.074\*10-8 | 4.14\*10-9 |
| (3) 10-1 | 0.42 | 2.234\*10-8 | 4.46\*10-9 |
| (4) 10-2 | 0.48 | 2.553\*10-8 | 5.10\*10-9 |
| (5) 10-3 | 0.52 | 2.765\*10-8 | 5.53\*10-9 |
| (6) 10-4 | 0.542 | 2.882\*10-8 | 5.76\*10-9 |

**Figure1 Absorbance Vs concentration**

from the table and the picture above it appears that the absorption increases with each time compared to the previous time, as well as the case with the concentration of p-nitrophenol, this indicates that the reaction occurred.

* **Discussion**

After isolating the lysosome, we aim to verify the presence of the enzyme ( acid phosphatase) in it, as well as the activity of that enzyme. In order to make sure the reaction takes place we used p-nitrophenyl phosphate as a substrate for the reaction, this will hydrolyzed and become p-nitrophenol (yellow coloured), These samples of the reaction, we measured the absorption of them using a spectrophotometer at a wavelength of 405 nm for 5 minutes as shown in Table 1. The absorption was increasing each time, and this indicates that the reaction was taking place well, that is, the enzyme is present in the lysosome fraction and is active.

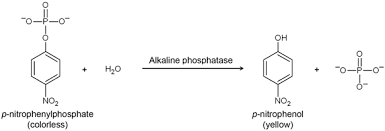


Figure 2 p-nitropheyl phosphate hydolysis to p-nitrophenol

* **Refrances**

.‏ Vincent, J. B., & Averill, B. A. (1990). An enzyme with a double identity: Purple acid phosphatase and tartrate‐resistant acid phosphatase. *The FASEB journal*, *4*(12), 3009-3014.‏

Dell'Angelica, E. C., Mullins, C., Caplan, S., & Bonifacino, J. S. (2000). Lysosome‐related organelles. *The FASEB Journal*, *14*(10), 1265-1278.‏

* **Appendix**

**A = εcl**

A it’s the absorbance

Ε its the coefficient 🡪18.8\*106

C it’s the concentration

L it’s the optical pathlength 🡪 1cm

EX🡪 tube 2

C = 18.8\*106 \*1 /0.39