# 17 th INTERNATIONAL BIOLOGY OLYMPIAD
# 9-16 JULY 2006
# Río Cuarto – República Argentina

## PRACTICAL TEST

## 3

Biochemistry

| Student code: | |
|---|---|

# 17 th INTERNATIONAL BIOLOGY OLYMPIAD
# 9-16 JULY 2006
# Río Cuarto – República Argentina

## General remarks about the practical tests

DEAR PRATICIPANS

The practical test are organized in four different laboratories.

Nº 1- Plant Anatomy, Systematics and Physiology

Nº 2- Animal Anatomy, Ecology and Systematics

Nº 3- Biocheminstry

Nº 4- Microbiology

- You have **1 hour** in laboratories Nº 1 and Nº 2.
- You have **1 hour 30 minutes** in laboratories Nº 3 and Nº 4.
- You can score maximum **40 points** in each laboratories, which means a total of 160 points for the practical test.

*Good luck !!!!!!!*

## Practical test № 3: Biochemistry

### Enzymatic determination of glucose

**TASK 1: You have to perform a calibration curve using a standard of glucosa, with known concentration. Then, plot the results as absorbance versus glucose concentration (15 points)**

**Important:** Raise the red card to call the lab assistant when you are ready to use the spectrophotometer.

**Introduction:**

Glucose oxidase (GOD) catalyzes the oxidation of (beta)-D-glucose to D-gluconic acid and hydrogen peroxide. It is highly specific for (beta)-D-glucose and does not act on (alpha)-D-glucose. The horseradish peroxidase (POD) breaks down hydrogen peroxide into water and oxygen, using the dye as an electron donor. At the same time, the dye is converted to its oxidized form, which is a colored compound. Since the amount of hydrogen peroxide produced indicates how much reaction has taken place, the formation of the red color can be used to follow the course of the reaction.

Its major use is in the determination of free glucose in body fluids. Although specific for (beta)-D-glucose, glucose oxidase can be used to measure total glucose, because as a result of the consumption of (beta)-glucose, (alpha)-glucose from the equilibrium is converted to the (beta)-form by mutarotation.

**PRINCIPLE**

The reaction system is as follows:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic Acid} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AP} + \text{4-Hydroxybenzoate} \xrightarrow{\text{POD}} \text{red quinoneimine}$$

**Glucose oxidase reagent:** solution containing glucose oxidase, peroxidase, 4-aminophenazone (4-AP), and phosphate buffer pH 7.0 containing hydroxybenzoate.

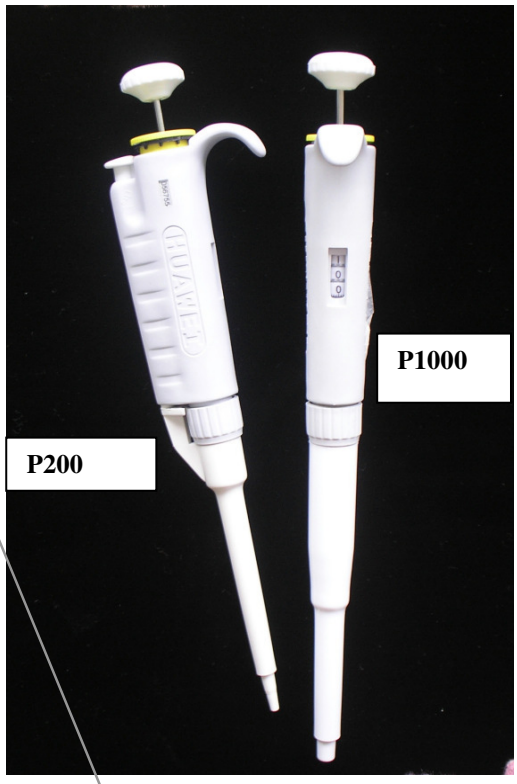
**Reagents:**

1. glucose oxidase reagent (ready to use).
2. glucose solution (unknown concentration).
3. glucose solution 5 mg. $ml^{-1}$.
4. distilled water.

**Equipment**

1. Lab gloves (1pair).
2. Marker pen (1).
3. 1.5 ml microtubes (18).
4. Pipettes (2).
5. Incubator at 37$^{o}$C .
6. Spectrophotometer (you will use it with lab assistants).
7. Spectrophotometric cuvettes (8).
8. Paper towels (3)
9. 1000 µl tips (30)
10. 200 µl tips (30)

**Instruments:**




Tip ejecting knob

Adjustable Wheel

Pushing Knob

Counter

**Adjustment method**

You have to pull up the Adjustable Wheel, then you can revolve the adjustable wheel or knob. Adjust the required volume and push down the Adjustable Wheel.

**Remember that minimal and maximal volumes for P100 are 10 µl and 100 µl respectively.**

**For P1000 minimal volume is 100 µl and maximal volume is 1000 µl.**

**Usage method:**

Please secure the suction tip, after that slightly push down the pushing knob to first stop, hold and immerse the tip into solution vertically. The immersed depth of the tip is 2-4 mm, then release the pushing knob slowly and make it return to the original position. Take off the pipette from the liquid and place the suction tip of the pipette into a special container receiving the dispensed liquid. The tip must be close to the inner wall of the container. Depress the pushing knob to the first stop and further more to discharge the solution completely from the tip. After that, you can take away the pipette and release the button. Eject the used tip to the trash recipient by pressing the Tip ejecting knob.

**EXPERIMENTAL PROCEDURE**

1) Label five 1.5-ml microtubes 1/2 through 1/32 with a marker pen. Using the glucose standard solution (5 mg. ml $^{-1}$) perform the following serial dilutions (in distilled water) in a final volume of 100 µl: 1/2, 1/4, 1/8, 1/16, and 1/32.

2) Mix well and perform (in a new 1.5-ml microtubes set) the enzymatic determination of glucose for each dilution according to the following squeme.

| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | Blank |
|---|---|---|---|---|---|---|
| Sample volumen | 10µl | 10µl | 10µl | 10µl | 10µl | 0 |
| Water volumen | 0 | 0 | 0 | 0 | 0 | 10 µl |
| Glucosa oxidase reagent volumen | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |

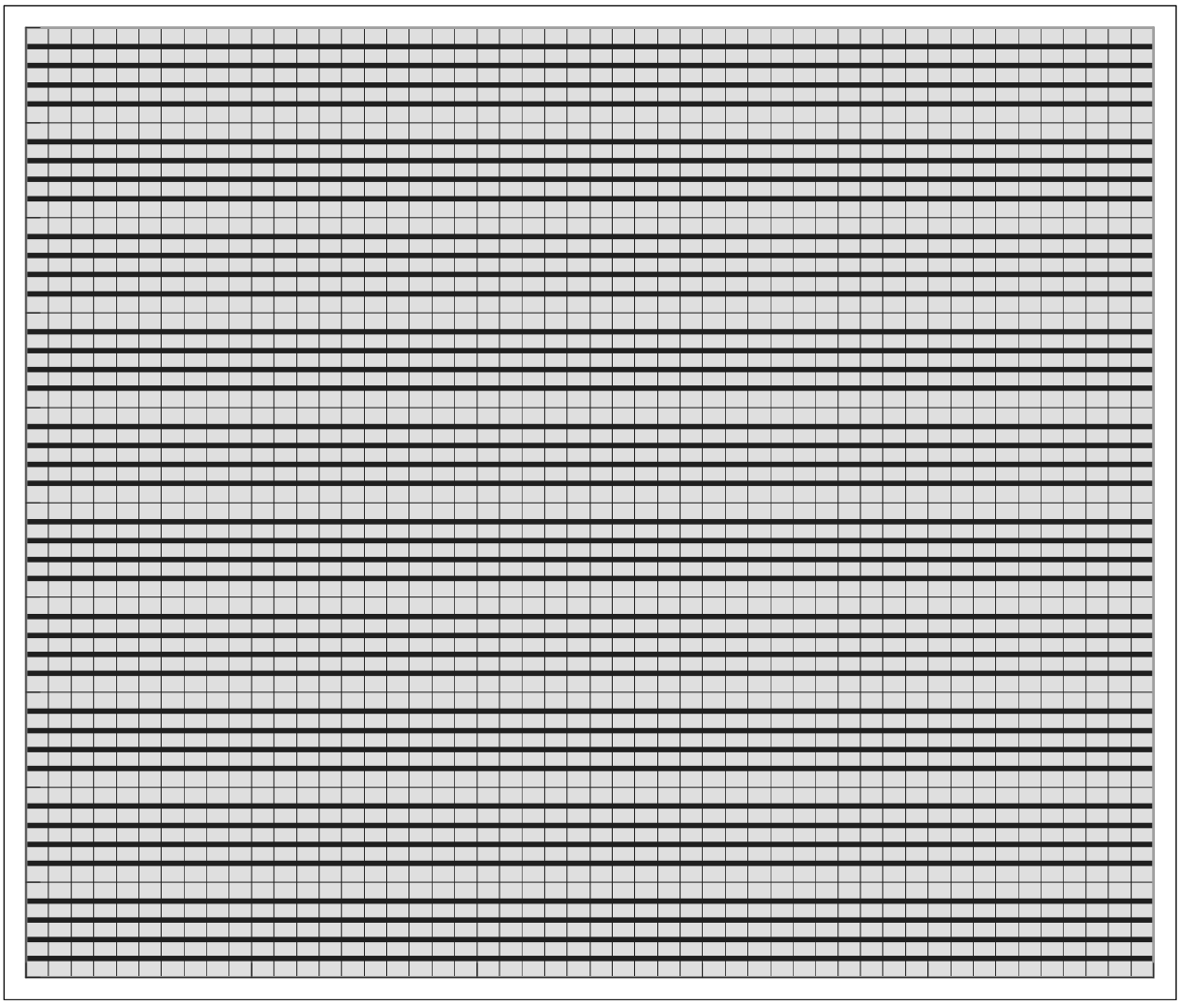
3) Mix well and incubate microtubes at 37°C for 5 min.

4) Put the content of each microtube in a spectrophotometric cuvette.

5) Read absorbance in a spectrophotometer at 505 nm (**When you are ready, to read in the spectrophotometer, please call the lab assistant)**

6) Plot the absorbance versus the amount of glucose (in µg) on the plotting paper below.

| | Dilutions | | | | |
|---|---|---|---|---|---|
| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 |
| glucose (µg in the reaction mix) | | | | | |
| Absorbance at 505 nm | | | | | |



**TASK 2: Determination of the glucose concentration in a sample employing the standard curve obtained before. (10 points)**

1) Perform the glucose oxidase reaction, to the glucose sample (unknown concentration), according to the follow squeme.

| | Sample | Blank |
|---|---|---|
| Sample volumen | 10µl | 0 |
| Water volumen | 0 | 10 µl |
| Glucosa oxidase reagent volumen | 1 ml | 1 ml |

2) Mix well and incubate microtubes at 37°C for 5 min.

3) Put the content of each microtube in a spectrophotometric cuvette

4) Read absorbance in the spectrophotometer at 505 nm **(When you are ready to read in the spectrophotometer please call the lab assistant)**

5) Using the calibration curve, calculate the glucosa concentration of the sample in µg. $ml^{-1}$.

| | |
|---|---|
| Absorbance of the sample | |
| Concentration of the sample (in µg . $ml^{-1}$) | |

**QUESTION 1:** As many glucose assays measure the peroxide produced by the glucose oxidase reaction, it is important that the enzyme used for these assays presents: **(1.5 points)**

A) a low catalase content.

B) a high catalase content.

C) a low peroxidase content.

D) a high peroxidase content.

WRITE DOWN THE LETTER CORRESPONDING TO CORRECT ANSWER

**Answer**:.....

**QUESTION 2:** If such a condition (question 1), is not taken in account the obtained results will give **(1.5 points)**:

A) underestimation of the glucose in the assay.

B) overestimation of the glucose in the assay.

C) not effect in the assay.

WRITE DOWN THE LETTER CORRESPONDING TO CORRECT ANSWER

**Answer**:.....

**QUESTION 3:** The most favorable pH value (the point at which the enzyme is most active) is known as the optimum pH. Extremely high or low pH values usually result in a complete loss of enzyme activity due to **(1 point)**:

A) The breakdown of the secondary structure of the protein.

B) The breakdown of the tertiary structure of the protein.

C) The breakdown of the primary structure of the protein.

SELECT THE CORRECT ANSWER, FILLING THE CORRESPONDING BOX

| | | | |
|---|---|---|---|
| A. | B. | C. | A, B |
| A, C | B, C. | | A, B, C. |

**QUESTION 4:** Glucose oxidase from *Aspergillus niger* was overexpressed in yeast. The recombinant glucose oxidase was purified and glycosylation pattern was analyzed by treatment with endoglycosidase H and α-mannosidase. After treatment, an aliquot was used for SDS-PAGE (electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate). The remaining enzyme was employed for determination of the *K*M (Michaelis-Menten constant) with glucose as the substrate. **(7 points)**

The values of the *K*M for each glycoforms are shown below the figure 1.

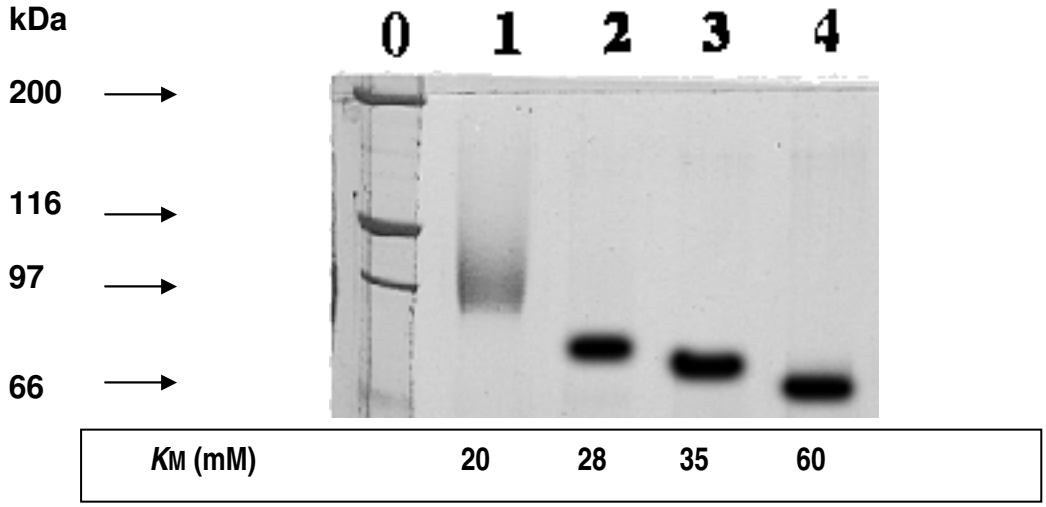



Figure 1: Analysis of the deglycosylation of Glucose Oxidase by 7.5% acrylamide SDS-PAGE gel electrophoresis. Lane 0 is the molecular mass standard. Lane 1 untreated enzyme. Lane 2 endoglycosidase H treated enzyme. Lane 3 α-mannosidase treated enzyme. Lane 4 endoglycosidase H and α-mannosidase treated enzyme (fully deglycosylated enzyme).

From the results obtained by treatment of the glucose oxidase with endoglycosidase H and α-mannosidase it is possible to arrive to the follow conclusions. Which of the following statements is/are correct?

A) Glucose oxidase is a homodimer with a molecular mass of 96 kDa.

B) The deglycosylated form has a molecular mass of 68 kDa.

C) Glucose oxidase is glycosylated since the treatment with endoglycosidase H and α-mannosidase results in a form with lower molecular mass.

D) The polysaccharide moiety of glucose oxidase contains *N*-acetylglucosamine and mannose.

MARK THE CORRECT ANSWER/ ANSWERS.

A B C D

From the results obtained in the determination of *K*M for each glycoform, the following conclusion could be made. Which of the following statements is/are correct?

A) The affinity of the fully glycosylated enzyme for glucose is higher than the affinity of the deglycosylated enzyme

B) The glucose oxidase activity is completely abolished in the deglycosylated form

C) The lack of the sugar moiety could cause changes in the structure of the active site of the enzyme resulting in the observed modifications of the *K*Ms.

MARK THE CORRECT ANSWER/ ANSWERS.

A B C

**QUESTION 5:** Another treatment consisted in the purification of the recombinant glucose oxidase under non-denaturating conditions and in presence of glutaraldehyde. The purified enzyme was analyzed by native PAGE (without SDS). Prior to loading, the samples were resuspended in loading buffer with (+) and without (-) DTT (a reducing agent). The obtained results are shown in figure 2 **(4 points)**

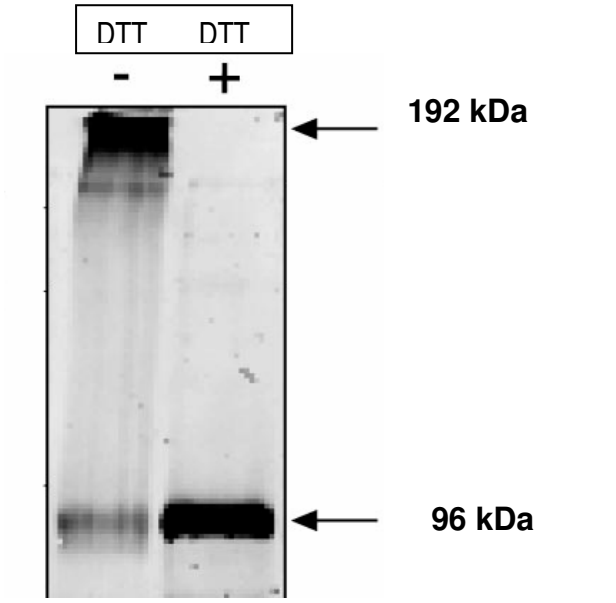



Figure 2: Native PAGE (non-denaturating electrophoresis in polyacrylamide gels) of recombinant glucose oxidase purified under non-denaturating conditions.

Taking in account the results obtained from figure 1 and figure 2 the most probable conformation of the recombinant glucose oxidase is:

A) A monomeric enzyme non-glycosylated.

B) A monomeric enzyme glycosylated.

C) An homodimer consisting of two monomers both glycosylated.

D) An heterodimer consisting of two subunits one of them glycosylated.

MARK THE CORRECT ANSWER.

A B C D