PRACTICAL EXERCISE: Determination of the Specific Activity of Catalase and Glutathione S-Transferase in the Gills and Hepatopancreas of the Mediterranean Mussel

1. Materials and Reagents

1.1 Biological Material

- Mussel gills (Mytilus galloprovincialis)
- Mussel hepatopancreas (Mytilus galloprovincialis)

1.2 Reagents

- Potassium dihydrogen phosphate (KH_2PO_4)
- Dipotassium hydrogen phosphate (K_2HPO_4)
- Hydrogen peroxide (H_2O_2)
- Distilled water
- MQ water

Think about it:

1. What are the main functions of the gills and hepatopancreas in mussels?
2. Why might CAT and GST activities be expected to differ between the gills and hepatopancreas?
3. What is oxidative stress, and how may it be associated with an organism's exposure to microplastics or pollutants?

1.3 Buffers and Reaction Mixture

1.3.1 Potassium Phosphate Buffer (100 mM, pH 7.4)

The buffer is used for tissue homogenisation and, when necessary, for sample dilution.

1. Prepare a 100 mM KH_2PO_4 solution by weighing 8.164 g of KH_2PO_4 and adding distilled water to a final volume of 600 mL.
2. Prepare a 100 mM K_2HPO_4 solution by weighing 10.45 g of K_2HPO_4 and adding distilled water to a final volume of 600 mL.
3. Mix the solutions by gradually adding the K_2HPO_4 solution to the KH_2PO_4 solution until a pH of 7.4 is reached.
4. If necessary, adjust the pH further using either of the two components.
5. Mix the buffer thoroughly and store at 4 °C.

1.3.2 Potassium Phosphate Buffer (50 mM, pH 7.0)

The buffer is used to prepare the reaction mixture for the determination of catalase activity.

1. Prepare solutions of KH_2PO_4 and K_2HPO_4 in distilled water.
2. Mix with MQ water at a ratio of 1:1 to obtain a final concentration of 50 mM.
3. Adjust the pH to 7.0.
4. Mix the buffer thoroughly and store at 4 °C.

1.3.3 Reaction Mixture (H_2O_2 in 50 mM Potassium Phosphate Buffer)

1. Add 11 µL of H_2O_2 to 10 mL of 50 mM potassium phosphate buffer (pH 7.0).
2. The final H_2O_2 concentration in the reaction mixture is 10.2 mmol/L.
3. Prepare the reaction mixture immediately before use, according to the number of samples, for example for 10–20 measurements.
4. Protect the solution from light by wrapping it in aluminium foil and keep it on ice until use.

2. Equipment and Laboratory Supplies

- Potter–Elvehjem homogeniser
- Centrifuge capable of cooling to 4 °C
- Eppendorf tubes or microcentrifuge tubes
- Pipettes and appropriate tips
- Analytical balance
- pH meter
- Refrigerator (4 °C)
- Freezer (−80 °C)
- Ice maker or container with ice
- UV-transparent microplate
- SPARK microplate reader

3. Procedure

3.1 Collection of Tissue Samples

1. Before starting the experiment, prepare and label microcentrifuge tubes for gill and hepatopancreas samples for all enzyme assays (SOD, CAT and GST).
2. For the enzyme assays, collect pooled samples containing tissues from ____ mussels from each aquarium.
3. Place the mussels on stainless-steel trays.
4. As soon as possible after removing the mussels from the water, dissect out the gills and hepatopancreas.
5. Drain the tissues on a piece of paper towel and transfer each tissue separately into a pre-labelled microcentrifuge tube.
6. Keep the samples on ice during collection, then store them in a freezer at −80 °C.
7. Discard the remaining parts of the mussels.

3.2 Tissue Homogenisation and Preparation of the Protein Extract

1. Preparation of the homogenisation buffer: Prepare 100 mM potassium phosphate buffer (KH_2PO_4/K_2HPO_4, pH 7.4) as described in Section 1.3.2. Store at 4 °C until use.
2. Homogenisation: Homogenise each tissue sample in potassium phosphate buffer at a ratio of 1:3 (tissue wet mass : buffer volume) using a Potter–Elvehjem homogeniser. Keep the samples on ice throughout the procedure.
3. Centrifugation: Transfer the homogenates to microcentrifuge tubes and centrifuge for 40 minutes at 10,000 rpm and 4 °C.
4. Preparation of the protein extract: After centrifugation, carefully pipette the supernatant into new, appropriately labelled microcentrifuge tubes. Avoid disturbing or aspirating the pellet.
5. Storage: Store the prepared protein extracts at −80 °C until catalase (CAT) activity is determined.

Think about it:

1. Why are tissues kept on ice during homogenisation and sample preparation?
2. What is the purpose of centrifuging the homogenate? Which fraction of the sample is used to determine enzyme activity, and why?
3. Why is it important to use an appropriate amount of homogenate or protein when comparing enzyme activities?

3.2 Preparation of Samples for Determination of Specific Catalase Activity

1. Thaw the supernatants on ice before measurement.
2. If necessary, dilute the samples with 100 mM potassium phosphate buffer (pH 7.4).
3. The recommended initial dilution of the homogenate is 1:3. If necessary, also test additional dilutions: 5-fold, 10-fold and 15-fold.
4. Test different combinations of homogenate and reaction-mixture volumes; the final reaction volume must be 300 µL.
5. Check the linearity of the measurements. An R^2 value above 0.96 is desirable, and ideally it should be above 0.98.

3.3 Determination of Specific Catalase Activity in a Microplate

Measurement Conditions

Parameter	Setting
Wavelength	240 nm
Temperature	25 °C
Total measurement time	1 minute
Measurement interval	10 seconds
Plate type	UV-transparent microplate
Final reaction volume	300 µL

Measurement Procedure

1. For each sample, prepare one blank measurement and three technical replicates of the sample.
2. Pipette 15 µL of sample into the appropriate wells of the microplate.
3. Place the plate in the SPARK reader.
4. Add 285 µL of reaction mixture containing H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) to each well.
5. Start the measurement immediately after adding the reaction mixture.
6. Measure absorbance at 240 nm every 10 seconds for a total of one minute.

Think about it:

1. What is the function of catalase in the cell? Write down the reaction it catalyses.
2. Why is absorbance measured at 240 nm when determining catalase activity?
3. Why does absorbance decrease during the reaction? What does the rate of this decrease tell us about catalase activity?
4. Why is it necessary to check measurement linearity and, if needed, use different sample dilutions?
5. What is the purpose of the blank and technical replicates?

3.3.1 Sample Preparation for Determination of Specific Glutathione S-Transferase Activity

The same protein extracts used to determine catalase (CAT) activity are used to determine GST activity. The homogenates must be prepared in 100 mM potassium phosphate buffer (pH 7.4).

Thaw the samples on ice before measurement.

3.3.2 Reagent Preparation for Determination of Specific Glutathione S-Transferase Activity

Prepare the reagents for one microplate.

Solution A – Cl-2,4-DNB

1. Dissolve 10.128 mg of Cl-2,4-DNB (Sigma, Cat. No. 237329-10G) in 1 mL of 96% ethanol.
2. Add 800 µL of the prepared Cl-2,4-DNB solution to 9.2 mL of 100 mM potassium phosphate buffer (pH 6.5).
3. The solution will turn white/cloudy.

Solution B – Reduced L-glutathione

4. Dissolve 3.07 mg of reduced L-glutathione (Sigma, Cat. No. G4251-5G) in 5 mL of 100 mM potassium phosphate buffer (pH 6.5).

To prepare reagents for three microplates, the following volumes are required:

- 30 mL of Solution B
- 15 mL of Solution A

3.3.3 Measurement of Specific Glutathione S-Transferase Activity

Measurement Conditions

- Wavelength: 340 nm
- Temperature: 25 °C
- Measurement duration: 10 minutes
- Measurement interval: 20 seconds

Procedure

1. Pipette the homogenate into the microplate wells:
2. hepatopancreas: 50 µL;
3. gills: 10 µL.
4. Add 100 µL of Solution B.
5. Then add 50 µL of Solution A.
6. Immediately after adding Solution A, start measuring absorbance at 340 nm and a temperature of 25 °C.
7. Measure absorbance for 10 minutes at 20-second intervals.

3.3.4 Calculation of GST Activity

Express GST activity as nmol of product/min/mg protein. Use an extinction coefficient of ε = 9600 L mol^{-1} cm^{-1} for the calculation.

Think about it:

1. What role does glutathione S-transferase play in cellular detoxification?
2. What roles do reduced glutathione (GSH) and the substrate CDNB play in the reaction being monitored?
3. Why is absorbance measured at 340 nm when determining GST activity, and how does it change during the reaction?
4. Why must the amount of protein in the sample be taken into account when calculating specific activity?

3.4 Determination of Protein Concentration Using the BCA Method in a Microplate

The BCA (bicinchoninic acid) method is based on the colorimetric determination of total protein concentration. Protein concentration is determined from absorbance at 562 nm and a calibration curve prepared using bovine serum albumin (BSA) standards. This protocol is based on the manufacturer's instructions for the Pierce™ BCA Protein Assay Kit.

3.4.1 Reagent Preparation

Working Reagent (WR)

1. Before preparation, calculate the required volume of working reagent based on the number of standards, samples and replicates.
2. Mix 50 parts of Reagent A with 1 part of Reagent B (ratio 50:1).
3. Mix the reagents thoroughly. When Reagent B is added to Reagent A, the mixture may initially become cloudy; this cloudiness disappears upon mixing.
4. Prepare enough working reagent to provide 200 µL for each well.

3.4.2 Preparation of BSA Standards

To prepare the calibration curve, use a bovine serum albumin (BSA) standard at a concentration of 2000 µg/mL. Prepare the standards by appropriate dilution in a diluent that is the same as, or as similar as possible to, the one used to prepare the samples.

Standard	Final BSA concentration (µg/mL)
A	2000
B	1500
C	1000
D	750
E	500
F	250
G	125
H	25
I (blank)	0

Prepare the standards according to the manufacturer's dilution scheme. For the blank, use the diluent only, without BSA.

3.4.3 Sample Preparation

1. Prepare the tissue protein extracts stored at −80 °C.
2. Thaw the samples and dilute them appropriately if necessary.
3. For the standard procedure, pipette 25 µL of each sample into the microplate. Analyse each sample and standard using an appropriate number of replicates.
4. Also load the BSA standards and the blank onto the plate.

If the sample volume is limited, 10 µL of sample may be used; however, this changes the applicable concentration range of the method.

3.4.4 Determination of Protein Concentration

Measurement Conditions

- Sample or standard volume: 25 µL
- Working reagent volume: 200 µL
- Total volume per well: 225 µL
- Incubation: 30 minutes at 37 °C
- Wavelength: 562 nm

Procedure

1. Pipette 25 µL of each BSA standard or sample into the appropriate microplate wells.
2. Add 200 µL of working reagent (WR) to each well.
3. Shake the plate on a microplate shaker for 30 seconds.
4. Cover the plate and incubate for 30 minutes at 37 °C.
5. After incubation, allow the plate to cool to room temperature.
6. Measure absorbance at 562 nm using a microplate reader.
7. Subtract the mean blank absorbance from the absorbance of each standard and sample.

3.4.5 Calculation of Protein Concentration

1. Use the mean blank-corrected absorbance values of the BSA standards to prepare a calibration curve showing the relationship between absorbance and protein concentration.
2. Determine the protein concentration in each sample using the calibration curve.
3. For diluted samples, multiply the calculated concentration by the dilution factor to obtain the protein concentration in the original sample.

Express protein concentration in µg/mL or mg/mL. Use the resulting values to calculate specific enzyme activity, expressed per mg of protein.

Think about it:

1. What principle does the BCA method use to determine protein concentration?
2. What does the calibration curve show, and how is it used to determine the protein concentration of an unknown sample?
3. Why is protein concentration determined when calculating the specific enzymatic activities of CAT and GST?

Discussion Questions

1. Plot absorbance against time for a selected CAT sample and a GST sample. How do the shapes of the curves differ between the two enzymes?
2. How can the initial reaction rate for CAT and GST be determined from the absorbance time course?
3. What could a non-linear change in absorbance during the measurement indicate? Give at least two possible causes.
4. Why can CAT and GST activities not be compared directly based only on the change in absorbance per minute?
5. Why is enzyme activity expressed per milligram of protein rather than only per gram of tissue?
6. If sample A had higher enzyme activity than sample B, would this necessarily mean that organism A had been exposed to greater oxidative stress? Explain.
7. If increased CAT activity, decreased GST activity, or the opposite pattern were observed in exposed mussels, how could these results be explained?
8. How can microplastics and pollutants bound to them affect the oxidative balance in mussels?
9. Can increased antioxidant enzyme activity indicate a protective response by the organism? Under what circumstances might enzyme activity be decreased?
10. Why is it useful to measure several biomarkers simultaneously, rather than only CAT or GST activity, when assessing the effects of pollutants?
11. What additional measurements would you include to better assess oxidative stress and possible cellular damage?
12. Could the overall health status of a mussel be inferred from a single CAT or GST measurement? Justify your answer.