Assay of hemolymph phenoloxidase activity

Reagents:

1. 10 mM Phosphate buffer: Na_2HPO_4 (dibasic, 10 mM, 138 mg in 100 ml) and NaH_2PO_4 (monobasic, 10 mM, 142 mg in 100 ml). Add monobasic to dibasic drop by drop to bring pH to 7.0.
2. DL-3,4, dihydroxyphenylalanine (DL-DOPA, FW 197.2) Make a solution of 3mg in 1ml of phosphate buffer. (since DL-DOPA is not readily soluble in water or phosphate buffer, it is necessary to dilute it first in a small volume of acetone and then make up the volume to 1ml with phosphate buffer).

Procedure:

3. 5 µl of whole hemolymph is diluted with 95 µl of 10 mM phopsphate buffer (pH 7.0).
4. Centrifuge at 10,000g at 4˚C for 5 min.
5. 40 µl of hemolymph-buffer supernatant is taken and mixed with 160 µl of L-DOPA in a microtiter plate.
6. The linear increase in absorbance at 492 nm over a 30 min period is recorded in a microtitre plate reader (ELISA reader).
7. Enzyme activity is expressed as au_{492} /min/ µl (absorbance units)

Determination of reaction rate in phenoloxidase assay:

1. Determine the change in absorbance (ΔA_{492}) per minute by a) plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve b) select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

ΔA_{492}/min = A_{492} (Time 2) – A_{492} (Time1)/ Time 2(min) – Time 1(min)

2. Determine the rate of ΔA_{492} /min for the background or non-enzymatic wells and subtract this rate from that of sample wells.