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ASSAY / ANALYTICAL PROCEDURE
PEROXIDASE_PYROGALLOL

1. METHOD OF ASSAY:

The method of assay measures the oxidation of pyrogallol to purpurogallin when catalysed by peroxidase at 420nm and at 20^{O}C.

2. UNIT DEFINITION:

One unit of peroxidase is defined as the amount of enzyme required to catalyse the production of 1 mg of purpurogallin from pyrogallol in 20 seconds at 20^{O}C under the assay conditions described.

3. REAGENTS:

3.1 1M NaOH

Dissolve 20 g NaOH [MM 40,0] in ± 450 ml distilled H_2O. Cool solution and adjust volume to 500 ml with distilled H_2O.

3.2 0,1M PHOSPHATE BUFFER pH6,0

Dissolve 13,6g of KH_2PO_4 [MM 136,09] in ± 800 ml of distilled H_2O. Adjust to pH 6,0 with 1M NaOH [3.1]. Adjust volume to 1000 ml [in a volumetric flask]. Buffer stable for up to one month at 2^{O}C to 8^{O}C.

3.3 SUBSTRATE [H_2O_2 SOLUTION]

Dilute 1 ml 30% H_2O_2 to a final volume of 75 ml with distilled H_2O. [Check A_{240} by diluting $^{1}/_{15}$. Reading should be ± 0,4]. Adjust if necessary. Prepare fresh daily.

3.4 ENZYME SOLUTION

For F/D POD, dissolve F/D material to a concentration of 10 mg/ml in buffer [3.2]

For POD PPT, dissolve a minimum of 300 mg PPT to a concentration of 10 mg/ml in buffer [3.2] Immediately prior to assay, dilute appropriately in buffer [3.2] to yield 0,5 – 1,5 u/ml [$0,6 < \Delta A_{420/min} < 1,8$].

3.5 5,33% PYROGALLOL SOLUTION

Dissolve 533 mg pyrogallol [MM 126,11] to a concentration of 53,3 mg/ml in H_2O. Store on ice and in an amber bottle. Prepare fresh daily.

4. PROCEDURE:

λ: 420nm; light path: 10 mm; temp.: 20^{O}C.

Pipette the following into a 10 mm cuvette:

Buffer [3.2]	2,40 ml
Pyrogallol solution [3.5]	0,30 ml
H_2O_2 solution [3.3]	0,20 ml

Equilibrate in a water bath until temperature reaches 20^{O}C.

Add enzyme [3.4]	0,10 ml
	3,00 ml

Start recording immediately.

Repeat procedure until each weighing has been assayed in triplicate. Rinse cuvette with chromic acid after each assay.

5. CALCULATION:

$$\text{Activity [u/mg]} = \frac{\Delta A_{420/min} \times TV \times DF}{12,0 \times SV \times EC \times 3}$$

Where:

12 = A_{420nm} of a 1 mg/ml solution of purpurogallin.
TV = 3,0 ml [cuvette volume].
DF = Dilution Factor.
SV = 0,10 ml [Sample Volume].
EC = Enzyme concentration in mg/ml.
3 = $\Delta A_{420/min} / \Delta A_{420/20sec}$

Determine the mean value for each lot of enzyme. [Reference: Genzyme Doc. No. AM155-1 Version 01].

Spectrophotometer Settings: Set total time to 120 seconds (2 minutes); Set max absorbance to 2,5

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ASSAY / ANALYTICAL PROCEDURE
PEROXIDASE GUAIAUCOL

1. METHOD OF ASSAY:

Based on that of Bergmeyer in which the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, is determined by measuring the rate of colour development spectrophotometrically at 436 nm and at 25°C.

$$4 \text{ guaiacol} + 4H_2O_2 \xrightarrow{\text{PEROXIDASE}} \text{Tetraguaiacol} + 8H_2O$$

2. UNIT DEFINITION:

That amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C.

3. REAGENTS:

3.1 0,1 M Potassium Phosphate Buffer pH 7,0

Dissolve 5,3g KH_2PO_4 [MM 136,09] and 10,6g K_2HPO_4 [MM 174,18] in distilled H_2O, check pH to 7,0 and dilute to 1000 ml. Store diluent on ice and equilibrate buffer at 25°C. Enter details in the reagent preparation book.

3.2 0,018 M GUAIACOL

Accurately weigh off ± 20 mg liquid guaiacol (MM 124,14) and dissolve (by stirring) in distilled water to a concentration of 2,23 mg/ml. Keep reagent on ice. Prepare fresh daily.

3.3 SUBSTRATE

Using a pipette, dispense one drop of 30% hydrogen peroxide in ± 10 ml distilled water. Check and adjust A_{240} versus distilled water. [$0,40 \leq A_{240} \leq 0,41$]

3.4 ENZYME SOLUTION

Dissolve 5 mg enzyme / ml 0,1M ice cold potassium phosphate buffer pH 7,0 (refer reagent 3.1 above). Immediately before assay, dilute to yield approximately 0,2 units/ml ice cold buffer. (0,040 to 0,045 ΔA_{436} / minute.)

4. PROCEDURE:

Into a 1cm quartz cell, pipette the following at:

Temperature = 25°C
Wavelength = 436 nm
Light Path = 1 cm

Buffer	2,80 ml
Guaiacol	0,05 ml
Substrate	0,05 ml

Equilibrate at 25°C and monitor ΔA/minute

Enzyme at zero time	0,10 ml
Total reaction volume	3,00 ml

Record the rate of increase in absorbance at 436 nm using the linear portion of the curve after the initial lag phase.

5. CALCULATION:

$$\text{Volume activity (U / ml)} = \frac{\Delta A_{436} / \text{min} \times 4 \times Vt \times \text{dilution factor}}{\varepsilon \times Vs}$$

$$U/ml = \frac{\Delta A_{436} / \text{min} \times 4 \times 3 \times \text{dilution factor}}{25,5 \times 0,1}$$

$$U/ml = \Delta A_{436} / \text{min} \times 4,7059 \times \text{dilution factor}$$

Where
- Vt = final volume of reaction mixture (ml) = 3,00
- Vs = sample volume (ml) = 0,10
- ε = micromolar extinction co-efficient of tetraguaiacol ($cm^2/\mu mol$) = 25,5
- 4 = derived from unit definition & principle

$$\textbf{Weight activity} \text{ (U / mg material)} = \frac{U / ml}{\text{mg enzyme / ml original solution}}$$

6. REFERENCE:

Bergmeyer H.U. : Methods of Enzymatic Analysis 1, Academic Press, New York 2nd Edition (1974), page 495