Peroxidase

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Peroxidase (POD) occurs in plants and in certain animal cells. The enzyme catalyses the oxidation of many organic compounds by hydrogen peroxide: amines (*o*-phenylenediamine[1], *p*-phenylenediamine[2], benzidine[3]), phenols (pyrogallol[4], guaiacol[5,6], *o*-cresol[7], hydroquinone[8,9]), leuco-dyes (leuco-malachite green[10], leuco-2,6-dichlorophenolindophenol[11–13]). Ascorbic acid can also act as a hydrogen donor in conjunction with other oxidizable compounds, for example, *o*-toluidine[14], as can mixtures of phenols and amines[15–17], for example, "Nadi reagent"[16] (α-naphthol + *p*-phenylenediamine). The specificity of peroxidase for peroxides is high. Only H_2O_2, methyl and ethyl hydroperoxides combine with peroxidase. On the other hand, the specificity of the peroxidase-hydroperoxide complex for the organic compounds mentioned above is low. A comparison of the activity of peroxidases of different origin is difficult if different substrates are used.

To measure the peroxidase activity the oxidation product or the unchanged substrate is determined chemically or spectrophotometrically at different reaction times. It should be noted that, in the absence of peroxidase, the oxidation of certain organic compounds can be catalysed by H_2O_2 and traces of heavy metals[18].

The methods employing *p*-phenylenediamine[2,19] and guaiacol[5,6] have proved successful even with turbid enzyme solutions.

Principle

Peroxidase catalyses the reaction:

(1) $$AH_2 + H_2O_2 \longrightarrow 2\,H_2O + A$$

AH_2 is a hydrogen donor and A is its oxidized form. In the method described here *p*-phenylenediamine is the hydrogen donor. It is oxidized by H_2O_2 and peroxidase to a coloured derivative which is probably a molecule formed from diamine and diimine. The POD activity is measured by the increase in colour (optical density at 485 mμ) per unit time.

Optimum Conditions for Measurements

The pH optimum depends on the hydrogen donor; it is generally at pH 7.0. Horse radish peroxidase is stable between pH 4.5 and 12, depending on the type of ions present. The rate of the peroxidative reaction depends on the hydrogen peroxide concentration: $>10^{-3}$ M H_2O_2 the activity usually decreases rapidly. Only with low hydrogen peroxide concentrations are the reaction curves linear from the start of the reaction.

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Reagents

1. Hydrogen peroxide *), about 30% (w/v)
2. *p*-Phenylenediamine, $H_2N-C_6H_4-NH_2$
3. Potassium dihydrogen phosphate, KH_2HPO_4, A. R.
4. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\,H_2O$, A. R.

Preparation of Solutions

Prepare all solutions with doubly distilled water to avoid the presence of trace metals.

I. Hydrogen peroxide (3×10^{-3} M):
Stock solution: dilute 0.66 ml. hydrogen peroxide (30% w/v) to 200 ml. with doubly distilled water. Adjust the solution to exactly 0.03 M (*e.g.* titrate against 0.1 N $KMnO_4$; 10 ml. 0.03 M H_2O_2 = 6 ml. 0.1 N $KMnO_4$).
Just before use dilute the stock solution 1:10 with doubly distilled water.

II. *p*-Phenylenediamine (1% w/v):
Dissolve 1 g. *p*-phenylenediamine in about 60 ml. hot doubly distilled water, filter and wash the filter with about 25 ml. hot doubly distilled water. Cool the filtrate quickly and dilute to 100 ml. with doubly distilled water.

III. Phosphate buffer (0.067 M; pH 7):
Dissolve 3.522 g. KH_2PO_4 and 7.268 g. $Na_2HPO_4 \cdot 2\,H_2O$ in doubly distilled water and make up to 1000 ml.

Stability of the solutions

The H_2O_2 stock solution keeps in a refrigerator for about a month. Prepare the dilute H_2O_2 solution and the *p*-phenylenediamine solution freshly each day. Store the *p*-phenylenediamine solution in a brown bottle protected from light. The buffer solution is stable indefinitely providing bacterial contamination is avoided (from time to time boil for a short period).

Procedure

Experimental material

Tissue: Homogenize tissue in a blendor (see p. 50) with 6.7×10^{-3} M phosphate buffer, pH 7 (solution III diluted 1:10 with doubly distilled water) and allow to stand in the cold for some hours with occasional shaking. Repeat the extraction once or twice.

Foodstuff: Treat foodstuff as for tissue or extract by grinding in a mortar with the buffer. Use milk directly after diluting 1:200 to 1:400[19]. To test for the pasteurization of milk, see the official description[20].

Spectrophotometric measurements

Wavelength: 485 mμ; (with photometers requiring filters use the appropriate filter, *e.g.* Zeiss S 49); light path: 1 cm.; final volume: *ca.* 3.5 ml. (in order to mix more quickly use cuvettes with glass lids); temperature: 20°C (room temperature); in comparative studies use a constant temperature cuvette holder; control cuvette: as for experimental cuvette, but containing doubly distilled water instead of H_2O_2. Read against this cuvette.

*) *e.g.* "Perhydrol" Merck.

20) *G. Schwarz, B. Hagemann, C. Hüttig, R. Kellermann* and *W. Staege:* Die chemischen und bakteriologischen Untersuchungsverfahren für Milch und Milcherzeugnisse und Molkereihilfsstoffe. Neumann-Verlag, Radebeul (Germany) 1950, p. 20.

Pipette successively into the cuvette:

2 ml. sample
1 ml. phosphate buffer (solution III)
0.1 ml. H_2O_2 solution (I)
0.1 ml. *p*-phenylenediamine solution (II).

Mix thoroughly, start a stopwatch and read the optical density every 30 sec. for the first 2 or 3 min.

Calculations

The rate constant k of zero order reactions can serve to determine the activity *), since the initial part of the reaction curve is linear:

$$k \approx \frac{\Delta x}{\Delta t} \approx k' \times a_0 \times e \tag{2}$$

where Δt = measured reaction time
Δx = amount of substrate or donor converted
k = zero order reaction rate constant
k' = proportionality factor
a_0 = initial concentration of the donor
e = enzyme concentration

With very low donor concentrations k is proportional to e and also to a_0, so that $k \approx k'a_0 \times e$. For relative measurements it is sufficient to substitute the change of optical density ΔE in place of Δx and so calculate $k'' = \Delta E/t$ per ml. enzyme solution or per mg. enzyme preparation. For more accurate studies the activity of the enzyme solution under examination should be compared with a peroxidase preparation of known activity.

The unit of activity of a peroxidase preparation is frequently given as the amount of purpurogallin [mg.] which is formed in 5 min. at 25°C from 1.25 g. pyrogallol in 500 ml. solution containing 12.5 mg. H_2O_2. The "purpurogallin number" (P. N.)[4] is

$$\text{P. N.} = \frac{\text{mg. purpurogallin}}{\text{mg. enzyme preparation}} \tag{3}$$

A conversion of k or k'' values to P. N. is not possible.

Example

The following optical densities were measured after 30, 60, 90 and 120 sec. with milk diluted 1:200: 0.079; 0.110; 0.135; 0.152. The optical densities (ordinate) were plotted against the time (abscissa). The first three points lay on a straight line, which cut the ordinate (0 sec.) at $E = 0.051$. The optical density increase in 90 sec. after the start of the reaction was therefore: $\Delta E = 0.135 - 0.051 = 0.084$. Therefore according to equation (2)

$$k'' = \frac{\Delta E}{\Delta t} = \frac{0.084}{90} = 0.00093$$

The activity per ml. milk is $k'' \times \frac{200}{2} = 0.093$. This means that the milk was not heated at a high enough temperature or that it contained raw milk.

*) The purity (purity number[21]) of a peroxidase preparation is defined by the quotient of the optical density of the haem component (Soret band, with horse radish peroxidase at 403 mμ) and the optical density of the protein (275 mμ). For crystalline horse radish peroxidase the purity number is 3.04[21]. The purity of a preparation gives no information on its activity.

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