Inorganic Peroxides

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Principle

Hydrogen peroxide is decomposed by peroxidase. The oxygen liberated in this process oxidizes a colourless hydrogen donor DH_2 to a coloured compound D:

(1) $$H_2O_2 + DH_2 \longrightarrow 2\,H_2O + D$$

Aromatic amines (*e.g.* *o*-dianisidine, *o*, *m*, or *p*-toluidine) are the usual hydrogen donors, but amine derivatives, phenols, quinones, *etc.*, can also be used. *o*-Dianisidine, which is used in this method, yields a red-brown dye with a broad absorption maximum at 460 mμ. The extinction coefficient depends on the experimental conditions, so therefore the measured optical density is related to the optical density of a H_2O_2 standard. The measurements are made at 436 mμ or an adjacent wavelength. The reaction is complete in 3 min. and the colour is stable for several hours.

Reagents

1. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot 2\,H_2O$
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\,H_2O$
3. *o*-Dianisidine hydrochloride
   Commercial preparations of *o*-dianisidine (free base) are usually strongly coloured. It can be recrystallized from 25% acetone with the addition of a little charcoal. To prepare the hydrochloride dissolve 10 g. of the recrystallized base in 200 ml. distilled water and 8.5 ml. conc. HCl, add about 1000 ml. acetone and allow to stand overnight in the cold. Suck off the crystals, wash with acetone and ether, and dry *in vacuo* over KOH.
4. Peroxidase, POD
   dry powder; commercial preparation, see p. 990.
5. Hydrogen peroxide*), A. R., *ca.* 35% (w/w)
6. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)

Purity of the enzyme preparation

The POD should have a k_4 value**) of at least 35000, which corresponds to a purpurogallin number (PN)***) of 70.

Preparation of Solutions (for *ca.* 20 determinations)

I. Buffer-enzyme mixture (0.12 M phosphate, pH 7; 40 μg. POD/ml.):
Dissolve 2.07 g. $Na_2HPO_4 \cdot 2\,H_2O$, 1.09 g. $NaH_2PO_4 \cdot 2\,H_2O$ and 6 mg. POD in distilled water and make up to 150 ml.

II. Chromogen (5 mg. *o*-dianisidine hydrochloride/ml.):
Dissolve 10 mg. *o*-dianisidine hydrochloride in 2 ml. distilled water.

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*) *e.g.* Perhydrol from E. Merck, Darmstadt, Germany.

**) According to[1] $k_4 = \frac{1}{a_0 \times e} \times \frac{x}{t}$ (a_0 = initial concentration of guaiacol in the assay, e = enzyme concentration, $\frac{x}{t}$ is the hydrogen peroxide decomposed per sec.).

***) According to[2] the purpurogallin number (PN) is the amount of purpurogallin (mg.) which is formed from pyrogallol in 5 min. by 1 mg. peroxidase in a 500 ml. assay mixture at pH 7.0.

1) *P. George*, J. biol. Chemistry *201*, 413 [1953]; *B. Chance* and *A. S. Maehly* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 764.

2) *R. Willstätter* and *A. Stoll*, Liebigs Ann. Chem. *416*, 21 [1917].

III. Peroxide reagent:
With vigorous stirring, add 0.5 ml. solution (II) to 50 ml. solution (I). Store the mixture in a dark bottle. Prevent the growth of bacteria by the addition of a few drops of chloroform.

IV. Hydrogen peroxide standard solution (20 μg. H_2O_2/ml.):

a) Dilute 1.00 ml. *ca.* 35% (w/w) H_2O_2 solution in a 250 ml. volumetric flask to the mark with distilled water. Check the H_2O_2 content: dilute 20 ml. of the solution with 30 ml. distilled water and 5 ml. *ca.* 1 N H_2SO_4, and titrate with 0.1 N $KMnO_4$ to a permanent pink colour. 1.00 ml. 0.1 N $KMnO_4$ solution is equivalent to 1.70 mg. H_2O_2.

b) According to the results of the titration, dilute the appropriate volume (between 10 and 20 ml.) of solution a) to 1000 ml. with distilled water.

V. Perchloric acid (*ca.* 0.6 M):
Dilute 5.2 ml. 70% $HClO_4$ to 100 ml. with distilled water.

Stability of the solutions

Prepare the peroxide reagent (solution III) freshly each day. In the preparation, pour solution I, do not pipette. If solution III becomes turbid, it can be filtered. Always prepare the H_2O_2 standard solution just before use by dilution of the *ca.* 35% (w/w) solution which is stable.

Procedure

Experimental material

Dilute solutions of hydrogen peroxide, sodium peroxide, magnesium peroxide, sodium perborate, percarbonate, urea peroxide, strontium peroxide, *etc.* to a concentration of 5—50 μg. H_2O_2/ml. Clarify turbid solutions, fat-containing fractions and extracts of foodstuffs by filtration; the fat remains on the filter paper. If the sample is coloured by protein, then it must be deproteinized with perchloric acid.

Deproteinization

Pipette successively into a 10 ml. centrifuge tube:

1 ml. perchloric acid (solution V)
1 ml. sample.

Mix thoroughly with a thin glass rod, centrifuge for 5 to 10 min. at *ca.* 3000 g, pour the clear supernatant into a test tube and use 0.2 ml. for the assay.

Colorimetric measurements

Wavelength: 436 mμ (430 to 480 mμ): light path: 1 cm.; final volume: 5.2 ml.; room temperature. Each series of measurements requires a reagent blank and a peroxide standard. Measure against the reagent blank.

Just before use bring the peroxide reagent (solution III) to room temperature.

Pipette successively into test tubes:

Reagent blank:	5.00 ml. peroxide reagent (solution III)
	0.20 ml. distilled water
Peroxide standard:	5.00 ml. peroxide reagent (solution III)
	0.20 ml. peroxide standard solution (IV b)

Sample 5.00 ml. peroxide reagent (solution III)
0.20 ml. sample.

Mix thoroughly, allow to stand for 5 min. at room temperature and read the optical densities E_{sample} and $E_{standard}$.

Calculations

Standard curves are linear up to *ca.* 10 μg. (*ca.* 0.3 μmoles) H_2O_2/assay mixture. With optical densities over 0.600 (measured against the blank) the accuracy of the colorimeter is too low. In this case, dilute the sample or deproteinized supernatant with distilled water and assay again. For the calculations the optical density of the sample is compared to that of standard. This contains 4 μg. H_2O_2/assay mixture; therefore with a sample volume of 0.2 ml.:

$$\frac{E_{sample}}{E_{standard}} \times 5 \times 4 = \mu g.\ H_2O_2/ml.\ sample.$$

Any preliminary dilution of the sample must be allowed for in the calculations.

Example

Assay of magnesium peroxide: 107.0 mg. MgO_2 was dissolved in 1000 ml. 0.1 N H_2SO_4 and 0.2 ml. of this solution was analysed.

The H_2O_2 standard solution was prepared as follows: 16 ml. 0.1 N $KMnO_4$ solution were required to titrate solution (IVa). The solution therefore contained $16 \times 1.7/20 = 1.36$ mg. H_2O_2/ml. 14.7 ml. were diluted to 1000 ml. Solution (IVb) contained $14.7 \times 1.36 = 20$ mg./1000 ml. or 20 μg. H_2O_2/ml.

The following optical densities were measured: $E_{sample} = 0.147$; $E_{standard} = 0.224$

$$\frac{E_{sample}}{E_{standard}} \times 5 \times 4 = 13.1\ \mu g.\ H_2O_2/ml.\ sample$$

or 21.7 μg. MgO_2/ml. sample or 21.7 mg. MgO_2/107 mg. or 20.2%

Specificity and Sources of Error

Peroxidase is specific for inorganic peroxidases. The analytical results are not reproducible with organic peroxides and therefore they cannot be determined with this method.