Catalase Activity Assays

Hydrogen peroxide (H_2O_2) is a mild redox signaling agent (i.e., a molecule that helps transfer information between biochemical pathways), and it is relatively stable until it reacts with transition metals, particularly ferrous iron, to form highly reactive and toxic hydroxyl radicals by Fenton chemistry (Fig. 7.7.1A). In most cells catalase decomposes H_2O_2 to oxygen (Fig. 7.7.1B), thereby preventing radical formation catalyzed by transitional metals.

Using intact hepatocytes, the authors have shown that endogenous H_2O_2 is mostly formed by a mitochondrial flavoprotein monoamine oxidase and by the superoxide dismutase (SOD) catalyzed dismutation of superoxide radicals (Fig. 7.7.1C) generated by the mitochondrial respiratory chain and by endoplasmic reticular cytochrome P450 electron transport. The addition of monamine to hepatocytes also increases H_2O_2 formation as a result of the action of mitochondrial flavoprotein monoamine oxidase (Siraki et al., 2002). In other cells, hypoxanthine or xanthine increased H_2O_2 formation generated by cytosolic xanthine oxidase (Figure 7.7.1D). H_2O_2 is found in exhaled air, and urinary H_2O_2 levels in normal, untreated humans range from 5 to 109 μM (Halliwell et al., 2004). Oxidative stress caused by high H_2O_2 levels is inherent in pathological conditions such as cancer, diabetes, cataracts, atherosclerosis, neurodegenerative disease, aging, and nutritional deficiencies.

Catalase (located mostly in the peroxisomes), together with superoxide dismutase (SOD) and glutathione (GSH; Fig. 7.7.1E) peroxidase (both located in the mitochondria and cytosol), plays an important role in preventing cellular oxidative stress, particularly in

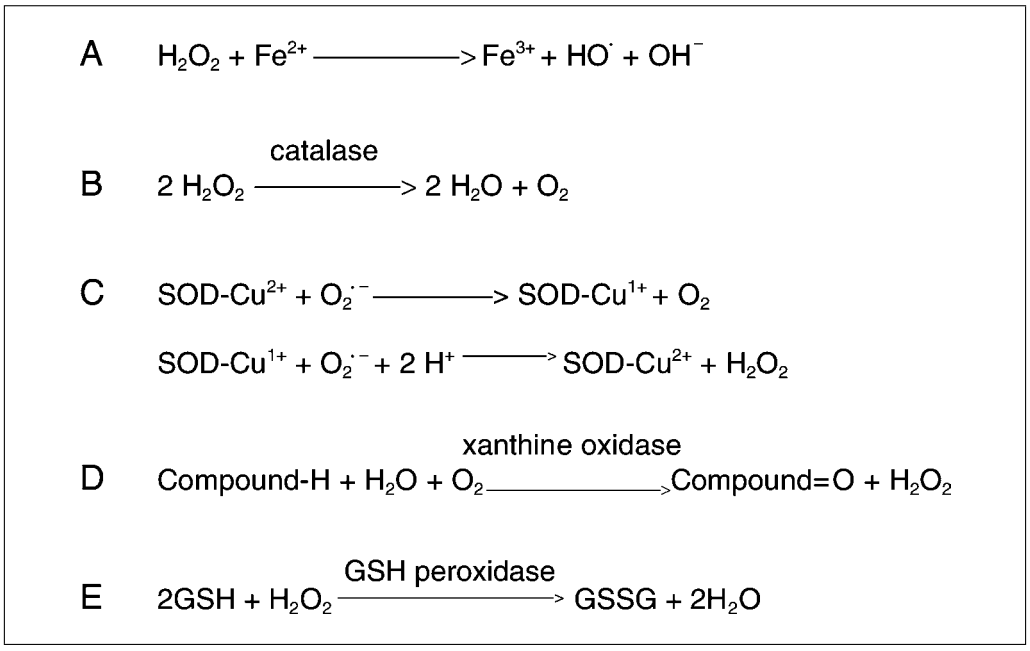

Figure 7.7.1 Chemical reactions relating to hydrogen peroxide (H_2O_2) metabolism in cells. (**A**) Toxic hydroxyl radicals are formed in the reaction of H_2O_2 with ferrous iron. (**B**) Catalase causes decomposition of H_2O_2 to H_2O and O^2. (**C**) Superoxide dismutase (SOD) produces H_2O_2 from superoxide radicals generated by the mitochondrial respiratory chain and cytochrome P450 electron transport. (**D**) Xanthine oxidase generates H_2O_2. (**E**) Glutathione (GSH) peroxidase reacts with H_2O_2 to produce oxidized GSH (GSSG).

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the peroxisomes that contain various H_2O_2-producing flavoprotein oxidases, e.g., fatty acid oxidases involved in fatty acid β-oxidation. Catalase and SOD, therefore, make an antioxidant partnership. Overexpression of catalase in cytosolic or mitochondrial compartments protects HepG2 cells against oxidative injury (Bai et al., 1999).

Catalase acts so quickly that the involvement of an enzyme-substrate complex cannot be verified. Catalase activity depends on the initial H_2O_2 concentration; furthermore, inactivation of catalase begins at 50 mM H_2O_2, an important consideration in choosing working concentration of H_2O_2 in assays.

The first method most likely used for measuring H_2O_2 decomposition by catalase was a continuous, direct spectrophotometric, kinetic assay following the disappearance of H_2O_2 at 240 nm in the 5 to 50 mM range. The method can be monitored continuously at 240 nm or discontinuously by sampling H_2O_2 at appropriate time intervals. The extinction coefficient of H_2O_2 at 240 nm is 39.4 M^{-1} cm^{-1}. The extinction coefficient represents the fraction of light that is lost due to scattering and absorption per unit distance in a participating medium. This method would be suitable if other cellular constituents were present in sufficiently low concentrations that they did not absorb strongly at 240 nm, e.g., for semipurified catalase preparations, but not for turbid cell suspensions or peroxidase-containing plant homogenates. However, there is also the problem of catalase undergoing autoinactivation, particularly at the high 50 mM H_2O_2 concentration. The catalase detection limit is 10 U/ml in this assay.

IMPORTANT NOTE: Before diluting a purchased batch of H_2O_2 to make the working solutions and standards used in the assays, always determine the actual concentration directly by measuring its absorbance at 240 nm. The extinction coefficient is 39.4 M^{-1} cm^{-1} at 240 nm.

BASIC PROTOCOL 1

DISCONTINUOUS SPECTROPHOTOMETRIC FERROUS OXIDATION ASSAY FOR H_2O_2

As discussed in the unit introduction, catalase can be monitored continuously by following the rate of decomposition of H_2O_2; however, it can also be measured discontinuously by sampling H_2O_2 at appropriate time intervals. The discontinuous ferrous oxidation assay has the advantage of using near physiological levels of H_2O_2 due to the high extinction coefficient of H_2O_2 in the FOX assay. Ammonium ferrous sulfate oxidation in xylenol orange is followed at 560 nm (Jiang et al., 1990; Ou and Wolff, 1996). An alternative is to measure ferrous thiocyanate oxidation to a reddish complex absorbing at 450 to 480 nm (Cohen et al., 1996). Both methods are suitable for measuring cell culture and subcellular organelle catalase activity.

Materials

30 % (v/v) H_2O_2 (Sigma-Aldrich; store at 2°C to 8°C)
Tissue samples (e.g., rat liver), primary cultures, or cell cultures (e.g., hepatocytes)
Homogenization buffer (see recipe)
Bradford protein determination kit (Bio-Rad; or see *APPENDIX 3H*)
Hypotonic cell lysis buffer (see recipe)
0.1 M phosphate buffer (either sodium or potassium phosphate; *APPENDIX 2A*) with or without 0.1% (v/v) Triton X-100, optional
BSA (Sigma-Aldrich; store at 2°C to 8°C), optional
2.2 mM H_2O_2
FOX 1 reagent (see recipe)

1.5-ml microcentrifuge tubes
Timer
Microcentrifuge
1-ml quartz cuvettes
Spectrophotometer with UV and visible light source

1. Prepare 10 mM H_2O_2 by diluting 30% (v/v) H_2O_2 in water and then serially diluting it (1/2 dilution) with water to give the desired concentrations of H_2O_2 standards (e.g., 0, 0.05, 0.25, 0.5, 1.0, 2.0 μM final concentrations after addition of FOX 1 reagent, i.e., a 1/20 dilution).

   For example, 10 mM H_2O_2 diluted 1/2 with H_2O = 5 mM H_2O_2.

   These standard solutions of H_2O_2 will be used to calibrate the FOX assay for H_2O_2 determination. The concentrations can be adjusted to the requirements of the assay.

2a. *To prepare tissue homogenates (e.g., rat liver)*: Remove liver from rat, chop liver into really small pieces with scissors and rinse several times with homogenization buffer to wash away blood. Transfer ~1 g of liver pieces with 3 to 5 ml homogenization buffer to a Dounce homogenizer and homogenize with 3 slow motions of the pestle. Store aliquots up to 1 year at −70°C. Determine protein concentration using a Bradford protein determination kit (or see *APPENDIX 3H*).

   The type buffer used for homogenization as well as volumes and amounts of tissue can vary according to the tissue being used.

   With tissue extracts, the amount of catalase will depend on the organ source of the tissue. Catalase activity is highest in liver and kidney, and lowest in connective tissues. Table 7.7.1 gives examples of dilutions and volumes for various samples and can be used as a guideline.

2b. *To prepare primary culture or cell lysates (e.g., hepatocytes)*: Add 1 ml hypotonic cell lysis buffer to 1×10^6 hepatocytes.

   Cell extracts from tissue culture are often assayed without dilution. Blood lysates show a relatively high amount of activity and therefore should be diluted accordingly (see Alternate Protocol).

   When samples are prepared by lysis with hypotonic buffer, 0.1 M phosphate buffer can be used to dilute the samples. If the sample is prepared in an isotonic buffer that keeps the peroxisome intact, dilute the sample with the 0.1 M phosphate buffer containing 0.1% Triton X-100. If the protein concentration of the sample is very low (less than 0.025 mg/ml), bovine serum albumin may be added to the buffer at a concentration of 0.5 mg/ml to stabilize the enzyme.

Table 7.7.1 Sample Dilutions of Catalase from Various Sources

Tissue	Tissue homogenate protein concentration	Volume of tissue homogenate added to reaction	Volume of 0.1 M phosphate buffer (μl) added to reaction
Blank	—	—	50 μl
HepG2 lysate	2.0 mg/ml	2–4 μl	48–46 μl
Rat liver lysates	0.3 mg/ml	5–10 μl	45–40 μl
Rat brain lysates	0.7 mg/ml	10–20 μl	40–30 μl
Rat spleen lysates	0.3 mg/ml	5–10 μl	45–40 μl
Rat kidney lysates	0.3 mg/ml	5–10 μl	45–40 μl

3. Add 100 µl of 2.2 mM H_2O_2 to 1 ml homogenate or lysate and rapidly mix to give an initial H_2O_2 concentration of 200 µM. Prepare a blank containing 200 µM H_2O_2 and buffer instead homogenate or lysate. Incubate at room temperature.

4. At varying time intervals between 0 and a maximum of 15 min, remove 50-µl aliquots of the incubation mixtures, blank, or H_2O_2 standards prepared in step 2. Rapidly mix with 950 µl of FOX 1 reagent in a 1.5-ml microcentrifuge tube.

   Organize experiments using a timer, and stagger sampling times for best results.

   The addition of the FOX 1 reagent stops the catalase reaction.

5. Incubate the tubes at room temperature for 30 min. Microcentrifuge 3 min at 12,000 × *g*, room temperature.

6. Read absorbance of the supernatant at 560 nm in a quartz cuvette.

7. Prepare a standard curve by performing a regression analysis using data obtained from absorbance by the H_2O_2 standards (see Fig. 7.7.2).

8. From the equation for the standard curve in Figure 7.7.2, calculate the concentration of H_2O_2 present in the sample.

   For Figure 7.7.2 the equation of the standard curve is: $y = 0.255x + 0.0189$ where y is absorbance at 560 nm and x is H_2O_2 concentration. Therefore, a sample with absorbance at 560 nm of 0.345 contains 1.28 µM H_2O_2.

   The higher the residual H_2O_2 concentration, the lower the catalase activity.

9. Calculate the residual H_2O_2 by reference to calibrated H_2O_2 standard and/or the extinction coefficient of H_2O_2 in the FOX 1 reagent of 2.35×10^5 M^{-1} cm^{-1}.

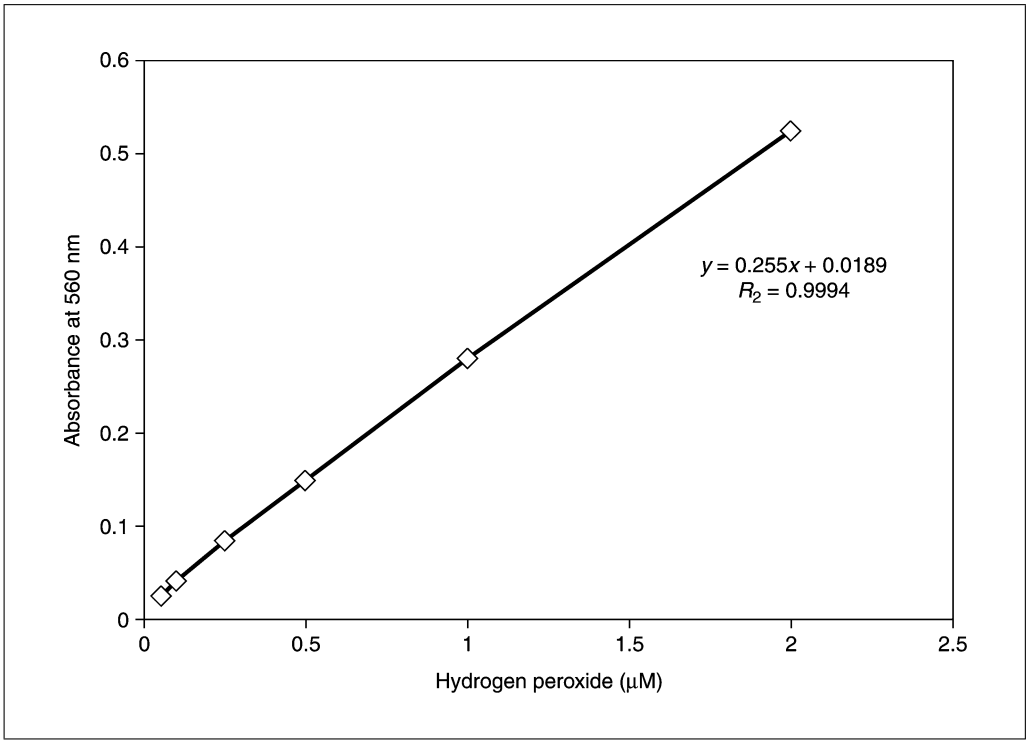

Figure 7.7.2 Standard curve for hydrogen peroxide measured with FOX 1 reagent.

10. Calculate units of catalase activity as follows:

$$\text{Activity } (\mu\text{moles/min/ml}) = \Delta\mu\text{moles } H_2O_2 \times d \times 20/(V \times t)$$

Δ μmoles H_2O_2 = difference between the amount of H_2O_2 added to the reaction and the amount at the end of the reaction
d = dilution of original sample for catalase reaction
t = catalase reaction duration (minutes)
V = sample volume in catalase reaction
20 = dilution of aliquot from catalase reaction in colorimetric reaction (50 μl from 1 ml).

$$\text{Units/ml} = [\Delta A/\text{min (blank)} - \Delta A/\text{min (sample)}] \times d \times v/(V \times \varepsilon)$$

ΔA = change in absorbance between
d = dilution of original sample
v = volume of reaction
V = Sample volume in catalase reaction
$\varepsilon = 2.35 \times 10^5\ M^{-1}\ cm^{-1}$.

MEASUREMENT OF CATALASE ACTIVITY IN RED BLOOD CELLS

ALTERNATE PROTOCOL

The assay described in Basic Protocol 1 must be modified for measuring red blood cell (RBC) catalase activity. RBCs have to be separated from other types of cells in whole blood (e.g., white blood cells), which contain other peroxidases that can interfere with the measurement of catalase activity (e.g., myeloperoxidase uses H_2O_2 to convert chloride to hypochlorous acid in order to destroy bacteria). Therefore, it is essential to separate RBCs from whole blood to avoid misleading results. Centrifugation steps are included in the protocol to purify RBCs from whole blood.

Additional Materials *(also see Basic Protocol 1)*

0.5 ml blood samples, collected in heparnized microcuvettes or tubes
Phosphate-buffered saline: 15 mM potassium phosphate/150 mM NaCl, pH 7.4, ice cold
10 mM potassium phosphate, pH 7.2: prepared by diluting 0.1 M phosphate buffer (*APPENDIX 2A*)

1. Centrifuge 0.5-ml samples of blood in 1.5-ml microcentrifuge tube 2 min at 750 $\times$ g, room temperature, to separate RBC from plasma and buffy layer.

   RBCs are found at the bottom after centrifugation.

2. Remove the plasma and buffy layers with a pipet or by aspiration and discard.
3. Resuspend the RBCs in an equal volume of ice-cold phosphate-buffered saline and centrifuge 15 min at 750 $\times$ g, room temperature. Repeat a total of three times.
4. Generate a 5% (v/v) suspension of packed cells by diluting the RBCs 20-fold into cold phosphate-buffered saline.
5. Lyse 15-μl aliquots of the suspension, in duplicate, by the addition of 985 μl of 10 mM potassium phosphate buffer, pH 7.2.
6. Add 100 μl of 2.2 mM H_2O_2 to the 1-ml lysate and rapidly mix to give an initial H_2O_2 concentration of 200 μM. Prepare a blank containing 200 μM H_2O_2 and buffer instead of RBC lysate. Incubate at room temperature.
7. Proceed as in Basic Protocol 1 steps 4 to 10.

BASIC PROTOCOL 2

SPECTROPHOTOMETRIC MOLYBDATE ASSAY FOR H_2O_2

Ammonium molybdate forms a yellow complex with H_2O_2 and is suitable for measuring serum and tissue catalase activity (Goth, 1991). Titanium oxide sulfate also forms a yellow complex (410 nm) with H_2O_2 and can be used to follow endogenous H_2O_2 formation by isolated rat hepatocytes (Boutin et al., 1989; also see Commentary).

Materials

Tissue samples (e.g., rat liver)
0.1 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*)
65 μmol/ml H_2O_2 in 6.0 mM sodium-potassium phosphate buffer, pH 7.4 (buffer concentrate; Sigma-Aldrich)
32.4 mM ammonium molybdate (Sigma-Aldrich)

Balance accurate to ±0.01 g
Dounce homogenizer
Spectrophotometer

1. Weigh the tissue samples after dissection.
2. Cut up tissue into really small pieces and immediately homogenize on ice in 0.1 M sodium phosphate buffer pH 7.0 at 0°C at a ratio 1:10 (e.g., 1 gram tissue per 10 ml buffer) with four strokes of a Dounce homogenizer.

   Cut the tissue quickly (as quickly as the surgery will allow). If samples cannot be homogenized immediately, snap freeze them in liquid nitrogen and store at −70°C until ready to be homogenized. Before homogenizing cut the tissue into very small pieces to minimize resistance in the homogenizing process. Tissue should be kept on ice at all times during the homogenization process.

   Do not homogenize more than 15 to 20 ml of tissue at a time.

   Keep everything on ice at all times.
3. Store homogenate up to 3 months at −20°C or up to 1 year at −70°C.
4. To analyze the catalase activity, incubate 0.2 ml of tissue homogenate with 1 ml 65 μM H_2O_2 in 6.0 mM sodium potassium phosphate buffer, pH 7.4 for 60 sec (sample 1). Prepare control reactions with 1 ml H_2O_2 plus 0.2 ml buffer (no enzyme control; blank 2) and 1.2 ml buffer (no enzyme/no substrate, blank 3).
5. Stop the reaction by adding 1.0 ml of 32.4 mM ammonium molybdate to sample and control reactions.
6. Determine the absorbance at 405 nm of the yellowish molybdate and H_2O_2 complex against the no enzyme/no substrate blank.
7. Calculate the catalase activity in kilounits (kU)/liter using the following equation:

   $$x = (A_{405} \text{ sample } 1 - A_{405} \text{ blank } 2 / A_{405} \text{ blank } 2 - A_{405} \text{ blank } 3) \times 271.$$

   The number 271 is a factor derived by Goth (1991).

BASIC PROTOCOL 3

EUROPIUM FLUORESCENCE ONE-STEP KINETIC ASSAY FOR H_2O_2

H_2O_2 markedly enhances the fluorescence of the weakly fluorescent europium(III)-tetracycline complex. Catalase-activity determination is based on the decomposition of H_2O_2 in the fluorescent Europium trichloride + tetracycline hydrochloride-H_2O_2 complex leading to a large decrease in its fluorescence due to the much less fluorescent Europium trichloride + tetracycline hydrochloride. The ideal H_2O_2 concentration to use for this assay is 400 μM. The assay can be performed using microtiter plates and plate

readers as a discontinuous or continuous assay (Wu et al., 2003). The catalase detection limit is 0.046 U/ml, and the H_2O_2 detection limit is 1.8 μM.

Materials

Europium-tetracycline (EuTc) solution (see recipe)
5 mM H_2O_2, prepared fresh
10 mM MOPS (see recipe)
Tissue, primary cultures, or cell cultures (for preparation see Basic Protocol 1, steps 2a and 2b)
11.2 mM 3-amino-1,2,4-triazole (AMT; Sigma-Aldrich)
100 U/ml catalase control and 5 to 50 U/ml catalase standards: prepared by diluting catalase from bovine liver (Sigma; 1,277,500 U/ml) with 10 mM MOPS (see recipe)
96-well microtiter plate with lid
30°C incubator
Multichannel pipettor
Microtiter plate fluorometer with 405-nm excitation and filters for collection of emissions at 612 or 620 nm

1. Place in each well of a 96 well microtiter plate:

   65 μl EuTc solution
   20 μl of 5 mM solution of H_2O_2 in water
   165 μl 10 mM MOPS to make a total of 250 μl.

   For the negative control wells, instead of 165 μl 10 mM MOPS add 115 μl 10 mM MOPS plus 50 μl 11.2 mM AMT (a catalase inhibitor). Total reaction volume in wells is 300 μl. See Table 7.7.2.

2. Incubate 10 min at 30°C.
3. Add 50 μl samples, 10 mM MOPS (blank), or 100 U/ml catalase standard (positive and negative controls) to all sample wells simultaneously, using a multichannel pipettor. Also include wells containing 50 μl of 5 to 50 U/ml for generating a standard curve. Incubate 25 min at 30°C.
4. Read the plates at 612 to 620 nm at 3-min intervals for 25 min. Program the microtiter plate reader for shaking before readings are taken.

   Use the fluorescence decrease between 0 and 10 min to quantify the activity of catalase.

   Readings are taken every 3 min to ensure the linearity of the reaction.

Table 7.7.2 Components for the Europium Assay for H_2O_2

	Blank	Sample	Positive control	Negative control
EuTc solution	65 μl	65 μl	65 μl	65 μl
5 mM H_2O_2 in water	20 μl	20 μl	20 μl	20 μl
10 mM MOPS	215 μl	165 μl	165 μl	115 μl
11.2 mM AMT	—	—	—	50 μl
Sample	—	50 μl	—	—
100 U/ml catalase	—	—	50 μl	50 μl
Total volume	300 μl	300 μl	300 μl	300 μL

5. Prepare a standard curve by performing a regression analysis using data obtained from the standards (e.g., see Fig. 7.7.2). Calculate activity of the samples using the equation for the standard curve.

BASIC PROTOCOL 4

CLARK OXYGEN ELECTRODE ASSAY FOR MEASURING THE OXYGEN PRODUCT OF CATALASE

This assay is suitable for assaying catalase activity in homogenates or cell suspensions (Del Rio et al., 1977; Zigman et al., 1998). A stream of nitrogen is bubbled through 50 mM phosphate buffer pH 7.0 in the reaction vessel until approximately zero oxygen is recorded. Then H_2O_2 is added, followed at 3 min by the catalase test sample. The rate at which oxygen is released is recorded, and activity is expressed as μmoles O_2/min/mg protein.

Materials

50 mM phosphate buffer, pH 7, air saturated: prepared by diluting 0.1 M phosphate buffer (*APPENDIX 2A*; either sodium or potassium phosphate) with Milli-Q-purified water and bubbling with air for ~10 min
Nitrogen gas tank with pressure regulator
0.08 U/ml catalase: prepared fresh by dissolving beef liver catalase (Sigma-Aldrich; 40,000 to 60,000 U/mg protein) in 50 mM phosphate buffer, pH 7.0
Tissue homogenates or cell culture lysates (see Basic Protocol 1, steps 2a and 2b)
33.5 mM H_2O_2: prepared fresh by diluting 30% H_2O_2 (Sigma-Aldrich) with water
10% bleach

Triton X-100 (optional)
Oxygen electrode (e.g., Fisher) with thermostated jacket, magnetic stirrer, and XY recorder (e.g., Pharmacia)
3-ml syringe
Spectrophotometer
20 × 30–cm sheets for the XY recorder

1. Add 2.95 ml air-saturated 50 mM phosphate buffer, pH 7.0, to the reaction cell of the oxygen electrode at 37°C. Turn on magnetic stirring and recording of electrode current with an applied potential of 0.8 V.

   The oxygen cathode is a micro-Clark electrode comprised of a platinum cathode, a silver anode, and a KCl solution held captive around the electrode by a Teflon membrane (Fig. 7.7.3).

2. Allow the system to equilibrate ~5 min.

3. Set the electrical zero of the oxygraph so that the distance of chart paper between electrical zero and full deflection is 185 mm.

   Pen deflections are converted to oxygen concentration on the basis of oxygen concentration in the air-saturated medium of 0.250 μmol of O_2/ml at 37°C.

   Assuming an oxygen concentration in the air-saturated buffer of 0.250 μmol of O_2/ml and considering the volume (3.10 ml), each 1-mm change in reading is represented as 0.00419 μmol of O_2.

4. Bubble a stream of nitrogen through the reaction cell by means of a hypodermic syringe connected to a gas pressure regulator. Continue until a maximum deflection is reached.

   The recorder tracing declines steadily, indicating that the oxygen dissolved in the cell is being displaced by nitrogen.

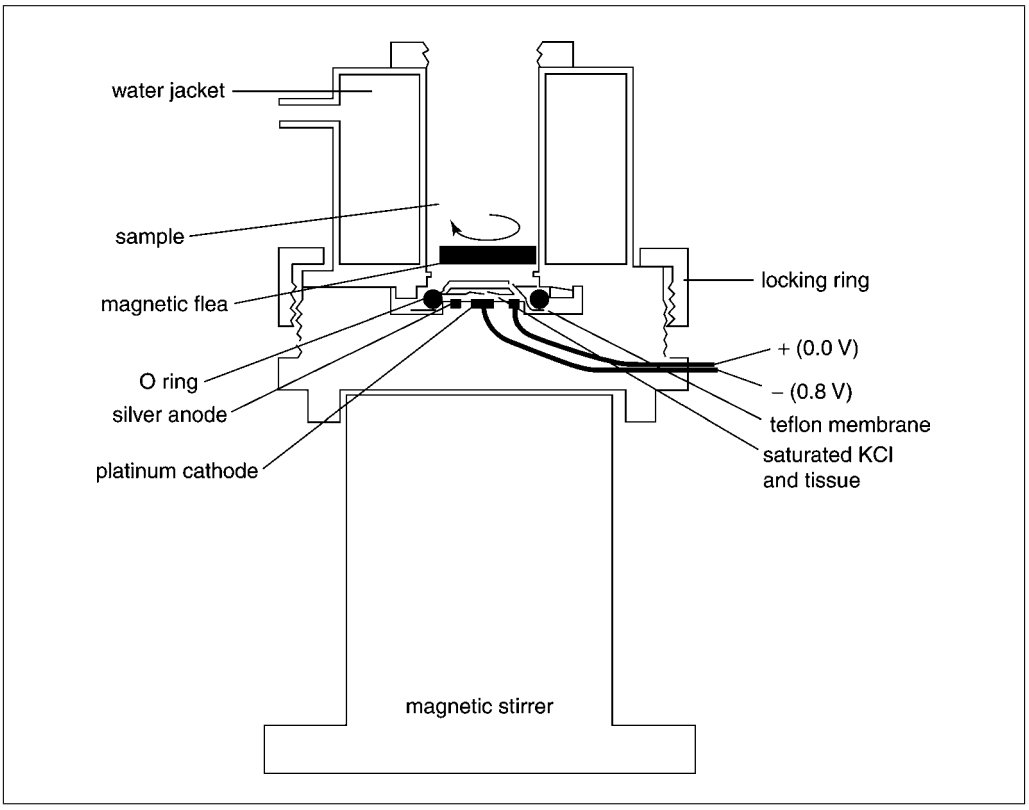

Figure 7.7.3 A diagrammatic representation of an oxygen electrode. The electrode measures the concentration of oxygen in a solution contained in the chamber of the apparatus. The base of the chamber is formed by a Teflon membrane permeable to oxygen. Underneath this membrane is a shallow compartment containing a saturated KCl solution and two electrodes, a platinum cathode, and a silver anode. A fixed polarizing voltage is applied between the electrodes and the resulting tiny current is proportional to oxygen concentration. The current is determined using a chart recorder. The method is essentially polarographic, oxygen being reduced at the platinum cathode. The saturated KCl allows the current to flow and silver chloride is formed on the silver anode. The current depends upon the rate of diffusion of oxygen to the cathode, and this rate is proportional to the dissolved oxygen concentration. To ensure that the oxygen concentration in the electrode compartment follows that in the main chamber, it is essential to stir the contents of the main chamber continuously. A closely fitting stopper is used in experiments when it is necessary to exclude atmospheric oxygen from the system. Its position may be carefully adjusted so that the liquid exactly fills the space under it. (Adapted from *http://www.isbu.ac.uk/biology/enzyme/oxelectrode.html*.)

5. Prepare tissue homogenates or cell culture lysates.

   Keep homogenates or suspensions on ice until ready to use to prevent proteases from denaturing proteins. Protein inhibitors may also be added.

   In the case of cells, if the plasma membranes have not been broken apart in the preparation procedure, it will be necessary to incubate the cells with detergents such as Triton X-100 (100 μl in 2 ml of 1 × 10^6 cell/ml for 5 min) before activity can be measured.

6. Add 100 μl 1.04 M H_2O_2 (final concentration 33.5 mM). After 3 min add 50 μl tissue homogenate or cell lysate from step 5 or 0.08 U/ml beef liver catalase.

   All reactions are run in 50 mM phosphate buffer, pH 7.0 at 37°C in a total volume of 3.10 ml.

   To start with in the reaction mix use 50 μl of either the lysed cell solution or the tissue homogenates. If the reaction proceeds very quickly (i.e., if a slope is not obtainable) use a 1:2 dilution to bring the reaction rate under control.

7. Transfer the reaction mixture using a 1-ml pipet to the cell of the oxygraph. Record for 120 sec (see Fig. 7.7.4).

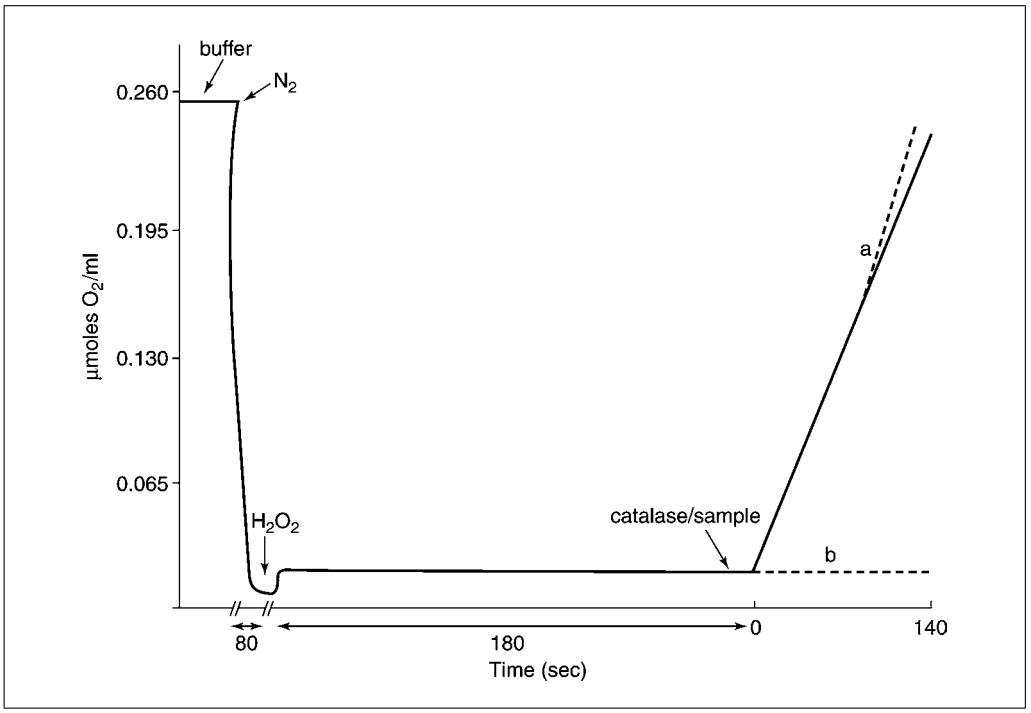

Figure 7.7.4 A Clark oxygen electrode recorder trace of catalase determination (adapted from Del Rio et al., 1977). The reaction is carried out at 37°C in 50 mM phosphate buffer, pH 7.0, with a final volume of 3.10 ml. The electrode reading is initially set at 100% deflection in air-saturated buffer, and the medium is flushed with nitrogen. H_2O_2 is added followed by sample or catalase standards. The arrows indicate the additions to the system. The broken lines are tangents drawn for measurement of initial velocity (a) and for correction of spontaneous decomposition of H_2O_2 (b).

8. At the end of the reaction remove the reaction mixture with a pasture pipet and discard in a solution of 10% bleach. Rinse cell with three times with 3 ml 50 mM phosphate buffer before using again.
9. Calculate the initial velocity of the reaction from the change in the slope by drawing the tangent to the first-order curve.
10. Correct this slope value for the contribution by the nonenzymatic photodecomposition of H_2O_2 during the reaction time by subtracting the slope corresponding to the equilibration period previous to the addition of the enzyme.
11. Determine catalase activity in µmole of O_2 evolved/min from the difference between the two slopes (refer to Fig. 7.7.4 for explanation).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Europium^{3+}, 6.3 mM

Dissolve 115.3 mg of $EuCl_3 \cdot 6H_2O$ (Sigma-Aldrich)
50 ml 10 mM MOPS (see recipe)
Store up to 1 week at room temperature

Europium/tetracycline solution

10.0 ml 6.3 mM europium^{3+} (see recipe)
10.0 ml 2.1 mM tetracycline (see recipe)
Dilute to 100 ml 10 mM MOPS (see recipe)
Make fresh before use and keep in dark or cover with aluminum foil.

FOX 1 reagent

250 μM ammonium ferrous sulfate (Sigma-Aldrich)
100 μM xylenol orange (Sigma-Aldrich)
0.1 M sorbitol (Sigma-Aldrich)
25 mM H_2SO_4
Make fresh before use and keep in dark or cover with aluminum foil.

CAUTION: Use caution when handling H_2SO_4; it can be corrosive to skin. Remember to add acid to water.

Homogenization buffer

21.38 g sucrose (125 mM)
11.39 g mannitol (125 mM)
0.190 g EGTA (1 mM)
0.596 g 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), pH 7.2 (5 mM)
Bring volume to 500 ml with H_2O
Store up to 2 months at 4°C

Final concentrations are in parentheses.

Hypotonic cell lysis buffer

1.19 g 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), pH 7.9 (10 mM)
0.15 g $MgCl_2$ (1.5 mM)
0.37 g KCl (10 mM)
250 μl 1 M dithiothrietol (DTT; 0.5 mM)
1 ml 100 mM phenylmethylsulphonylfluoride (PMSF) dissolved in isopropanol (0.2 mM)
Bring volume to 500 ml with H_2O
Store up to 4 weeks at 4°C

Final concentrations are in parentheses.

MOPS, 10 mM

2.3 g of MOPS sodium salt
800 ml of distilled water
Adjust pH to 6.9 with 1.0 M HCl
Bring volume to 1 liter with distilled water
Store up to 4 weeks at room temperature
Always check pH before use

Tetracycline, 2.1 mM

50.5 mg of tetracycline·HCl (Sigma-Aldrich)
50 ml 10 mM MOPS (see recipe)
Store up to 4 weeks at room temperature

COMMENTARY

Background Information

Catalase (hydrogen peroxide/hydrogen peroxide oxidoreductase) is a widely distributed enzyme that is found in the peroxisomes of all aerobic microorganisms and plant and animal cells (Mueller et al., 1997). The physiological function of catalase is to catalyze H_2O_2 disproportionation to water and O_2. Catalase is relatively unique compared to other members of the peroxidase family because it displays both catalase and peroxidase activity. Typical substrates of peroxidase activity include ethanol, methanol, formate, nitrite, phenol, and others (Wu et al., 2003). Another enzyme that can

metabolize H_2O_2 is glutathione (GSH) peroxidase. It has been suggested that the catalase pathway catalyzes the decomposition of H_2O_2 generated in the peroxisomes while GSH peroxidase plays a major role in the cytosol. Because H_2O_2 diffuses freely within the cell and the capacity of GSH peroxidase to metabolize H_2O_2 is limited, at higher rates of H_2O_2 generation the role of catalase may become predominant (Boutin et al., 1989).

Catalase is a primary component of the antioxidant defense system of the cell against oxidative stress. Recently, there has been a lot of interest in oxidative stress because it has been implicated in pathogenesis of diseases such as diabetes, cancer, atherosclerosis, neurodegenerative disease, and arthritis (Vendemiale et al., 1999). It has been shown that genetic overexpression of catalase protects against oxidative injury, whereas a catalase deficiency can increase susceptibility to oxidative stress–induced diseases (Kyle et al., 1987; Ohmori et al., 1991; Wang et al., 1996). Serum catalase is increased in a wide variety of diseases, including pancreatic and liver diseases, hemolytic anemia, renal, skin, and respiratory diseases, acquired immunodeficiency, and oxidant-mediated vascular diseases (Slaughter and O'Brien, 2000). On the other hand, catalase levels are decreased in certain cancers, psychiatric diseases, and alcoholism (Hosono et al., 1996; Tarasova, 1998). Furthermore, the peroxisomal location of catalase makes it a good biomarker for peroxisome proliferation, which is induced in rats by a wide range of drugs such as fibrates, thiazolidinediones, and certain nonsteroidal anti-inflammatory drugs (Slaughter and O'Brien, 2000). Therefore, catalase can be used as biomarker not only for drug-induced toxicity but also for disease states.

Continuous and discontinuous measurement of catalase

Catalase activity is commonly measured by the rate of H_2O_2 disappearance using an oxygen electrode/polarographic measurement of evolved oxygen (Basic Protocol 4; Del Rio et al., 1977; Cohen et al., 1996). Catalase can also be monitored continuously by following the rate of H_2O_2 decomposition at 240 nm or discontinuously by sampling H_2O_2 at various time intervals. Both spectrophotometric methods have the advantage of being direct kinetic measurements while other methods usually involve two or more steps or reagents. With the continuous approach of measuring catalase there are some technical difficulties as very high concentrations of catalase are needed. These high concentrations of catalase can cause autoinactivation of catalase in <30 sec, and they are highly toxic to cells (Mueller et al., 1997). Furthermore, many cellular constituents absorb strongly at 240 nm so that low levels of enzyme activity are being measured against very high background absorbance levels (Ou and Wolff, 1996). As mentioned earlier the high extinction coefficient of the H_2O_2 in the FOX assay (discontinuous method) allows the measurement of catalase activity at low concentrations of H_2O_2.

H_2O_2 probes

FOX 1 reagent

Xylenol orange is used to detect peroxide-generated Fe^{3+}. Ferrous ion (Fe^{2+}) is a relatively stable ion towards autoxidation in dilute acid. It can, however, be oxidized by peroxides to yield Fe^{3+} and hydroxyl/alkoxyl radicals in a reaction that proceeds rapidly at room temperature as illustrated below.

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\cdot} + OH^{-}$$

The detection of Fe^{3+} ions that are formed in a reaction mixture consisting of ferrous sulfate in dilute H_2SO_4 can be achieved using the dye xylenol orange, which binds Fe^{3+} forming a complex that absorbs between 540 and 580 nm in dilute acid (Michaels and Hunt, 1978). Jiang and coworkers determined that the extinction coefficient of the Fe^{3+}-xylenol complex at 560 nm (the absorbance maximum) is $1.5 \times 10^4\ M^{-1}\ cm^{-1}$ in 25 mM H_2SO_4 at room temperature (Jiang et al., 1990).

Transition metal complexes

In the early 1990's Goth (1991) developed ammonium molybdate as a probe to measure H_2O_2 as a means of measuring catalase activity in clinical laboratories instead of using polarographs. The yellow complex formed between H_2O_2 and ammonium molybdate is used in this reaction and it is measured at 405 nm.

Boutin and coworkers developed a method for measuring H_2O_2 in freshly isolated rat hepatocytes based on the irreversible inhibition of catalase by AMT. In this system, H_2O_2 is complexed with titanium oxide sulfate ($TiOSO_4$) in sulfuric acid. The H_2O_2-$TiOSO_4$ complex is measured at 410 nm (Boutin et al., 1989).

In the past, metals such as manganese in permanganate complexes have been used to form adducts with H_2O_2. However, when a portion of permanganate is reduced to Mn^{2+} by

H_2O_2 and a portion remains unreacted, a precipitate of MnO_2 starts to form slowly within 30 to 60 sec of the reactions so the samples have to be read quickly, otherwise turbidity develops. Therefore, this method is not used widely with high-throughput sampling (Cohen et al., 1996).

Thiocyanate (SCN^-)

An alternative method for assessing H_2O_2 colorimetrically is with ferrous ions and thiocyanate. Thiocyanate and ferric ions form a reddish complex with a peak in the 450 to 480 nm range. Thiocyanate has been widely used to measure either H_2O_2 or iron levels. This method was scaled down recently by Cohen and coworkers so that it can be carried out with plate readers and high-throughput screening (Cohen et al., 1996).

Fluorescent probe: Europium (Eu)-Tetracycline

Recently, Wu and coworkers have introduced the weakly fluorescent Eu^{3+}–tetracycline complex (EuTc) as a promising new fluorescent probe for detection of H_2O_2. EuTc acts as a fluorescence-enhancing ligand (Wolfbeis et al., 2002). As little as 1.8 μmol/liter of H_2O_2 can be detected at near-neutral pH. This effect has been used previously to improve the limits of detection in the measurement of the drug tetracycline but it appears this activity has a much greater potential in the direct measurement of catalase activity (Wu et al., 2003). The determination of catalase activity is based on the finding that, with the H_2O_2 in the fluorescent system, EuTc-H_2O_2 can be decomposed by catalase, thus leading to a large decrease in its fluorescence due to the formation of the much less fluorescent EuTc.

Clark oxygen electrode

Since the 1960s the measurement of oxygen concentration in solution with a Clark oxygen electrode has been used for assaying catalase activity. The earlier methods used oxygen concentrations greater than that of an air-saturated solution, i.e., oversaturated oxygen. This condition diminished both the sensitivity in the lower limit of measurable catalase activity and the reaction time of linear oxygen production. Furthermore, oxygen bubbles may appear in the reaction cell forming two phases, and, as a result, part of the oxygen released by catalase would not be measured by the Clark oxygen electrode (Del Rio et al., 1977). Del Rio and coworkers described a method to measure the initial velocities of oxygen released by the catalase in a medium that has been previously flushed with nitrogen to make it practically oxygen free. This method is a rapid and sensitive way of measuring the oxygen released during the metabolism of H_2O_2 by catalase.

Other methods

Continuous luminol chemiluminescence assay

In this assay, luminol is oxidized by HOCl to a diaziquinone which is converted by H_2O_2 to an excited aminophthalate. The assay is particularly useful for very low H_2O_2 concentrations. The ideal H_2O_2 concentration to use is 20 to 40 μM. However kinetic continuous detection techniques are rather difficult with this assay due to the short luminescence signal. The catalase detection limit is ~0.2 U/ml (Mueller et al., 1997).

Discontinuous spectrophotometric/fluorescent peroxidase assays

This technique relies on adding horseradish peroxidase (HRP) to compete for H_2O_2 with catalase. A fully automated spectrophotometric method for catalase antioxidant activity has been described involving 3,5-dichloro-2-hydroxy-benzenesulfonate as the chromogenic peroxidase substrate that is oxidized to a 505-nm absorbing dimeric product (Slaughter and O'Brien, 2000). The limit of catalase detection is ~0.1 U/ml. Homovanillic acid is oxidized by H_2O_2 and HRP, generating a fluorescent dimer (λ_{ex} = 312 nm and λ_{em} = 420 nm). It is commonly used to measure cell or mitochondrial H_2O_2 formation (Barja, 2002). While convenient, the involvement of peroxidase in the detection procedure will change the H_2O_2 concentration in the system and affect the activity of catalase.

Critical Parameters and Troubleshooting

Ferrous oxidation assay

The high extinction coefficient of H_2O_2 in the FOX assay allows the measurement of catalase activity at low concentrations. The levels of H_2O_2 detected by this assay are close to physiological. Incubation at room temperature with the FOX 1 reagent is essential for detecting the H_2O_2. This reaction should ideally be carried out in the dark; however, the samples should remain at room temperature. If the samples contain flocculated proteins or other debris, it should be removed by centrifugation before reading the absorbance at 560 nm (Ou and Wolff, 1996).

Molybdate assay

The molybdate-H_2O_2 complex is yellow in color and is detected at 405 nm. This complex is stable for at least 60 min. Concentrations of ammonium molybdate as high as 48.5 mM can be used for the assay. Ammonium molybdate absorbs between 352 and 360 nm, and decreased absorption at these wavelengths can be monitored to verify its complexing with the H_2O_2 and to help determine whether the concentration needs to be adjusted. A concentration of 65 μM H_2O_2 is used in the assay; this does not inactivate serum catalase and it yields a linear relationship for the measured catalase in the range of 0 to 100 kilounits (kU)/liter. If the catalase range is higher than 100 kU/liter then a new measurement should be made by either diluting the sample 2- or 10-fold with 0.9 g/liter NaCl or using a shorter incubation time (Goth, 1991).

Europium fluorescence one-step kinetic assay

The fluorescent complex formed between H_2O_2 and EuTc (referred to as EuTc-H_2O_2) is reversibly decomposed by catalase. Hence, H_2O_2 cannot oxidize one of the components of the EuTc complex, but rather must be present in a form that makes it acceptable to this highly specific enzyme. This reaction can be quenched by fluoride, which can act as a weak quencher if present in a concentration higher than 0.1 mM. Phosphate and copper (II) ions are strong quenchers of this system. Furthermore, the fluorescence of the EuTc-H_2O_2 system is weakly quenched by molecular oxygen. Ascorbate, uric acid, bilirubin, and glutathione interfere in displaying a quenching effect on the fluorescent complex system if present in concentrations of >6, 40, 7, and 16 mM, respectively, in the wells. These species usually are present in constant concentrations in catalase assays. If needed, calibration may be performed in presence of these interfering compounds (Wolfbeis et al., 2002; Wu et al., 2003).

Clark oxygen electrode assay

This method measures the initial velocities of oxygen released by catalase in a medium that has been previously flushed with nitrogen to make it practically oxygen free. It is a rapid and sensitive way of measuring the oxygen released during the metabolism of H_2O_2 by catalase. The sensitivity of this method can be increased by suitable amplification of the oxygraph settings to measure an upper limit of 0.002 μmol of oxygen/min. It is a reproducible method for the measurement and kinetic studies of catalase (Del Rio et al., 1977).

Anticipated Results

Ferrous oxidation assay

The decomposition of H_2O_2 in the presence of varying concentrations of catalase should be linear over a reasonable range (2 to 16 units of catalase). The same should be true when varying amounts of samples are taken from RBCs, liver, or other tissues/organs. There should be an almost linear relationship between H_2O_2 depletion and enzyme activity. The decomposition of H_2O_2 by catalase is curvilinear over 5 min (Ou and Wolff, 1996).

Molybdate assay

The reaction between H_2O_2 and molybdate ions is fast and the three-step process yields a complex that is stable for 60 min. This complex is dependent on both molybdate and H_2O_2 concentrations. The kinetics of catalase does not obey a normal pattern. Goth (1991) has shown a semi-zero order reaction range of serum catalase between 45 μM and 65 μM of H_2O_2. Therefore, 65 μM of H_2O_2 is suggested for this reaction. Serum catalase levels in normal individuals are 40 to 60 kU/liter as determined by the calculations made using the formula listed in the protocol (Goth, 1991).

Europium fluorescence one-step kinetic assay

Compared to the above methods for catalase detection, this method is easy to use for kinetic analysis, works at a clearly defined H_2O_2 concentration at neutral pH, and does not require the addition of other substrates or enzymes. The assay can be performed using microtiter plates and is fully compatible with plate readers. The fluorescence of the EuTc-H_2O_2 system decreases faster with increasing activity of catalase (the slope represents units/ml of catalase activity) thus reflecting the actual activity of the enzyme. The most sensitive range of EuTc to H_2O_2 concentration is at less than 0.6 mM as higher H_2O_2 concentrations result in higher fluorescence, the decrease in fluorescence due to the consumption of H_2O_2 is smaller. Therefore, smaller changes are observed at high H_2O_2 levels. The assay works only in the range where consumption of H_2O_2 makes a difference to the total amount of EuTc-H_2O_2. The activity of the catalase is proportional to the concentration of H_2O_2. The linear range of the catalytic activity of catalase is from 1 to 10 U/ml (Wu et al., 2003).

Table 7.7.3 Activity of Catalase in the Tissues of Mice[a]

Tissue	Catalase activity (μmol/min/mg protein)
Brain	8.9 ± 0.8
Kidney	377 ± 22
Lung	116 ± 6
Muscle	7.0 ± 1.5
Liver	883 ± 68
Heart	62.6 ± 6.8

[a]Ibrahim et al. (2003).

Clark oxygen electrode assay

The effect of enzyme concentration is directly related to the initial velocity of the reaction. For cells, the reaction rates can be enhanced by the incubation with a detergent like Triton X-100 as the detergent breaks open the plasma membrane of the cell. Furthermore, for incubation times up to 60 sec there is no significant decrease in the catalase activity. It is important to use appropriate enzyme dilutions and final H_2O_2 concentrations of 33.5 mM to achieve linear rates within the first 60 sec. This method is applicable over an 840-fold range of catalase concentrations and has sensitivity of at least 20 times higher than the other assays utilizing the oxygen electrode (Del Rio et al., 1977).

Catalase activity has been found to be elevated in the liver of aged mice as compared to young healthy mice (Wozniak et al., 2004). Erythrocytes are the cells with the highest amount of catalase (377 μmol/min/mg of protein; Ibrahim et al., 2003). The organ that has the highest catalase activity is the liver (833 μmol/min/mg of protein), and the activity in the kidney is 377 μmol/min/mg of protein and the lung is 116 μmol/min/mg of protein, while the heart, brain, and muscle have the least amount of catalase (7.0, 8.9 and 62.6 μmol/min/mg of protein, respectively; Ibrahim et al., 2003; see Table 7.7.3).

Time Considerations

The protocols mentioned in this unit should take <4 hr to complete. This time takes into account preparation of reagents, samples, and standards and carrying out the experiment. For the discontinuous assay for catalase activity with the FOX 1 reagent there is a 30-min incubation with the reagent that has to be accounted for as well in the assay time. Instrument set-up (oxygen electrode/polarograph or spectrophotometer) should take 5 min. However, the protocol may take longer depending on the number of samples that have to be read. Each sample should be read either on an oxygen electrode/polarograph or UV/Vis spectrophotometer depending on the protocol being used. Analysis of each sample may require 5 to 10 min depending on which protocol is being used.

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