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Selenium Nutrition

Assessment Using Glutathione Peroxidase

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1. Introduction

The enzyme glutathione peroxidase [glutathione: hydrogen peroxide (H_2O_2) oxidoreductase, EC 1.11.1.9] contains four moles of selenium per mole of enzyme, and is the major biochemical role of selenium in animals. Activity of this enzyme in blood and other tissues is directly related to dietary selenium intake (Ganther *et al.*, 1976) and the severity of several selenium deficiency diseases has been correlated with low blood glutathione peroxidase activities. More than 97% of blood glutathione peroxidase is associated with the erythrocyte fraction, where it accounts for a large proportion of erythrocyte selenium, and activity of this enzyme has been correlated with blood selenium concentrations in a number of species (Paynter, 1979; Paynter *et al.*, 1979; Caple *et al.*, 1980). Blood activity of this enzyme provides a rapid, convenient and sensitive measure of selenium nutrition, with a number of advantages over the direct estimation of selenium. Because of the inherent toxicity of many selenium compounds, it is important that an accurate assessment of selenium nutrition be made before animals are treated with selenium.

The method for the measurement of glutathione peroxidase activity in blood described in the remainder of this paper, is a modification (Paynter, 1979) of the coupled enzyme assay described by Paglia and Valentine (1967) and is suitable for automation (Andrewartha *et al.*, 1979). Activities of glutathione peroxidase associated with adequate and inadequate selenium nutrition in a number of domestic species are tabulated. A brief outline of a method for the direct measurement of selenium in animal tissues and in feedstuffs is also included. This method is based on the fluorimetric method of Watkinson (1966) and has been used to correlate blood glutathione peroxidase activities with blood selenium concentrations in livestock (Caple *et al.*, 1980).

2. Principle of the Glutathione Peroxidase Assay

Glutathione peroxidase catalyses the reduction of both inorganic and organic peroxides with the oxidation of glutathione. Activity of the enzyme is determined by inclusion into the assay of an excess of NADPH-dependent glutathione reductase, allowing the oxidation/reduction of glutathione to be coupled to the oxidation of NADPH. The oxidation of NADPH is measured at 340 nm.

$$2GSH + ROOH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

As the concentration of glutathione is maintained constant in the assay, the reaction rate is linear, and glutathione peroxidase activity is measured directly as a rate reaction.

In the assay to be described, hydrogen peroxide is used as the peroxidase substrate, and the activity of the enzyme in blood is expressed relative to the haemoglobin concentration.

3. Materials Required for the Glutathione Peroxidase Assay

3.1. Samples

Assays can be performed on whole blood or the cell fraction remaining after removal of plasma. Heparin or EDTA (see 3.2.) are both suitable anticoagulants for the collection of blood samples.

The minimum requested volume of blood is 2 mL. The enzyme is stable for seven days at 4°C, and for several months at -20°C.

3.2. Chemicals

(a) Potassium dihydrogen orthophosphate (KH_2PO_4) analytical reagent (AR) grade.
(b) Ethylenediaminetetraacetic acid, disodium salt (EDTA) [$EDTA(Na)_2.2H_2O$] laboratory reagent (LR) grade.
(c) Sodium azide (NaN_3) LR grade.
(d) Potassium cyanide (KCN) AR grade.
(e) Potassium ferricyanide [$K_3Fe(CN)_6$] AR grade.
(f) Hydrogen peroxide 30% (w/v) AR grade.
(g) Glutathione, reduced (GSH), e.g. Boehringer crystallised, 98%.
(h) $NADPH(Na)_4$, e.g. Sigma type 1, Boehringer, 98%.
(i) Glutathione reductase (EC 1.6.4.2), e.g. Sigma type III, Boehringer 120 IU/mg, 5 mg/mL.

3.3. Reagents

All reagents are prepared in distilled, deionised water.

3.3.1. Reaction buffer

KH_2PO_4, pH 7.0	50.0 mmol/L
EDTA	5.0 mmol/L

Add 3.402 g potassium dihydrogen orthophosphate and 0.930 g of EDTA to about 400 mL water. Titrate to pH 7.0 with KOH, and adjust final volume to 500 mL. This buffer is stable for two weeks at 4°C.

A concentrated reaction buffer (10 times) can be prepared for convenient storage at -20°C. For this buffer, add 17.0 g potassium dihydrogen orthophosphate and 4.65 g EDTA to about 230 mL water. Titrate to pH 6.85 with potassium hydroxide and adjust final volume to 250 mL. Dilute 1 in 10 with water when required for use. This concentrated buffer is stable for several months at -20°C.

3.3.2. Sodium Azide Solution

Sodium azide, NaN_3 1.125 mol/L

Add 3.657 g sodium azide to water to a final volume of 50 mL. This solution is stable for several months at 4°C.

3.3.3. Hydrogen Peroxide Solution

H_2O_2 6.0 mmol/L

Add 0.34 mL of 30% (w/v) hydrogen peroxide to 500 mL of water. This solution is stable for 12 hours at 25°C.

3.3.4. Cyanide Solution (Quadruple Strength)

$K_3Fe(CN)_6$	2.4 mmol/L
KCN	3.2 mmol/L
KH_2PO_4	20.0 mmol/L

Add 0.395 g potassium ferrocyanide, 0.104 g, potassium cyanide and 1.361 g potassium dihydrogen orthophosphate to water to final volume of 500 mL. For use, this solution is diluted 1 in 4 with water to give a final normal strength solution. These solutions are stable for several months at 4°C in amber bottles.

3.3.5. Reaction Solution

This solution is normally prepared in bulk. For 10 assays, each of 3.0 mL final volume, the following mixture is prepared:

Reaction buffer	27.8 mL
$NADPH(Na)_4$	7.0 mg
NaN_3, 1.125 mol/L	0.1 mL
GSH	46.0 mg
Glutathione reductase	10 units

Final assay concentrations are:

KH_2PO_4	50.0 mmol/L
EDTA	5.0 mmol/L
NADPH	0.28 mmol/L
NaN_3	3.75 mmol/L
GSH	5.0 mmol/L
Glutathione reductase	0.33 units/mL

This reaction solution is incubated for 15 min at 25°C and is then stable for about 12 hours at 4°C.

4. Procedure for the Assay of Glutathione Peroxidase

4.1. Blood Dilution

Blood is diluted with normal strength cyanide solution before addition to the reaction solution. For samples of unknown activity, an initial dilution of one in 20 blood in cyanide solution is used. A minimum of five minutes should be allowed before use in the assay, and the enzyme is stable for several hours in this solution.

4.2. Preincubation

Add 0.1 mL of blood-cyanide solution to 2.8 mL of reaction solution. Mix and incubate for five minutes at 25°C.

4.3. Hydrogen Peroxide Addition

Initiate the reaction by the addition of 0.1 mL of 6.0 mmol/L hydrogen peroxide (25°C) and transfer to a temperature controlled (25°C) cuvette with a 10 mm light path.

4.4. Rate Measurement

Measure absorbance of the solution at 340 nm, 30 s to two minutes after initiating the reaction with hydrogen peroxide, and again exactly four minutes after the first reading.

4.5. Non-enzymatic Control

There is a need for a non-enzymatic control (NEC). A significant non-enzymatic reaction occurs in the absence of glutathione peroxidase, after addition of hydrogen peroxide. This non-enzymatic rate is determined by replacing blood with water in a reaction system equivalent to that used for the test assay. The NEC is subtracted from the test reaction rate when sample activity is calculated.

5. Procedure for the Assay of Haemoglobin

An aliquot of the blood diluted in cyanide, previously used for the glutathione peroxidase assay, is further diluted with normal strength cyanide solution such that the final dilution of blood is one in 200. The absorbance of this solution is then read at 546 nm against a reagent blank of cyanide solution only (van Kampen and Zijlstra, 1961).

6. Calculations

Glutathione peroxidase activity is expressed as µmol NADPH oxidised/min.g Hb (U/g Hb). Using the general formula for converting ΔA/min into International Units, i.e.

$$U/L = \frac{\Delta A \times RV \times 1000}{6{\cdot}2 \times SV \times P \times T}$$

where:
RV=Reaction volume in millilitres
SV=Sample volume in millilitres
6.2=millimolar absorption coefficient of NADH at 340 nm
P=cuvette path length in centimeters
T=time (min) over which reaction is measured.

$$Activity\ (U/L) = \frac{(\Delta A^{340}_{Test} - \Delta A^{340}_{NEC}) \times 3{\cdot}0 \times 1000}{6{\cdot}2 \times 0{\cdot}005 \times 1{\cdot}0 \times 4}$$

$$= \Delta A^{340}_{Test} - \Delta A^{340}_{NEC} \times 24194$$

In this example, SV assumes that 0.1 mL of a 1 in 20 dilution of blood is used for the activity determination i.e. 0.005 mL of the original blood sample, with a reaction time of four minutes.

For haemoglobin, the conversion factor of van Kampen and Zijlstra (1961) is used. At a final blood dilution of 1 in 200, the calculation becomes:

$$Hb\ (g/L) = A_{Hb}^{546} \times 294$$

Final activity (U/g Hb), therefore, becomes:

$$U/gHb = \frac{\Delta A_{Test}^{340} - \Delta A_{NEC}^{340}}{A_{Hb}^{546}} \times \frac{24194}{294}$$

$$= \frac{\Delta A_{Test}^{340} - \Delta A_{NEC}^{340}}{A_{Hb}^{546}} \times 82{\cdot}24$$

Where other dilutions of blood are used for the activity and haemoglobin determination, the general calculation is:

$$Activity = \frac{\Delta A_{Test}^{340} - \Delta A_{NEC}^{340}}{A_{Hb}^{546}} \times \frac{Dilution\ of\ blood\ for\ enzyme\ assay}{Dilution\ of\ blood\ for\ Hb\ assay} \times 822{\cdot}4$$

7. Interpretation of Results

Activities of blood glutathione peroxidase representing adequate, marginal and deficient selenium nutrition are tabulated in Table 1. Activities of this enzyme have been correlated with selenium responsive conditions in a range of domestic animal species (Paynter and McDonald, 1976; Caple *et al.*, 1978; Paynter *et al.*, 1979).

It should be noted that several dietary factors may affect the selenium requirements of animals. Diseases such as myopathy (white muscle disease) and unthriftiness may also be caused by factors other than selenium deficiency. Glutathione peroxidase measures only selenium nutrition and does not measure these additional factors.

Selenium appears to be incorporated into erythrocyte glutathione peroxidase only during erythropoiesis. Blood activities are, therefore, a reflection of the dietary selenium intake several months previously, and of erythrocyte turnover.

Blood glutathione peroxidase activity has been correlated with blood selenium concentrations in several species (Caple *et al.*, 1980).

Table 1. Activities of blood glutathione peroxidase (gp) in domestic animals

Species	*Activity of gp associated with:*		
	Adequate selenium	*Marginal selenium*	*Selenium deficiency*
Sheep	Up to 550	<50	<20 Unthriftiness <30 Myopathy
Cattle	Up to 300	<40	<10 Unthriftiness <20 Myopathy
Horses	Up to 150	<35	<15 Myopathy
Goats	Up to 240	<40	<30 Myopathy
Pigs	Up to 140	<30	<20 Hepatosis dietetica

8. Notes on the Assay

(a) Interferences in the assay system by catalase and NADPH-dependent methaemoglobin reductases are prevented by inclusion of azide and cyanide in the reaction mixture. Glutathione peroxidase is unaffected by these compounds, even at concentrations several times higher than those employed in the assay.

(b) If the reaction rate (ΔA^{340}) is greater than 0.5 per four minutes, then the assay should be repeated using a greater dilution of blood in cyanide solution, or the result reported as being greater than this activity.

(c) The NEC rate of reaction increases with increasing hydrogen peroxide concentration, pH and temperature. In the assay system described, this rate should be in the range of 0.120–0.165 per four minutes; within-run variation in the NEC should be small (<2%).

(d) Hydrogen peroxide may undergo autocatalytic decomposition. The concentration of the concentrated stock should, therefore, be established prior to use. The following procedure (Anonymous, 1977) has been found to be suitable:
1.0 mL 30% (w/v) hydrogen peroxide diluted in 625 mL water. To 50 mL of this solution, add 5 mL of 0.5 mol/L sulfuric acid (H_2SO_4)(28 mL concentrated sulfuric acid per 1 L water). Titrate with 0.02 mol/L potassium permanganate ($KMnO_4$) (3.16 g potassium permanganate/L water) to a permanent pink colour.
1.0 mL 0.02 mol/L $KMnO_4$ = 1.70 mg H_2O_2

(e) Other peroxide substrates may be used in the glutathione peroxidase assay. However, because of its ready availability, relative stability and ease of handling, Hydrogen peroxide has been chosen as the standard substrate.

(f) Commercial quality controls suitable for this assay are not readily available. To monitor between and within-run assay variation, it is recommended that internal controls, covering a range of activities, be included in each assay run. These controls can be prepared from samples of whole blood containing high and low glutathione peroxidase activities, diluted with an equal quantity of glycerol prior to aliquoting and storage at -20°C. Activities of these samples should be checked by interlaboratory comparison.

(g) Automation of this assay using a Philips ACI autoanalyser has been described (Andrewartha *et al.*, 1979). The assay has also been adapted for automation on Cobas Mira, Kone Specific and Gilford SBA 300 autoanalysers (D. I. Paynter, unpublished 1987). For the latter, 0.02 mL of 1 in 20 dilution of blood in cyanide solution, is added to 0.3 mL of a 1.65 times concentrated reaction solution. After a five minute preincubation,

0.2 mL of 0.52 mmol/L hydrogen peroxide is added and the reaction rate measured over 30 s, beginning 30 s after hydrogen peroxide addition. In all automated methods, final assay reagent concentrations are as described in the manual method, and there is a five minute preincubation of sample with reaction solution prior to hydrogen peroxide addition.

(h) Preferred assay and reaction temperature is 25°C. At higher temperatures, the NEC rate of reaction increases substantially. The assay may be performed at 30°C or even 37°C, by shortening the lag time between hydrogen peroxide addition and the start of the rate recording period, and reducing the overall rate measurement period. Activities in ovine and bovine samples measured at temperatures other than 25°C can be converted to 25°C by using the factors (30°C/0.75) or (37°C/0.61).

9. Fluorimetric Determination of Selenium

9.1. Principle of the Assay

Selenium within the biological material is released by digestion with nitric (HNO_3)/perchloric acid ($HClO_4$) mixture and converted to the selenite form. Selenite is complexed with 2,3-diaminonaphthalene and the resultant piazselenol is extracted into cyclohexane. The fluorescence of the cyclohexane extract is measured and the concentration of selenium calculated by comparison with a standard curve (Watkinson, 1966).

9.2. Reagents

All reagents are prepared in distilled deionised water and all glassware acid washed before use.

9.2.1. Acid Mixture

Mix 500 mL 70% nitric acid (AR) with 100 mL 72% perchloric acid (AR).

9.2.2. 0.1 mol/L Hydrochloric Acid

Add 8.5 mL 36% hydrochloric acid (AR) to water, to a final volume of 1 L.

9.2.3. 3.0 mol/L Hydrochloric Acid:

Add 250 mL 36% hydrochloric acid(AR) to water, to a final volume of 1 L.

9.2.4. 0.04 mol/L Ethylenediaminetetraacetic Acid

Add 15 g EDTA (AR) to water, to a final volume of 1 L.

9.2.5. Cresol Red Indicator

Dissolve 0.1 g cresol red in 26.2 mL of 0.01 mol/L sodium hydroxide and make up to 250 mL with water.

9.2.6. 7 mol/L Ammonium Hydroxide

Mix 250 mL 25% ammonia solution (AR) with 250 mL water.

9.2.7. 0.1% 2,3-Diaminonaphthalene Reagent

Diaminonaphthalene (DAN) reagent is photosensitive and all preparative and reaction steps must be performed in diffuse light. Dissolve 0.1 g DAN (DAN hydrochloride, 99%, Aldrich Chemical Co.) and 0.5 g hydroxylamine hydrochloride (ClH_4NO) (AR) in 100 mL of 0.1 mol/L hydrochloric acid. Incubate for 30 min in a water bath at 50°C; cool.

Extract the solution three times with 25 mL cyclohexane (C_6H_{12}), discarding each cyclohexane layer. Store DAN reagent under a layer of cyclohexane in an amber bottle. Stable for four weeks at 4°C.

9.2.8. Selenium Standard

A stock solution (1000 mg Se/L = 12.66 mmol/L) is prepared by dissolving 1.000 g elemental selenium (AR) in 5 mL of 70% nitric acid with heat and making up to 1 L with 0.1 mol/L hydrochloric acid. This stock solution is diluted as required with 0.1 mol/L hydrochloric acid. For assays of blood, standards ranging from 0 to 1 mg Se/L are generally suitable.

9.2.9. Reference Standards

Suitable reference standards should be included with each assay run to check accuracy and precision. National Bureau of Standards [NBS; Office of Standard Reference Materials, National Bureau of Standards, Washington, DC 20234, USA. Tel. (301) 921 2045] reference materials (e.g. NBS liver, NBS citrus leaves) are routinely used for this purpose.

9.3. Method

This method has been adapted to use minimum reagent volumes and glassware. Digestion, reaction and extraction steps are completed within a single 150 x 18 mm borosilicate glass test tube fitted with a ground glass socket.

9.3.1. Acid Digestion

Samples to be analysed (containing <250 ng selenium and <0.5 g dry weight, particularly if samples are high in fat), appropriate standards and blanks are added to the test tubes. Add 6 mL of acid mixture, 0.05 mL kerosene and anti-bumping granules. Digest overnight at room temperature. Heat gradually to 190°C until white fumes of perchloric acid are evolved. Increase temperature to 210°C for 30 min. Cool, add 1 mL water and boil until perchloric acid refluxes in the tube again. Cool.

9.3.2. Selenium VI – Selenium IV

Add 1 mL of 3 mol/L hydrochloric acid to each tube and heat to boiling. Cool and add 1 mL 0.04 mol/L EDTA. Mix.

9.3.3. Titration

Add three drops of cresol red indicator and titrate with 7 mol/L ammonium hydroxide (NH_4OH) to a yellow colour. Add 4 mL of 0.1 mol/L hydrochloric acid and mix.

9.3.4. Diaminonaphthalene Reaction

The DAN reaction is performed in diffuse light. Add 1.0 mL of 0.1% DAN, mix and incubate at 50°C for 30 min. Cool, add 2 mL of cyclohexane. Stopper and shake tubes vigorously for 30 s. Allow phases to separate and sample cyclohexane into fluorimeter microcuvette.

9.3.5 Fluorimeter

The fluorimeter should be set at an excitation wavelength of 364 nm and emission wavelength of 523 nm, or fitted with appropriate filters. For a Turner model III Fluorimeter fitted with 6 x 50 mm round microcuvettes, an excitation filter type 7-60 (NP 366 nm), with emission filter type 58 (NP 515 nm) have been found satisfactory.

9.4. Calculations

Selenium concentrations of unknown samples are calculated by graphical interpolation from the standard curve.

10. References

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