EXPERIMENT : 8

DETERMINATION OF ANTIOXIDANTS (DPPH,GLUTATHION)

a) Determination of antioxidant activity

Antioxidants are considered important nutraceuticals on account of many health benefits. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay.

Determination of scavenging potency of DPPH radicals

Experimental Design

The free radical-scavenging activity of the samples will be evaluated by 1,1,-diphenyl-2-picryl-hydrazil (DPPH). Prepare DPPH solution (100 µM) in 70% methanol. Mix 3 mL of this solution with 0.5 mL of sample (final concentration 0.001M). After incubation in a dark place for 30 min at room temperature, measure the absorbance of the mixture at 515 nm against methanol as blank using spectrophotometer The activities of the samples were evaluated by comparison with a control (containing 100 µM DPPH solution). The activity was calculated according to the following formula:

$$DPPH\ \ scavenging\ \ activity\ \ (\%)\ =\ [(Ac–As)/Ac]\ \times\ 100$$

where A_C is the absorbance value of the control and A_S is the absorbance value of the added test samples solution.

b) Determination of Glutathione Levels

Glutathione

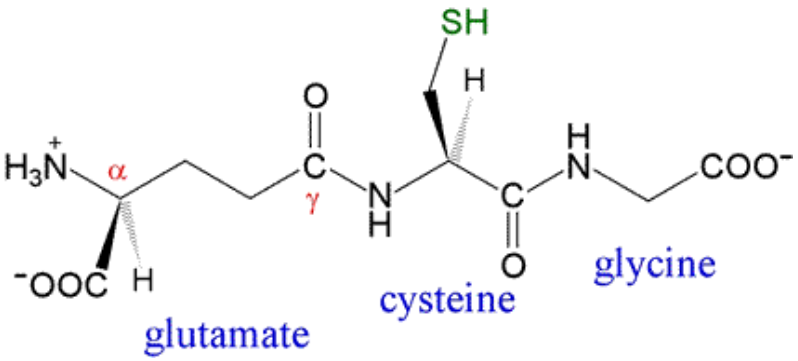

Figure: Reduced gluthation (GSH)

Gluthatione is an isotripeptide (γ-Glu-Cys-Gly, FW 307.3) that serves as an antioxidant in erythrocytes (red blood cells) and as a sulphydryl buffer in all types of animal cells.

Glutathione can be oxidized by formation of a disulfide bond between the two cysteine residues, and this form is denoted GSSG, while (reduced) glutathione is denoted GSH.

Glutathione contains the amino acids glutamate and glycine, in addition to cysteine. The figure at left shows the structure of reduced glutathione (GSH). Glutamate is linked in an isopeptide bond (via its γ-carboxyl group) to cysteine, which in turn forms a peptide linkage with glycine.

When glutathione is used as the reductant of cellular substrates, it must be regenerated using the reducing power of NADPH. The enzyme glutathione reductase [EC 1.8.1.7] carries out the NADPH-linked reduction of oxidized glutathione:

$$GSSG + NADPH + H+ \rightarrow 2\ GSH + NADP+$$

Glutathione is particularly important in erythrocytes (red blood cells), which are under considerable oxidative stress due to the high levels of oxygen they carry. Mutations in the enzyme catalyzing NADPH-producing first step of the oxidative branch of the pentose phosphate pathway, called glucose 6-phosphate dehydrogenase [EC 1.1.1.49], result in susceptibility to oxidative stress.

Measurement of GSH

The assay is based on the reduction of 0.01 M 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) by sulfhydryl groups of GSH to form 2-nitro-5-mercaptobenzoic acid per moles of GSH. The 2-nitro-5-mercaptobenzoic acid is yellow color and can be measured by spectrophotometric method.

Figure: Reaction of DTNB with a thiol (R-SH)

Preperation of Solutions

10% Trichloroacetic acid (TCA): Add 5 g TCA into 50 mL distilled water

0.4 M Tris buffer: Add 48.4 g and 7.4 g EDTA into 800 mL distilled water, adjust pH to 8.9 with 6M HCl, complete the total volume to1 L.

10mM DTNB: Dissolve 99 mg DTNB in 25 mL methanol.

GSH standart solutions: Dilute 1mM stock GSH solution and prepare 25,50,100 µM standard GSH solutions.

Experimental Design

Add 10%TCA into the tissue homogenate tube at the same volume (0.5 mL)

Mix the tubes 10-15 minutes

Centrifuge the tubes 15 minutes at 3000g

Carefully pipette 0.4 mL of supernatant to an other tube and add 0.8 mL tris buffer and 0.02 mL 10 mM DTNB solution, mix

Measure the yellow color absorbance at 412 nm by using spectrofotometer.

Create a standard curve by plotting the absorbance for each standard concentration on the ordinate. To determine the sample values the slope of the curve is determined. Because of the samples have been diluted, the standard curve must be multiplied by the dilution factor.