CHAPTER II

MATERIALS AND METHODS

1. Chemicals

All chemicals used in this study were analytical grades and listed as following.

Chemicals	Companies
Acids	
Acetic acid	Merck
Hydrochloric acid, concentrated	"
Phosphoric acid, 85%	"
Phosphotungstic acid	"
Sulfuric acid, concentrated	J.T. Baker
Organic solvents	
Ethanol	Merck
Methanol	Mallinekrodt
Buthanol	Lab scan
Chloroform	Merck
Ethyl acetate	Lab scan
Formal aldehyde	Merck
Buffers	
HEPES (N-[12-Hydroxy Ethyl]Piperazine-N'-[2-Ethan Sulfonic acid])	Sigma
Tris (tris[Hydroxy methyl]amino methane	"
Glycine	"
K_2HPO_4	M&B
$KH_2 PO_4$	"

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Chemicals	Companies
Chelating agents	
EDTA (Ethylene diamine tetraacetic acid)	Sigma
EGTA (Ethylene glycol-bis [-aminoethyl ether]-N,N,N',N'-tetraacetic acid)	"
HEDTA (N-Hydroxyethyl ethylene diamine triacetic acid)	"
Enzyme and Proteins	
Bovine Serum Albumine (BSA)	Sigma
Calmodulin (CaM)	"
Lactate dehydrogenase (LDH)	Boehringer
Pyruvate kinase (PK)	"
Xanthine oxidase (XOD)	Oriental
Metmyoglobin	Sigma
Nucleotides and High-Energy Compounds	
ATP, disodium salt	Fluka
β-NADH	Sigma
PEP (phosphoenolpyruvate)	"
Oxidants	
Hypoxanthine (HPX)	Wako
ABTS (2,2'-Azino-bis(3-ethylbenz thiazoline-6-sulfonic acid)	"
NBT (Nitro-blue tetrazolium)	"
$FeCl_3$	ucb
H_2O_2	Merck
Tert- Butyl peroxide	Aldrich

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Chemicals	Companies
Antioxidants	
Trolox (6-hydroxy-2,5,7,8-tetramethyl chlorma n-2-carboxylic acid)	Aldrich
L-ascorbic acid	BDH
α-tocopherol (vitamin E)	Wako
Curcumin	Sigma
Proanthocyanidin from pine bark	Maritime Prime
Proanthocyanidin (French paradox) from grape seed	Arkopharma
Miscellaneous	
Ouabain (g-strophanthin)	Boehringer
Coomassie blue G250	Serva
$(NH_4)_2SO_4$	Carlo Erba
KOH	"
$CaCl_2$	Merck
KCl	M&B
NaCl	Carlo Erba
NaOH	J.T.Baker
$MgCl_2$	"
$KMnO_4$	BDH
Bromine solution	Merck
2-Thiobarbituric acid	Sigma
Dimethyl sulfoxide (DMSO)	"
TEP (1,1,3,3-Tetraethoxypropane)	Wako

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2. Instruments

Instruments	Companies
Spectrophotometer UV-2401PC	Shimadzu
510-FT-IR Spectrometer	Nicolet
Super speed refrigerated centrifuge, RC5	Sorval
Microcentrifuge (micron13)	Herolab
pH meter	Corning
Water bath, 37 °C	Lab-line
Water bath, 100 °C	Memmert
Balance	Precisa
Vacuum rotating evaporator	Buchi

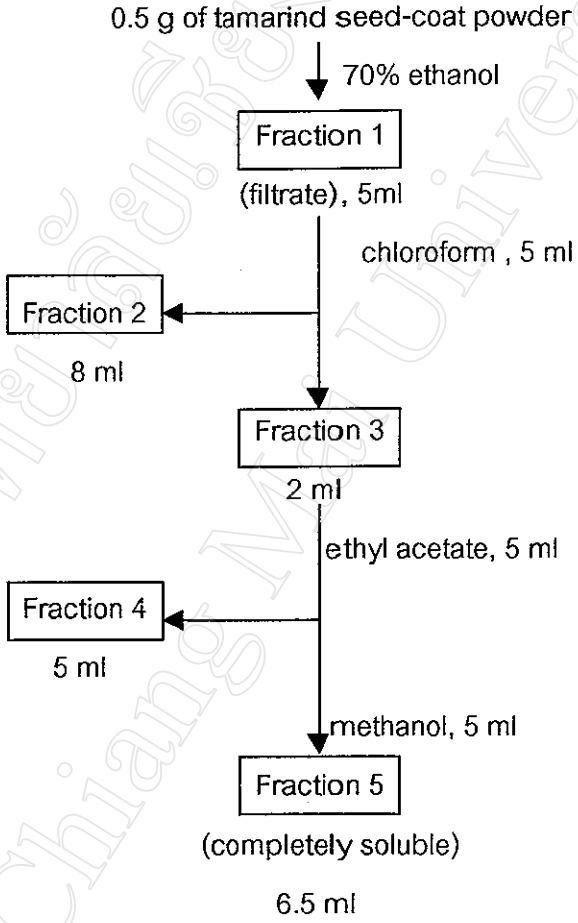
3. Preparation of tamarind seed coat powder.

Tamarind seeds were obtained from ripen tamarind fruits after removing the edible parts. The good seeds were heated in a hot air oven at 140 °C, 45 minutes, cooled and readily cracked to separate their outside brown layer. Only brown-red seed coats were collected and ground into fine powder.

4. Extraction of antioxidative substances from tamarind seed coat powder.

The method was modified from the report of Abdel Alim, 1997(71). The tamarind seed coat powder weighed 0.5 gram were added to 10 ml of 70% ethanol in a separating funnel . The mixture was vigorously shaken for 10 minutes and then filtered through a filter paper. The extraction was repeatedly done until the filtrate became colorless all. The filtrates were pooled, and five ml of the pooled filtrates, so-called Fraction 1 or ethanol fraction, were further fractionated in separating funnel by addition 5 ml of chloroform. The mixture was shaken well and left it until the 2 layer were separated apart. The lower layer or chloroform layer, called Fraction 2, was firstly collected approximately 8 ml of volume, and the rested upper layer of aqueous layer in funnel, so-called Fraction 3 having amount as equal as 2 ml, was added ethyl acetate, 5 ml. The

mixture was shaken and left to separate into 2 layers. The lower layer or Fraction 4 containing a large amount of ethyl acetate was collected at 5 ml, and the remaining layer was totally dissolved in 5 ml of methanol and was called Fraction 5 (6.5 ml). of volume. Antioxidant activity and dry weight of each fraction were determined. The procedure is described in the subsequent protocol:



5.Determination of total antioxidant activity.

Oxidation reaction in almost all cases by plant peroxidase can be represented by the following reactions, in which SH_2 is the substrate and $SH^{\bullet}$ is substrate-derived radicals (1):

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + SH_2 \longrightarrow SH^{\bullet} + \text{Compound II}$$

$$\text{Compound II} + SH_2 \longrightarrow SH^{\bullet} + \text{Peroxidase}$$

In this study the antioxidant activity was measured in the ABTS/H_2O_2/White-radish peroxidase (WRP) system. ABTS is oxidized in the presence of H_2O_2, a peroxidase substrate in a typical peroxidative reaction, generates an intermediate, a metastable radical form of oxidized ABTS, with a characteristic and maximum absorption at 414 nm (72).

By the principle, antioxidant activity is measured by an inhibition assay. All the reagents are mixed together and the reaction is started by the addition by hydrogen peroxide at the time 0. The reaction rate is observed and the antioxidant potency of the extract as % inhibition of the formation of ABTS radical was calculated as the following:

$$\%\text{Inhibition} = \frac{(\text{Reaction rate}_{\text{control}} - \text{Reaction rate}_{\text{sample}}) \times 100}{\text{Reaction rate}_{\text{control}}}$$

Reagents

50 mM glycine-HCl buffer, pH 4.5

2 mM ABTS in 50 mM glycine-HCL, pH 4.5

20 mM H_2O_2

3.0 units of lyophilized white-radish peroxidase (WRP).

Procedure

In a plastic cuvette, 2 ml of 2 mM ABTS solution, 10 µl of WRP solution and 10 µl of either control (solvent) or sample (antioxidant) was mixed. The reaction was started by the addition of hydrogen peroxide, and the solution was mixed quickly. Time course program of the UV-2401PC spectrophotometer used for recording the kinetic reaction rate; the machine was started by pressing auto-zero button and followed with the start button. The reaction was monitored for 2 minutes at 414 nm with a cuvette containing ABTS solution in the reference socket and determined in triplication. The reaction rates of both control and sample tests were calculated and total antioxidant activity values were compared.

6. Preparation of peroxidase .

Peroxidase utilized in the determination of the antioxidant activity in ABTS/H_2O_2/peroxidase method, was prepared from white radish. The enzyme was partially purified and dried by lyophilization as final product (73).

Reagents

0.1M Dipotassium phosphate

Saturated ammonium sulfate

Procedure

Chopped white radish roots, 1 kg, were blended with 100 ml of 0.1M dipotassium phosphate. The homogenate was filtered through cotton white gauges and the filtrate was added with saturated ammonium sulfate up to 35% saturation and let stand at room temperature on a working magnetic stirrer for about 1 hour. The solution was centrifuged at 2000 rpm, 10 minutes in a rotor. The supernatant was made to 90 percent saturation of ammonium sulfate and the following method was done as previously described until the pellet was finally obtained from the rotor. The certain pellet was dissolved in 5 ml of water and dialyzed at 4 °C. The desalted dialysate of solution was centrifuged at 2000rpm,10 minutes in the rotor, and the supernatant was lyophilized and eventually the lyophilized powder was stored at 4 °C. The kinetic property of the prepared enzyme as determined by ABTS/H_2O_2/ method was shown subsequently.

Specific activity = 1.04 dA(414nm)/min/mg Protein

Km = 4.93

Vmax = 0.5514 dA(414nm)/min

The enzyme unit of peroxidase in this experiment was defined as 1 unit of the enzyme is the amount of peroxidase that can change the optical density 0.1 dA (at 414nm) within 1 minute in ABTS/ H_2O_2 system.

7. ABTS/ H_2O_2/Metmyoglobin method.

The method derives from the principle that when ABTS is incubated with a peroxidase and hydrogen peroxide, the relative long-lived radical, the oxidized ABTS is formed. Metmyoglobin containing Fe(III) heme ring is replaced peroxidase. The principle of the reaction as described in previous study of P. George and D.H. Irvine, 1952 (74) and Nicholas J. Miller et al,1993 (75) inferred that:

$$\text{Heme}(Fe^{III}) + H_2O_2 \longrightarrow \text{ferryl myoglobin} + X^{\bullet}$$

$$X^{\bullet} + \text{ABTS} \longrightarrow \text{intermediate radical of ABTS}$$

$X^{\bullet}$ is an oxidizing agent, transient; only during the actual formation of the complex is its oxidizing ability observed. When metmyoglobin is used to replace peroxidase, the ABTS radical formed by ferryl myoglobin shows absorption peaks at 650, 734 and 820 nm, beyond the region of absorption of heme proteins.

Reagents

500 μM ABTS in 0.05 mM phosphate buffer saline, pH7.4,	500 μl
76 μM Metmyoglobin,	70 μl
0.05 mM Phosphate buffer saline, pH 7.4,	980 μl
500 μM Hydrogen peroxide,	450 μl
Final volume	2000 μl

Procedure

This method was modified from that reported by Nicholas J. Miller et al,1993 (74). All reagents were mixed and the reaction was initiated by the addition of 500 μM hydrogen peroxide, 450 μl. The reaction rate was recorded on time course program of UV-2401PC spectrophotometer for 120 seconds at 734 nm and determined in triplication. The reaction rates were used for calculation as the antioxidant activity.

8. ABTS/ H_2O_2/$FeCl_3$ method: Fenton reaction.

Hydrogen peroxide can readily react with transition-metal catalysts to generate the hydroxyl radical ($OH^{\bullet}$). The sequence of the reactions was basically described by Fenton in 1894 as shown below:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^{\bullet-} + 2H^{+}$$

Even more reaction was possible:

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

This so-called iron-catalyzed Haber-Weiss reaction, was first proposed as one of several possible reaction in 1934 (1). In this study, the reaction of hydroxyl radical occurred transiently; the reaction rate was detected by the changes of intermediate radical of ABTS instead.

Reagents

500 µM ABTS in 0.05 mM phosphate buffer saline, pH 2.0	2 ml
20 mM H_2O_2	50 µl
0.2 mg/ml $FeCl_3$	10 µl
Final volume	2060 µl

Procedure

All reagents were mixed and the reaction was started by the addition of 10 µl ferric chloride to start the reaction. The rate of reaction was recorded on time course program of UV-2401PC spectrophotometer for 120 seconds, and the change rate of the absorbance at 414 nm was determined in triplication and calculated for the antioxidant activity.

9. **NBT/HPX/XOD: Neotetrazolium method.**

Xanthine oxidase (XOD) catalyses the oxidation reaction of O_2 to hypoxanthine(HPX) and xanthine with the generation of superoxide anion ($O_2^{\bullet-}$) as shown below:

$$HPX + O_2 \xrightarrow{XOD} xanthine + O_2^{\bullet-}$$

$$Xanthine + O_2 \xrightarrow{XOD} uric\ acid + O_2^{\bullet-}$$

In this study, superoxide anions were generated enzymatically in a hypoxanthine-xanthine oxidase system to generate superoxide anions, and monitored the products of the reduction of nitro blue tetrazolium (NBT) as shown below (76-77).

$$NBT + O_2^{\bullet-} \longrightarrow NBT^{\bullet}\ radical + O_2$$

NBT which is reduced by superoxide anion to $NBT^{\bullet}$ radical, forming a deep-blue colored formazan at 560 nm maximum absorption.

Reagents

0.2 mM hypoxanthine

0.05 mM EDTA

0.6 mM NBT

0.5 M sodium-phosphate (Na-Pi) buffer, pH 7.5

10 unit/ml XOD

Procedure

The assayed mixture contained 0.2 mM HPX and 0.05 mM EDTA in 0.5 M Na-Pi buffer (pH 7.5) including 0.6 mM NBT. The reaction was started by addition 10 µl of 10 unit/ml of XOD. The reaction rate was recorded on time course program of UV-2401PC spectrophotometer for 120 seconds at 560 nm determining in triplication, and calculated for the antioxidant activity.

10. Preparation of calmodulin-deficient erythrocyte membranes.

In order to study the basal ATPase activity and the effect of calmodulin on the erythrocyte membrane Ca^{2+} ATPase, the calmodulin-deficient erythrocyte membranes were required. Preparation of the membranes was performed by the method described by Niggli, et al (78).

Reagents

Buffer 1;	130 mM KCl
	10 mM Tris-HCl, pH 7.4
Buffer 2;	1 mM EDTA
	10 mM Tris-HCl, pH 7.4
Buffer 3;	130 mM NaCl
	0.5 mM $MgCl_2$
	0.05 mM $CaCl_2$
	10 mM HEPES, pH 7.4

Procedure

The whole procedure was carried out at 4°C and the specimen was kept in an ice bath. Whole blood sample was spun in SC-5 Sorvall centrifuge machine at 5,000 rpm, 10 minutes, 4°C, using a SS- 34 rotor. Plasma and buffy coat were discarded by aspiration. Packed red blood cells were re-suspended in volumes of buffer1, and the cells were washed 3 times to clean up the plasma and white blood cell.

One volume of washed erythrocytes was added to 10 volumes of the lysis buffer 2. The mixture was shaken vigorously for 1 minute prior to centrifugation at 13,000 rpm for 30 minutes. The supernatant and the dense creamy brown "button" at the bottom of the centrifuge tube were discarded by aspiration. The ghost membranes were repeatedly washed twice by the same procedure using buffer 2.

The cleaned ghosts were washed once in buffer 3. The calmodulin-deficient membranes were re-suspended in buffer 3 to the final protein concentration of about 4

mg/ml. The membrane suspension could be stored at –80 °C up to 1 month without significant difference in the enzyme activity.

11. Protein assay

According to the purpose of rapid estimation of protein concentration and to avoid the interference of HEPES buffer in the membrane preparation to Lowry's method, the dye-binding method using Coomassie brilliant blue G-250 as described by Read and Northcote (79) was used.

Reagents

95% Ethanol,	94	ml
Coomassie Serva blue G-250	0.1	g
85% *o*-Phosphoric acid	200	ml
Stock solution bovine serum albumin(BSA)	1	mg/ml

Procedure

Preparation of the Bradford's reagent.

Serva blue G-250 (0.1 g) was stirred in 94 ml of the 95% ethanol for 30 minutes or until the dye was completely dissolved. *o*-Phosphoric acid, 200 ml, was added with stirring and followed by distilled water to the final volume of 2 liters. The solution was filtered through Whatman no. 1 filter paper. The reagent was kept in a dark brown bottle at room temperature.

Setting up a standard curve.

The stock standard solution BSA (10 mg/ml) was diluted to give final concentrations of 0.1 to 1.0 mg/ml. Aliquot of 50 µl protein sample was mixed with 2.0 ml Bradford's reagent in a test tube. The tube was left 10 minutes at room temperature. The absorbance at 596 nm was read within 10 minutes by UV-2401PC spectrophotometer.

Triplicate measurements were performed and the standard curve was drawn from the average values. The protein standard curve is shown in Figure 9.

Determination of membrane protein.

The erythrocyte membrane suspension was diluted 1: 50 prior to protein determination. The volume proportion of the diluted membrane to Bradford's reagent was the same as the working standard BSA solution.

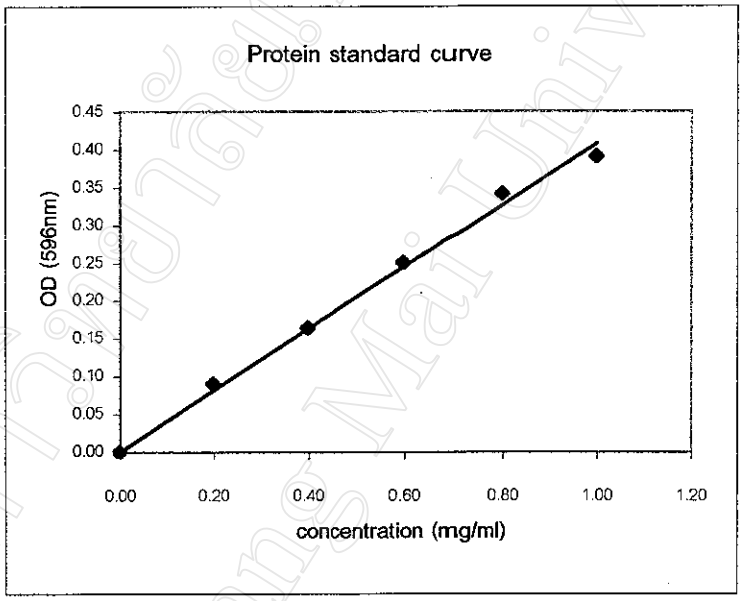


Figure 9: The standard curve of protein. Protein was assayed by the Coomassie brilliant blue dye-binding as described in "Materials and Methods". Bovine serum albumin (BSA) was used as the standard protein.

12. Determination of the antioxidative effect of tamarind seed-coat extract on erythrocyte membranes.

In order to determine the effects of the extract to protect lipid peroxidation and Ca^{2+}-ATPase on erythrocyte membrane, the calmodulin-deficient erythrocyte membranes were incubated with the extract prior to exposure to reactive free-oxygen radicals. The experiments were determined in comparison to vitamin E and curcumin.

Reagents

10% tert-Butylperoxide

0.1 mM $FeCl_3$

Buffer 3; 130 mM NaCl

0.5 mM $MgCl_2$

0.05 mM $CaCl_2$

10 mM HEPES, pH 7.4

Procedure

Aliquots, 1 ml, of the calmodulin-deficient erythrocyte membrane suspension at 4 mg/ml protein in mricocentrifuge tubes, were added with antioxidants, such as the tamarind seed coat extract, vitamin E and curcumin, and incubated on the water bath for 20 minutes at 37 °C. The suspension were centrifuged in a microcentrifuge at 13,000 rpm, 5 minutes, and the supernatant was discarded approximately 700 µl, then the equivalent volume of buffer 3 was added to the membrane to recover the original volume.

The membrane suspension were subsequently added 50 µl of 10% tert-butylperoxide and 50 µl of 0.1 mM $FeCl_3$ and incubated in water bath under the same condition. Then, the membranes were washed 3 times with buffer 3. The supernatant was discarded and the membranes were used for the determination of lipid peroxidation and Ca^{2+}- ATPase activity.

13. Thiobarbituric acid – reactive substances (TBARS) measurement.

Malondialdehyde (MDA) is the most abundant aldehyde arising from lipid peroxidation. It is determined by measurement of colored product formed upon reaction with thiobarbituric acid (TBA). TBARS determination is one of the most common assays used in lipid peroxidation studies. The tested sample is treated with TBA in acidic pH, and a pink chromogen is measured. In the TBA reaction, one mole of MDA reacts with two moles of TBA to produce a pink pigment with an absorption maximum at 532 nm(33).

Reagents

4 μM Tetraethoxypropane(TEP), stock solution

10%(w/v) Phosphotungstic acid

0.67%(w/v) TBA in 1: 1 water and glacial acetic acid

n-Butanol

4 M H_2SO_4

Procedure

Preparation of standard curve

Standard MDA solution was prepared from 4 μM stock TEP solution (range 0.1-1.0 μM). aliquots of 2.0 ml of diluted standard solution was added with 0.5 ml of TBA reagent in sealed glass tubes. The mixture was heated in a water bath at 95 °C for 60 minutes, after cooling on ice, 2.5 ml of butanol was added and mixed vigorously for 30 seconds. The test tubes were centrifuged in a microcentrifuge bench-top at 3,000 rpm for 10 minutes and the absorbance at 532 nm of the butanol layer was determined by UV-2401PC spectrophotometer. The standard curve is shown in the Figure 10.

Determination of TBARS in the oxidized erythrocyte membranes

Aliquots of 10 μl of oxidized membrane suspension were added to 850 μl of 4 M H_2SO_4 and 150 μl of 10% phosphotungstic acid, mixed and left standing at room temperature for 5 minutes. The mixture was added with 1 ml of distilled water and 500 μl

of TBA and mixed well. Then the reaction was proceeded by the same procedure as described in standard curve preparation and determined in triplication.

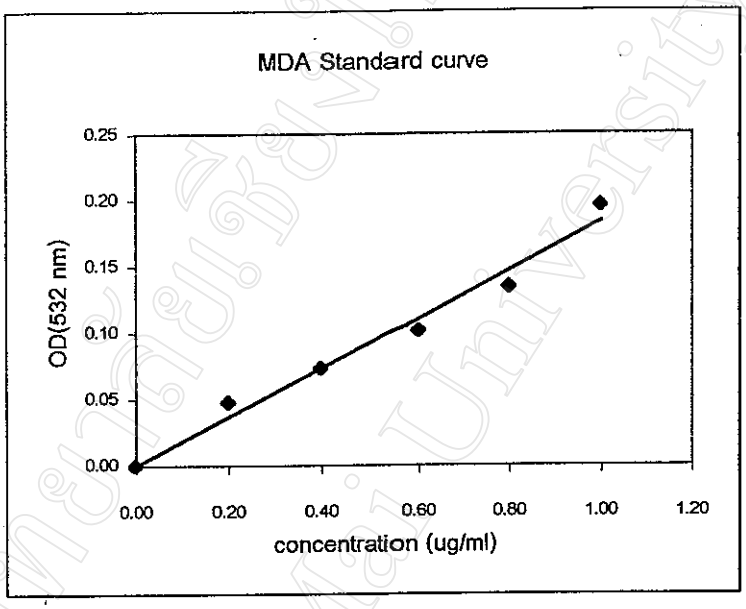
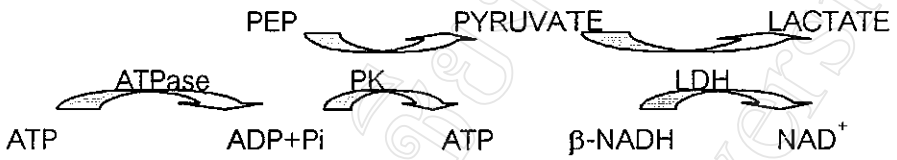


Figure 10: The MDA standard curve. MDA was assayed by the TBARS reaction as described in "Materials and Methods". 1,1,3,3-Tetraethoxypropane (TEP) was used as the standard MDA.

14. Determination of the Ca^{2+}- ATPase activity.

Ca^{2+}-ATPase activity was assayed by the coupling enzyme method. The assay involves the coupling of the ATPase reaction with the reaction of pyruvate kinase (PK) on phosphoenolpyruvate (PEP), and lactate dehydrogenase (LDH) with β-NADH. The reaction sequence is as followed:



Since PK and LDH amounts are in excess, the rate limiting step in the coupled enzyme assay is the ATPase-catalyzed conversion of ATP into ADP+Pi. The oxidation of β-NADH is measured at 340 nm. This is directly related to the ATPase activity. For each mole of β-NADH oxidized, one mole of ATP is hydrolyzed.

The composition of the coupled enzyme medium was prepared followed the procedure described by Sarkadi, et al (80).

Reagents

1 μg/ 5 μl Bovine brain calmodulin (CaM) solution

coupled enzyme medium:

50 Mm HEDTA	1.0 ml
50 mM EGTA	1.0 ml
10 mM Ouabain	1.0 ml
50 mM $CaCl_2$	1.2 ml
3 M KCl	4.0 ml
1 M HEPES	3.0 ml
100 mM $MgCl_2$	2.5 ml
ATP	0.0870 g
PEP	0.0293 g

The pH was adjusted to 7.4 with 2 M KOH

β-NADH	0.0140 g
PK (5,000 U)	56 µl
LDH (10,000 U)	11 µl

This medium contained 10 µM free Ca^{2+} ions.

Procedure

The UV-2401PC spectrophotometer equipped with kinetic program software and controlled chamber which was set at 37°C was used in this investigation. A volume of 1.0 ml of the coupled enzyme medium was pipetted into a quartz cuvette. The cuvette was placed in the chamber of the UV-2401PC spectrophotometer and incubated for 10 minutes. Aliquot of 10 µl of the membrane suspension was added into the medium and mixed well. The rate of β-NADH oxidation was monitored by following the declining slope of the absorbance at 340 nm against the coupled enzyme medium placed in the reference chamber of the spectrophotometer. The kinetic program was used in the analysis.

The calmodulin solution, 5 µl, was added to 10 µl of the membrane suspension and mixed well. The mixture was incubated for 5 minutes at 37°C prior to addition of the coupled enzyme medium.

$$\text{Fold of calmodulin response} = \frac{Ca^{2+}\text{- ATPase activity in the presence of CaM}}{Ca^{2+}\text{- ATPase activity in the absence of CaM}}$$