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Modulation of cellular calcium adenosine triphosphatase in tissues of rats fed with protein-deficient diet fortified with *Moringa oleifera* leaves

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Abstract

Calcium Adenosine Triphosphatase is a ubiquitous enzyme and key protein for the maintenance of calcium homeostasis in various cellular compartments. Moringa leave has been reported to be a very good source of dietary protein as well as pool of minerals. So far the effect of varying dietary protein on the activities of Ca^{2+} pump across cell membranes is yet to be fully explained. In this study, the effects of protein deficiency on Ca^{2+}-ATPase activities were investigated. Twenty four Wistar rats (average weight 119±0.3 g) randomised into four groups of six (6) rats each were used for the study. Group A rats were treated with normal animal diet, group B were placed on protein-deficient diet, Group C were treated with protein deficient diet fortified with fish while Group D were administered with protein deficient diet fortified with *Moringa oleifera* leaves powder. They were maintained on respective diets for 6 weeks then sacrificed. The skeletal muscle, brain and liver were removed and homogenised. Vanadate is a known cellular Ca^{2+}-ATPase inhibitor and was used to confirm the sensitivity of the enzyme in the homogenate. The protein concentration in each formulated diet was determined to be 9.71%, 2.19%, 23.92% and 31.54% for group A, B, C and D respectively. The enzyme's activities were highest in group D and lowest in Group B. In the same line, the activities were also highest in the skeletal muscle and lowest in the liver of all the groups. The $K_{m\ (ATP)}$ and V_{max} of the enzyme in all the studied organs were lowest in group D and highest in group B. The results indicate that protein deficiency reduces the activities of Ca^{2+}-ATPase in the entire tissues' cells and that *M. oleifera* leave powder is a better activator for the enzyme and therefore a good modulator of Ca^{2+}-ATPase activities.

Keywords: Cellular Ca^{2+}-ATPase Activities, Protein deficiency, *Moringa oleifera*, Protein concentration.

Introduction

Effect of dietary protein level on the activities of membrane-bound enzymes is just receiving attention and has been reported to have caused physiological and pathological changes in the body system [1]. This may result in protein-energy malnutrition which is characterized by growth retardation and wasting of subcutaneous fat and muscle as a result of an inadequate intake of protein with reasonable caloric intake. Proteins are essential components of a balanced diet. They are important sources of amino acids and these amino acids are reported to be important in a variety of biochemical pathways [2]. Alterations in diet have been reported to result in changes in the internal milieu and the extracellular fluid, which surround individual cells. Changes in the extracellular fluid often lead to changes in concentration, composition, and configuration of membrane transporters [1].

Maintenance of electrochemical gradient across the plasma membrane is very important [3]. Calcium pumps are therefore important in the sense that they direct the flow of calcium ions through the cellular membrane and the resulting gradients are used in a variety of signaling systems mediated by gated ion channels [4]. They are ubiquitous protein located in various tissues cells. Calcium pumps are ATPases that transport ions across membranes using energy obtained from the hydrolysis of ATP [3]. The Ca^{2+}- ATPase activity in erythrocytes of malnourished patient has been reported to be reduced. The affinity of the pump for ATP was lower in the membranes of kwashiorkor children in comparison to normal membranes [5].

It has been reported that *Moringa oleifera* leaves meal could be used as a source of plant protein since it was highly accepted even at high inclusion levels in the diet [6]. However, for optimum utilization 10% inclusion is recommended [6, 7] observed a high pepsin and total soluble protein in *Moringa oleifera* leaf meal than other parts of the plant. It has been reported that leaves and pods of *M. oleifera* are a good source of important minerals and these plant organs might be explored as a viable supplement and ready source of dietary minerals in human food [7]. *M. oleifera, Lam* is the most widely cultivated species of a monogeneric family, the Moringaceae.

The tree is grown worldwide in the tropics and sub tropics of Africa

and Asia. All parts of the Moringa tree are edible and have long been consumed by humans. This plant is specially promising as a food source in the tropical area. The nutritional properties of Moringa are now so well known that substantial health benefits are realised. Its leaves and green fresh pods are used as vegetables by humans and are rich in carotene and ascorbic acid with a good profile of amino acids, and have appreciable crude protein levels (8-11].

However, like the huge amount of reports on the nutritional importance of Moringa, there is little or no information on the effects of its consumption on the activities of cellular Ca^{2+}-ATPase in animals. Been an important protein in the regulation of cellular Ca^{2+} homeostasis, this research is aimed at investigating the effects of protein deficiency and its amelioration with *Moringa oleifera* leaves on the cellular Ca^{2+}-ATPase activities in some tissues of Wistar rats. The research was carried out in the cellular membrane of some rats' tissues because Ca^{2+}-ATPase is a ubiquitous enzyme present in the selected tissues that perform crucial functions in the body.

Methodology

Chemicals

Adenosine Triphosphate (ATP), Trizma base and Sodium dodecyl sulphate, were products from sigma Chemical Company, St Louis, M. O., U. S. A. All other reagents and materials were of analytical grade and prepared with distilled water.

Experimental animals

The care of the animals was in accordance with the U.S. Public Health Service Guidelines [12] and approved by the Ethical Committee, Kwara State University, Malete, Kwara State, Nigeria. Twenty four male wistar rats (average wieght 119.39±0.13 g and age 10.17±0.11) obtained from the animal house, Department of Biochemistry, University of Ilorin, Nigeria were used in the study. They were acclimatised for 7 days in an environmentally controlled animal laboratory with 12 h light and dark schedule and had free access to clean drinking water. The rats were then completely randomised into four (4) groups of six rats each.

Group A –Animals in this group were fed with normal experimental animals' diet.

Group B –Animals in this group were fed with formulated protein deficient diet.

Group C –Animals in this group were fed with formulated protein deficient diet fortified with fish.

Group D–Animals in this group were fed with formulated protein deficient diet fortified with *Moringa oleifera* leaves powder.

Table 1: Formulated Iso-Caloric Protein Diets* [13, 14]

Food components (g)	Animal Groups		
	Protein-deficient diet (B)	Formulated diet + fish (C)	Formulated diet + Moringa (D)
Moringa leaves	_	_	250
Fish meal	12.5	250	_
Maize chaff	512.5	275	275
Sucrose	375	375	375
Vegetable oil	50	50	50
Minerals/vitamin	30	30	30
Wheat olfa	20	20	20
Total (g)	1000	1000	1000

*The normal experimental animal diet was not formulated but purchased from market in Ilorin, Nigeria and was administered without alteration.

The composition of the normal experimental diet purchased from market was not known and therefore only its percentage protein concentration was determined. The dietray composition of the rats was calculated based on 1 Kg feed formulation model (Table 1). They were maintained on respective diets for 6 weeks then sacrificed by cervical dislocation. Immediately, the brain, liver and skeletal muscle of the rats were removed and then put in ice-cold 0.25M sucrose solution buffered with 10 mM Tris (pH 7.4) in a beaker to maintain the integrity of the enzymes in the tissues.

Preparation of Tissues Homogenate

The brain, skeletal muscle and liver of the rats were homogenised separately in ice-cold 0.25 M sucrose buffered with 10 mM Tris, at pH 7.4 using Teflon homogeniser. The resulting homogenate was quickly transferred into small sample bottle and stored in the refrigerator at below 4^0 C. The homogenate was used within five days of constant electricity supply.

Determination of Protein concentration

The protein content of the homogenate was determined using biuret reagent [15] using bovine serum albumin (BSA) as standard as described by [16].

Determination of Standard Phosphate Curve

The inorganic phosphate released was estimated using standard phosphate curve [17] based on colour changes reaction using 2% ammonium molybdate and 9% ascorbic acid as described by [16].

Vanadate Inhibition Experiment

Vanadate and Thapsigargin are inhibitors of Ca^{2+}-ATPase and can be used to confirm the presence of the enzyme in a sample. In this research work, an inhibitory effect of vanadate on $Ca^{2+}$was tested in all the tissues. 1 mg/ml of orthovanadate (Na_3VO_4) was prepared by dissolving 1 mg of the reagent in 100 ml of distilled water. The assay medium consist of Tris buffer (30 mM) at pH 7.4, $MgCl_2$ (2 mM), ATP (1 mM), KCl (120 mM) and $CaCl_2$ (0.2 mM). ATPases activity was assayed for by spectrophotometric determination of inorganic phosphate released from ATP hydrolysis as described by [16]. Graph of Ca^{2+}ATPase activity against the vanadate concentration is shown in Figure 1.

Assays for Ca^{2+}ATPase Activity

Ca^{2+}ATPase activities were assayed as described by [16]. The assay medium consists of Tris buffer (30 mM) at pH 7.4, $MgCl_2$.$6H_2O$ (2 mM), ATP (1 mM), KCl (120 mM) and $CaCl_2$ (0.2 mM). The reaction was started with the addition of 10 µg of the enzyme fraction. After 30 minutes of incubation at 37 C with constant shaking, the reaction was stopped with 0.2 ml of 5% (w/v) Sodium Dodecyl Sulphate (SDS). The absorbance was read with Spectronic-20 spectrophotometer at the wavelength of 820 nm. All the assays were carried out in triplicate and individual experiment repeated three times to confirm the results.

Statistical Analysis

All the statistical analyses were performed using the SPSS Version 16 (SPSS Inc, Chicago Illinois, USA 2006 edition) and the numerical values were expressed as means ± SEM. For the Lineweaver-Burk graphs and ATP-dependent activities of Ca^{2+}-ATPase, enzyme kinetics software, ENZFITTER for windows XP was use. A statistically significant association was taken at $P < 0.05$.

Results

Table 2 depicts the results of the summary of Ca^{+}-ATPase activities in the three organs studied after treatment. The result indicates an increase in activities of Ca^{+}-ATPase in all the organs studied in the manner of increasing protein concentrations of the diet (Figure 1). The activities of Ca^{2+}-ATPase was inhibited in a concentration dependent manner by vanadate at constant ATP concentration in the homogenate of all organs investigated (Figure 2). The activity of the enzyme was observed to be higher in the skeletal muscle compared with the brain and lowest in the liver. When compared among the treated groups, cellular Ca^{2+}ATPase activity was observed (in all the tissues) to be lowest in the protein deficient rats, even than normal animal diet. But in the rats fed with diet fortified with *M. oleifera*, Ca^{+}-ATPase activities were even higher than those of the rats placed on fish supplemented diet.

The results of the kinetic constants of Ca^{2+}-ATPase in the skeletal muscle, brain and liver of the rats after treatment are shown in Table 4-6 and Figure 6-8. The $K_{m(ATP)}$ was observed to be significantly raised in the brain of rats placed on protein deficient diet to a value of 32.94 ± 2.54 compared with the observed $K_{m(ATP)}$ of 16.75 ± 5.73 in the brain of rats placed on fish supplemented diet. A significantly lowered K_m of the enzyme (4.90 ± 1.85) was observed in the brain of protein deficient rats when diet was supplemented with *M. oleifera.* The observed $K_{m(ATP)}$ value in Moringa supplemented diet was lowered than even that of normal animal diet. The V_{max} was also observed to be lowest in the brain of protein deficient rat placed on *M. oleifera*. The highest Vmax (9.10±1.80) was observed in the brain of protein deficient rats, a value higher than that obtained with normal experimental animal diet.

Ca^{2+}-ATPase $K_{m(ATP)}$ value of 179.60 ± 12.80 was observed in the skeletal muscle of protein deficient diet rats supplemented with *M. oleifera* (Table 5 and Figure7). The observed $K_{m(ATP)}$ value was significantly lowered than 304.80 ± 14.00 observed in the skeletal muscle of fish diet rats. A much higher $K_{m(ATP)}$ value of 349.41 ± 0.62 was however observed in the skeletal muscle of protein deficient rat. A similar result was observed in the V_{max} of all the rats after treatment. The same trend in the variations in $K_{m(ATP)}$ and V_{max} was observed in the liver of the rats after treatment (Table 6 and Figure 8).

Table 2: Percentage of protein concentration in the four groups of diets

GROUPS	PROTEIN CONCENTRATION
Normal Animal Diet	9.71^{a}
Protein Deficient Diet	2.19^{b}
Protein Deficient Diet + fish	23.92^{c}
Protein Deficient Diet + Moringa	31.54^{d}

Values carrying superscripts a to d are significantly different down the column ($P< 0.05$).

Table 3: Ca^{2+} - ATPase Activities in Some Tissues of Rats after Treatment

	Specific activity (µmole Pi /mg protein/hr)			
	Protein Deficient Diet	Protein Deficient Diet + Fish	Protein Deficient Diet +*M. oleifera*	Normal Animal Diet
Brain	0.32 ± 0.28[a]	0.42 ± 0.34[b]	0.47 ± 0.31[c]	0.36±0.11[d]
Skeletal Muscle	0.30 ± 0.20[a]	0.36 ± 0.31[b]	0.40 ± 0.37[c]	0.34±0.19[d]
Liver	0.18 ± 0.10[a]	0.21 ± 0.14[b]	0.25 ± 0.12[c]	0.19±0.01[d]

Results are Mean ± SEM of 6 determinations. Values carrying superscripts a to d are significantly different across the rows for each organ (P< 0.05). While those with the same superscript for each diet group are not significantly different (P> 0.05).

Table 4: Kinetic Parameters of Ca^{2+}-ATPase in the Rats' Brain after treatment

Animal Diet Grouping	$K_{m(ATP)}$(mM)	V_{max} (µmole Pi /mg protein/hr)
Normal Animal Diet	23.10 ± 0.10[a]	6.10 ± 0.11[a]
Protein Deficient Diet	32.94 ± 2.54[b]	9.10 ± 1.80[b]
Protein Deficient Diet + Fish	16.75 ± 5.73[c]	2.02 ± 0.49[c]
Protein Deficient Diet + *M. oleifera*	4.90 ± 1.85[d]	1.04 ± 0.18[d]

Results are Mean ± SEM of 6 determinations. The values were derived from the Lineweaver-Burk plots of figure 6. Values carrying superscripts a to d down the column are significantly different (P< 0.05).

Table 5: Kinetic Parameters of Ca^{2+}-ATPase in the Rats' Skeletal Muscle after treatment

Animal Diet Grouping	$K_{m(ATP)}$ (mM)	V_{max}(µmole Pi /mg protein/hr)
Normal Animal Diet	321.01± 0.06[a]	4.01 ± 0.12[a]
Protein Deficient Diet	349.41 ± 0.62[b]	5.45 ± 0.04[b]
Protein Deficient Diet + Fish	304.80 ± 14.00[c]	3.46 ± 0.29[c]
Protein Deficient Diet + *M. oleifera*	179.60 ± 12.80[d]	2.67 ± 0.38[d]

Results are Mean ± SEM of 6 determinations. The values were derived from the Lineweaver-Burk plots of figure 6. Values carrying superscripts a to d down the column are significantly different (P< 0.05).

Table 6: Kinetic Parameters of Ca^{2+}-ATPase in the Rats' Liver after treatment

Animal Diet Grouping	$K_{m(ATP)}$ (Mm)	V_{max}(µmole Pi /mg protein/hr)
Normal Animal Diet	103.03 ± 9.01[a]	0.98 ± 1.02[a]
Protein Deficient Diet	114.11 ± 3.96[b]	1.26 ± 0.02[b]
Protein Deficient Diet + Fish	89.70 ± 40.97[c]	0.65 ± 0.18[c]
Protein Deficient Diet + *M. oleifera*	45.93 ± 18.36[d]	0.52 ± 0.10[d]

Results are Mean ± SEM of 6 determinations. The values were derived from the Lineweaver-Burk plots of figure 6. Values carrying superscripts a to d down the column are significantly different (P< 0.05).

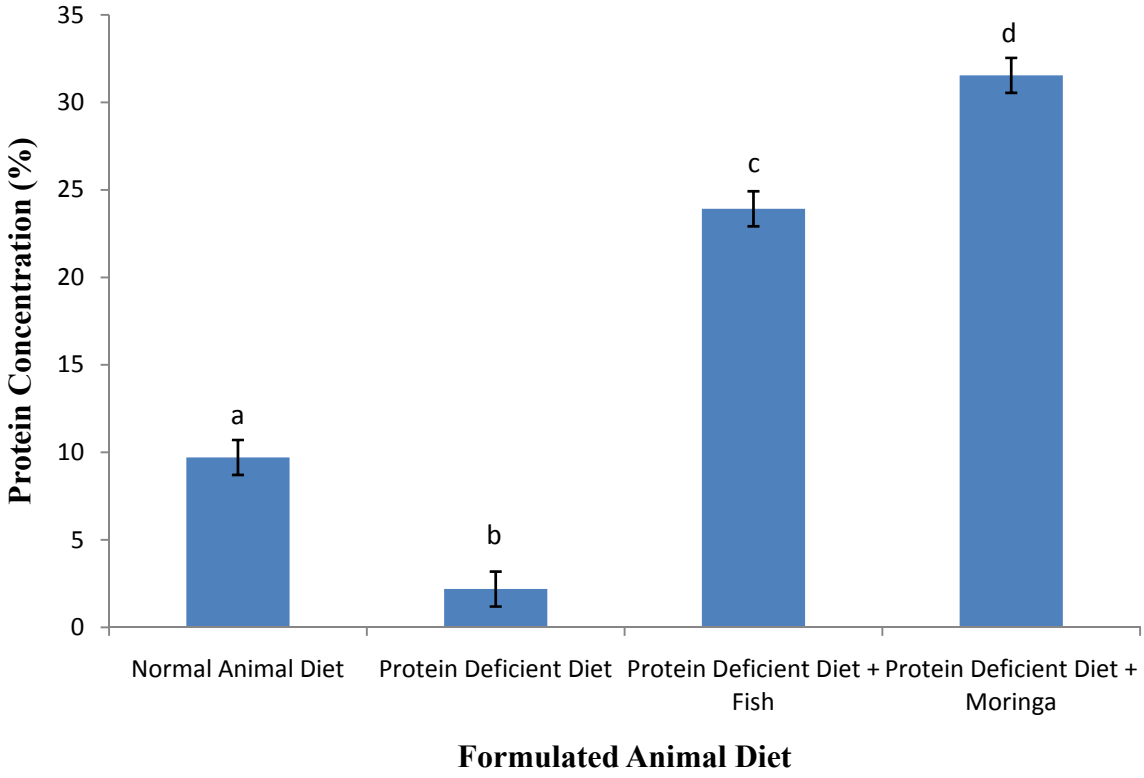

Figure 1: Percentage protein concentration in the diet administered to the four groups of rats. Values are Mean ± SEM.

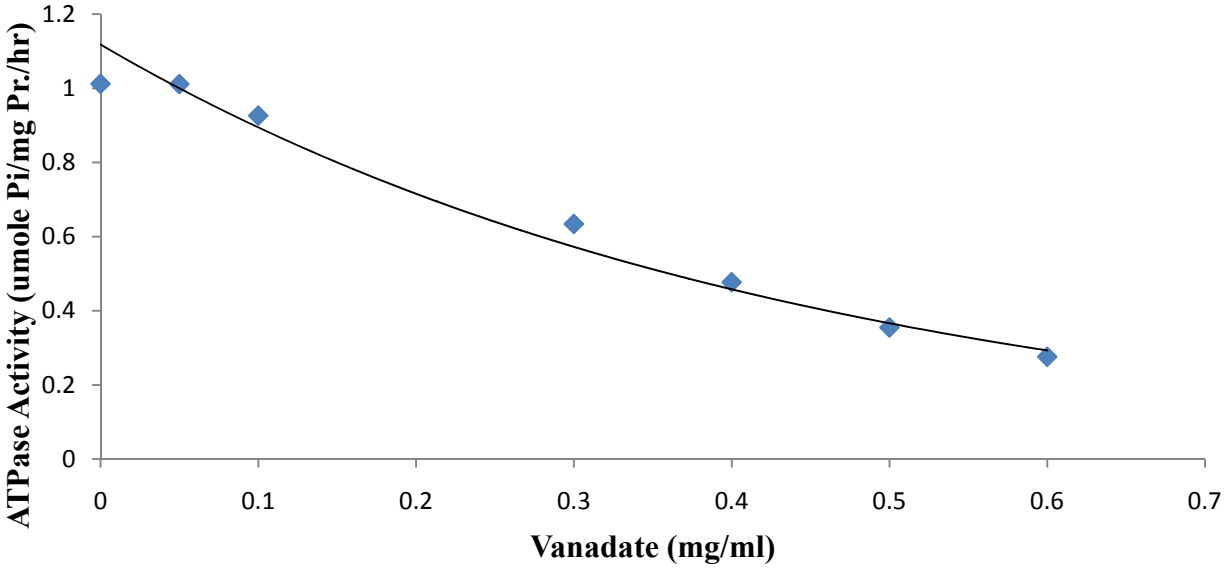

Figure 2: Effects of Vanadate on cellular Ca^{2+}-ATPase Activity in rats' tissue. This pattern was obtained for all the tissues investigated.

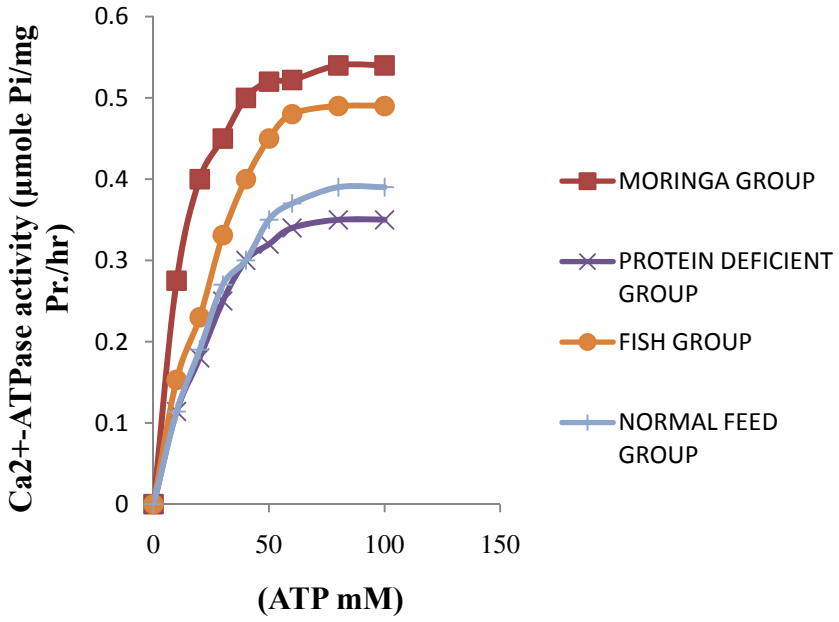

Figure 3: Graph of ATP-Dependent Ca^{2+}-ATPase Activity in the Brain of Rats Fed with Diet of Different Protein Sources and concentrations

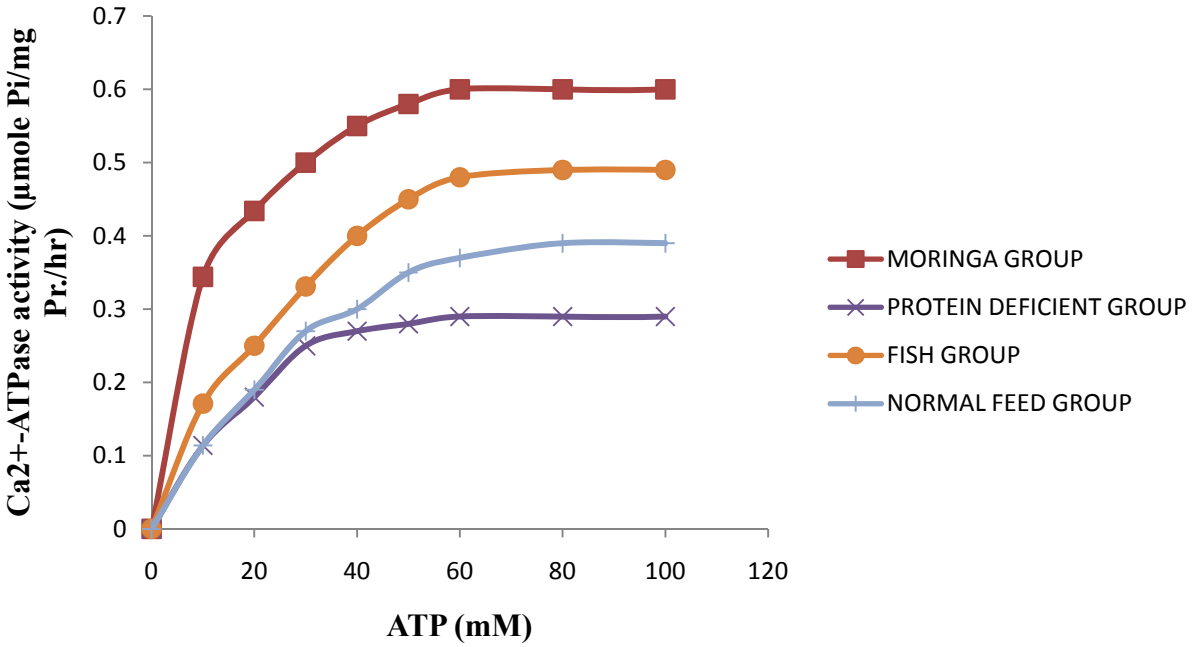

Figure 4: Graph of ATP-Dependent Ca^{2+}-ATPase Activity in the Skeletal Muscle of Rats Fed with Diet of Different Protein Sources and Concentrations

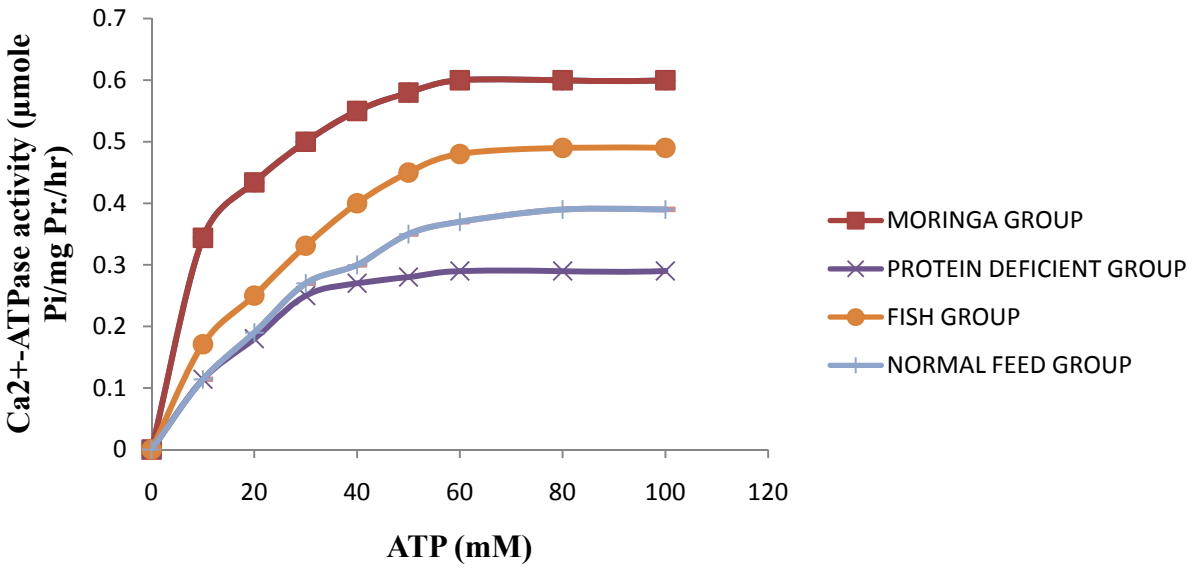

Figure 5: Graph of ATP-Dependent Ca^{2+}-ATPase Activity in the Liver of Rats Fed with Diet of Different Protein Sources and Concentrations

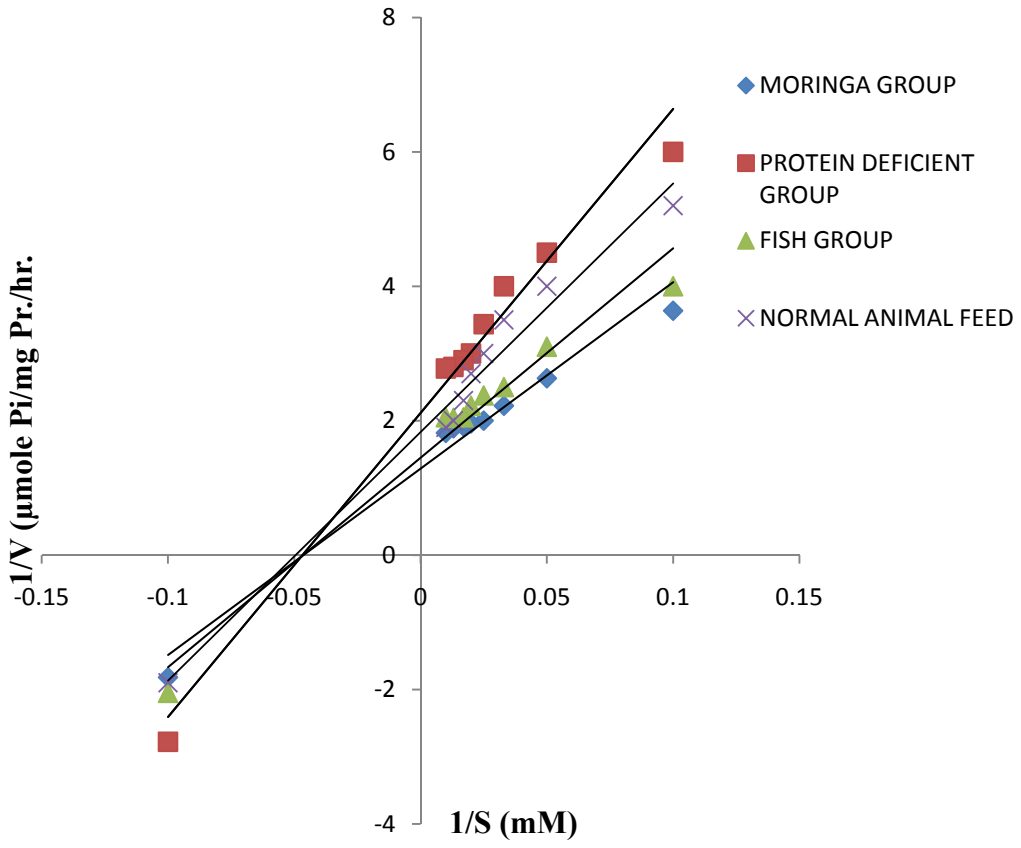

Figure 6: Lineweaver-Burk Plot of Ca^{2+}-ATPase Activities in the Brain of Rats after Treatment

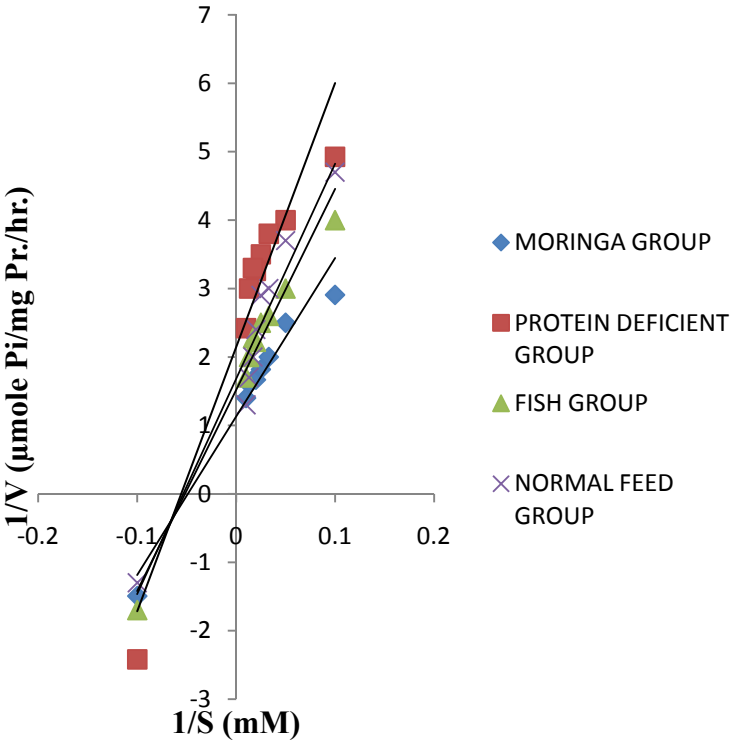

Figure 7: Lineweaver-Burk Plot of Ca^{2+}-ATPase Activities in the Skeletal Muscle of Rats after Treatment

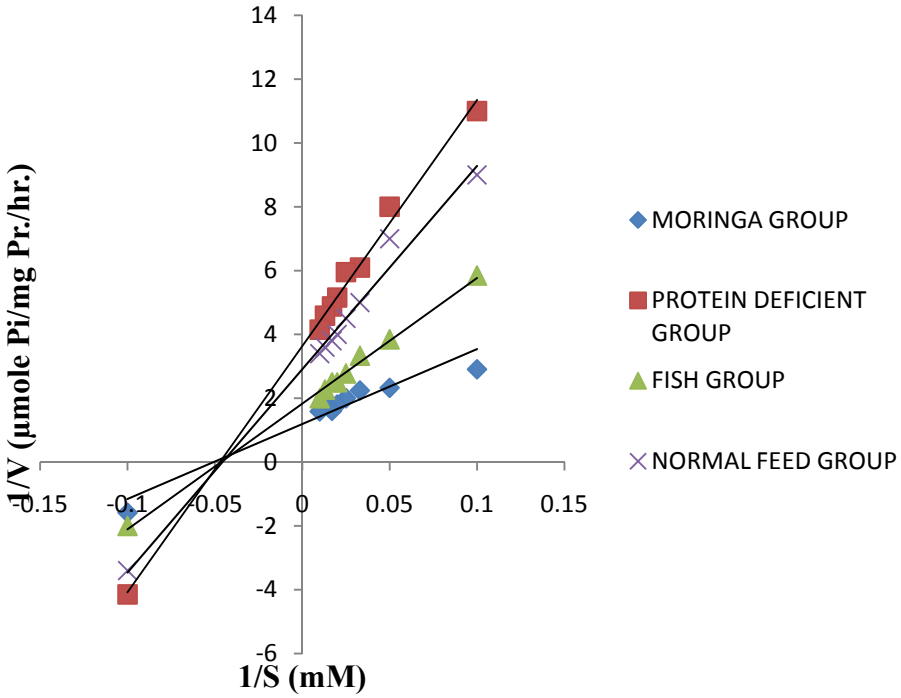

Figure 8: Lineweaver-Burk Plot of Ca^{2+}-ATPase Activities in the Liver of Rats after Treatment.

Discussion

Intracellular Ca^{2+} level are essential elements for normal cellular activities which are closely connected with the development of cells, mitotic activity, immune response, muscle contraction, endo- and exocytosis, or modulation of neuronal cells processes [18, 19]. The precise regulation of the Ca^{2+} homeostasis in cells is a result of the concerted functioning of transporters located in the various cellular compartments i.e. endo/sarcoplasmic reticulum, mitochondrion, and nucleus. Ca^{2+}-ATPase is a ubiquitous protein that removes Ca^{2+} ions from cells. One molecule of ATP is hydrolysed for every Ca^{2+} ion transported with concomitant release of energy. So the activity of cellular Ca2+- ATPase is proportional to molecules of ATP hydrolysed which is also proportional to molecules of Ca^{2+} ion transported. Calcium efflux from excitable cells occurs through two main systems, an electrochemically driven Na^{+}/Ca^{2+} exchanger with a low Ca^{2+} affinity, and a plasma membrane Ca^{2+}-ATPase, with a high Ca^{2+} affinity [20].

Data from our study indicate that all the three organs exhibited measurable cellular Ca^{+} -ATPase activity in all the groups of animal, however the specific activity of the enzyme differ among the organs. This could be an indication of expression of different isoforms of Ca^{2+}-ATPase which differ in specific activities by the organs. This, in our opinion, suggests that biochemically different cellular Ca^{2+}ATPase are localised in the organs of animals. [21] in their study, reported cytochemical distribution of Ca^{2+}-Mg^{2+}-ATPases which was said to be representing biochemically different ATPases including endoplasmic reticular ATPase involved in intracellular calcium regulation, oligomycin-sensitive mitochondrial ATPase, dynein-like ATPase associated with centrioles and an ectoenzyme associated with cell surface specializations. Based on functional studies, [22] have also shown that there are different isoforms of Ca^{2+}ATPases and that these isoforms and variants differ in their regulatory properties. Data from our study agrees with this observation.

The lowest activity of the enzyme was observed in rats placed on protein deficient diet whereas supplementation of protein deficient diet with *M. oleifera* leaves significantly increased cellular Ca^{+}-ATPase activities in all the organs. The observed increase in activity of cellular Ca^{2+} ATPase when the *M. oleifera* was used as supplement was higher than when fish was used in the diet. This result suggests that *M. oleifera* contains nutritive principles at a proportion and quality that is better than fish. *M. oleifera* enhances cellular Ca^{2+}-ATPase activity and thus increases the affinity of the enzyme for ATP thereby increasing the ability of the protein to regulate calcium homeostasis. Proteins form an essential component of a balanced diet because they are an important source of amino acids for the body. These amino acids are important in a variety of biochemical pathways. Alterations in protein diet consumed by individual have been reported to lead to changes in calcium metabolism [23, 24, 25]. Protein rich diets lead to increased absorption of calcium from the gut, Hypercalciuria and subsequent loss of calcium in urine [25].

Diets low in protein on the other hand has been reported to result in reduced calcium absorption and hyperparathyroidism [25]. Calcium is essential for many important processes in the body which include bone formation, nerve depolarisation, signal transduction, muscle contraction and neuronal function 930. Result from our study therefore suggest that protein energy malnutrition will cause a reduction in the activity of cellular Ca^{2+}ATPase, thereby increasing the concentration of Ca^{2+} in the cell which may predispose to several cellular degeneration. This may be the biochemical basis for the numerous symptoms of PEM, including growth failure, hypoproteinemia, odema, fatty liver and decreased immune defense. Our study also revealed that *M. oleifera* supplemented diet supplies enough protein of better quality which we consider better than fish and thus makes it a better and cheaper source of protein. Another interesting result from our study is the fact that we observed that the Ca^{2+} ATPase activity was higher in skeletal muscle than the brain and was least in the liver (except in the protein deficient rat.

We have estimated the kinetic constants, $K_{m(ATP)}$ and V_{max} in order to effectively compare the affinity of the enzyme for the substrate in all the organs studied among the groups of rats. A lower $K_{m(ATP)}$ in brain of protein deficient rats placed on *M. oleifera* when compared with the protein deficient rat group that was not supplemented may be due to an increase affinity of the enzyme for ATP when the diet was supplemented with *M. oleifera*. Our result also indicates that this affinity is higher than that observed in rats fed with fish supplemented diet. Result of the kinetic constant of the Ca^{2+} ATPase in the skeletal muscle and the liver indicate that inclusion of *M. oleifera* in protein deficient diet of rat increased the affinity of the enzyme for ATP in these organs. We observed in our study that the increased in the affinity of the enzyme for the substrate was greater than that which was obtained with fish supplemented diet. This result further suggests that *M. oleifera* is more effective in the skeletal muscle as a protein source than fish.

Conclusion

We have reported based on the result from our study that *Moringa oleifera* is a good dietary supplement for regulating cellular Ca^{2+}ATPase. Since the enzyme is very crucial in maintaining cellular homeostasis, the plant could be a readily available dietary supplement especially for developing countries of the world.

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