EFFECT OF AFLATOXIN B¹-CONTAMINATED FEED ON GOAT ERYTHROCYTE MEMBRANE CALCIUM ADENOSINE TRIPHOSPHATASE IN VITRO

C. A. EZEOKONKWO

Department of Biochemistry, University of Nigeria Nsukka, Nigeria.

Received 23 September 1997; Accepted 18 March 1999

ABSTRACT

Aflatoxin B1 (AFB1) 2, a food/feed contaminant in the tropics, stimulated the activity of goat erythrocyte membrane ion motive calcium adenosine triphosphatase (Ca-ATPase). The stimulatory action was concentration-dependent, typical of a saturation kinetic effect. The degree of stimulation varied from 20% to more than 90% between the lowest (0.1 M) and highest (1000 M) contentrations. AFB1 induced changes in the apparent kinetic parameters, Km and Vmax, of the erythrocyte membrane-bound enzyme. This could affect erythrocyte structure, and thus, the erythrocyte function in oxygen and delivery to the cells.

Key words: Aflatoxin B1, goat, erythrocyte membrane, Ca-ATPase

INTRODUCTION

The potential feed value and nutritional limitations of certain nutrient sources for animal feeds have been reported (Ravindran and Blair, 1991). By-products from soybean, sorghum, millet, barley and groundnut have been used as alternatives for animal feeds (Reddy, 1987; Punjr, 1988; Gupta, 1988). Agro-industrial by-products and food waste materials incorporated into broiler feeds gave feed efficiencies and weight gains better than the control diet (Castillo et al., 1986). The use of plant products as components of livestock feed in the tropics is affected adversely by their susceptibility to contamination by toxin-producing organisms (WHO, 1979).

Aflatoxin B1 (AFB1), a bisfuranocoumarin, is a mycotoxin produced by two species of Aspergillus, namely, A.flavus and A. parasiticus which are common contaminants of foods in the tropics (WHO, 1979). AFB1 production is favoured during storage under high humidity and ambient temperatures. The contamination of food resources by aflatoxin is a major problem for those countries where the meteorological and sanitary conditions allow the growth of A. flavus and the contaminated food is not discarded because of the critical economic and social situation (Gilli et al., 1989). Some of the pharmacological effects already associated with aflatoxin B1 ingestion include interference with several aspects of metabolism including mitochondrial electron transport and labilization of cellular and subcellular membranes (Heathcote and Hibbert, 1978).

Calcium adenosine triphosphatase (Ca-ATPase) is one of the distinct ATPases of the erythrocyte membrane (Drickamer, 1975). It functions in the maintenance of the intracellular calcium (Ca) ion concentration. It pumps Ca ion out using adenosine triphosphate (ATP) as a source of energy. Ca plays a role in erythrocyte structure and function.

The investigation on the effect of AFB1 on goat erythrocyte membrane ion motive Ca-ATPase is intended to determine the implications of the ingestion of AFB1-contaminated feed on the physiological functions of goat erythrocyte.

AFB1 was obtained from Sigma Chemicals, St. Louis, USA and ATP (disodium salt) was a product of Merck Chemicals, Germany. All other products were Analar grade products of May and Baker Ltd., Dagenham, England and British Drug Houses (BDH) Chemicals Ltd., Poole, England. The goat blood was from the abattoir in Nsukka, Nigeria.

Preparation of erythrocyte ghost

The goat blood was collected with an anticoagulant-containing bottle in an ice-pack. After removing the plasma, the red cells were washed three times with buffered saline (0.1 M, pH 7.4) at 2°C. Unsealed ghosts were prepared by rapid introduction of one volume of washed red cells at 50% haematocrit into 15 volumes of ice-cold NaH2PO4 (5.0 M, pH 7.7). The membrane ghosts were washed with Tris-HCl (10mM, pH 7.7) at 4°C according to the method of Hamlyn and Duffy (1978).

Determination of protein and ATPase activity assay

The protein content of the erythrocyte ghost was determined using the Biuret reagent with bovine serum albumin (BSA) as standard (Plummer, 1971). The Ca-ATPase activity was assayed by the method of Hesketh et al (1978). The reaction mixtures consisted of Tris-HCl buffer, the ghost suspension, the operational ions (Ca and Mg ions, and Mg ion, respectively) and varying concentrations (from 0.1 M to 1000 M) of AFB1. The AFB1 was absent in the control reaction mixture. The reaction was initiated by the addition of ATP and continued for 1 h at 37°C. Then it was terminated by adding ice-cold 10% trichloroacetic acid to the reaction mixtures. The mixtures were centrifuged at 4000 g for 5 min after standing for 20 min at 4°C, the supernatant was used for the estimation of inorganic phosphate released according to Keleti and Lederer (1974). The activity of the enzyme is expressed as mol Pi/mg protein/h which was also the unit used for the Vmax, while Km was in millimoles. The enzyme activity due to Mg ion alone, defined as MgATPase was subtracted from that due to Ca and Mg ions (Ca-Mg-ATPase) to obtain the Ca-ATPase activity (Hesketh et al., 1978). Also, the effect of a fixed concentration of AFB1 (20μM) on the enzyme activity with varying concentrations (5 - 20mM) of ATP was determined.

Statistical analysis

A linear regression analysis of the data was done using Statgraphics, a statistical software. Analysis of variance (ANOVA) was also used in the statistical evaluation of the data. Fisher's Least Significant Difference (F-LSD) was used in the mean separation analysis for the determination of AFB1 concentration that produced significant effect on the enzyme activity.

RESULTS

The effect of AFB1 on goat erythrocyte membrane Ca-ATPase is shown in Fig. 1. AFB1 significantly, ($P<0.01$) stimulated the ATPase activity in a concentration-dependent manner. The activity of the enzyme increased with the AFB1 concentration up to a maximum and subsequently, remained virtually constant, typical of a saturation kinetic effect. There was a hyperbolic relationship between the enzyme activity and AFB1 concentration. As shown in Fig. 2, the degree of stimulation varied from about 20% to more than 90% between the lowest (0.1M) and the highest (1000μM) concentrations of AFB1 used. From the F-LSD calculations it was observed that significant stimulatory effect of AFB1 on the enzyme started from 10 M concentration (Table 1).

At varying ATP concentrations (5-20mM) with 20u M AFB1 the ATPase activity increased with increase in the concentration of ATP (Fig. 3). This pattern was not altered by AFB1 although the degree of increase in the ATPase activity was more in the presence of AFB1.

Using a split-plot design it was observed that the effect of the ATP concentrations on the activity of the enzyme was statistically highly significant ($P<0.01$), from the main-plot analysis. However, the stimulation of the Ca-ATPase by AFB1 was to a statistically non-significant level ($P>0.05$) from the sub-plot analysis. Also, there was no significant interaction ($P>0.05$) between the effect of AFB1 and that of varied ATP concentrations.

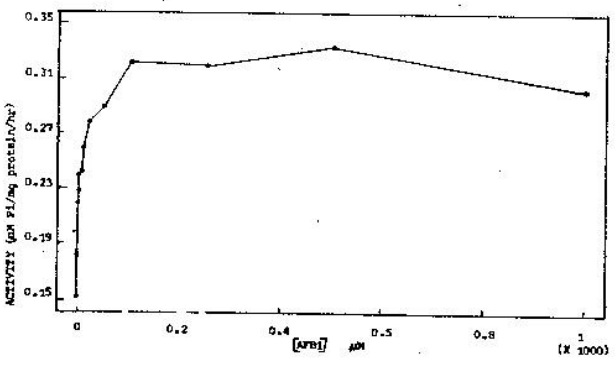

Figure 1.Effect of AFBI and Ca-ATPase activity. Each point represents mean activity of threee independent replicate experiments each assayed in duplicate

TABLE 1: MEAN SEPARATION OF THE CONCENTRATIONS OF AFB1 THAT PRODUCED SIMILAR STATISTICAL EFFECT ON THE GOAT ERYTHROCYTE MEMBRANE (GEM) CALCIUM ADENOSINE TRIPHOSPHATASE ACTIVITY

AFBI concentration(M)	Ca-ATPase activity	SEM umol/mg protein/h
0	0.152	0.008
0.1	0.181	0.006
0.25	0.220	0.003
0.5	0.229	0.002
1.0	0.240	0.002
10.0	0.259	0.000
20.0	0.278	0.001
50.0	0.290	0.002
100.0	0.322	0.005
250.0	0.319	0.005
500.0	0.333	0.006
1000.0	0.303	0.006

$F\text{-}LSD_{0.01} = 0.111$
$F\text{-}LSD_{0.05} = 0.092$

Where the F-LSD is greater than the difference between any two means, then those means are not significantly different at that-level. Each value represents mean activity of triplicate experiments assayed in duplicates.

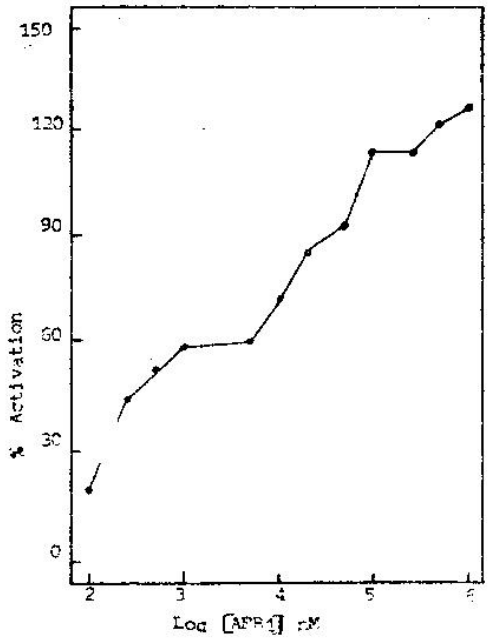

Fig 2: Degree of activation of Ca-ATPase activity by varying concentration of AFBI . Each point represents the mean percent activation of the enzyme from triplicatye experiments each assayed in duplicate

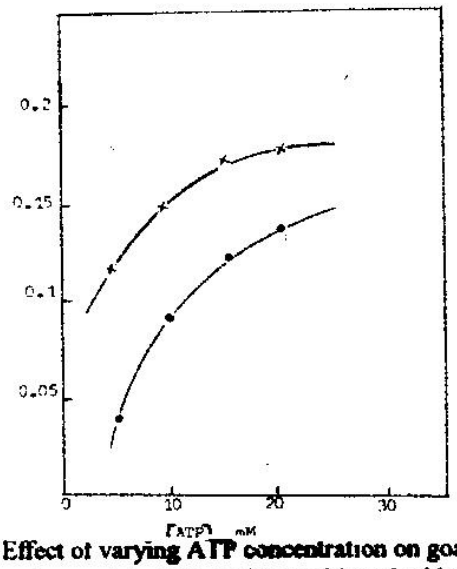

Fig 3: Effect of varying ATP concentration on goat erythrocyte membrane Ca-APTase with and without AFBI
= Control x =AFB_1

A linear regression analysis of the data for a double reciprocal plot obtained with the varying ATP concentrations was used to obtain accurate intercept and slope values (Fersht, 1977), while a direct linear plot analysis of the same data was used to estimate the Km and Vmax of the enzyme (Tipton, 1978). The result (Table 2) showed an increase in the Vmax from 0.134 in the control experiment to 0.196 by AFB1 and a reduction in the Km from 1.60 (control) to 1.46 by AFB1. There were also changes in the slope and intercept values in the presence of AFB1 with respect to the control (Table 2). The slope was changed from 0.099 (control) to 0.145 by AFB1.

DISCUSSION

The stimulatory effect of AFB1 on Ca-ATPase activity may be due to an increase in the velocity of the reaction or a shift in the equilibrium reached or both (Dixon and Webb, 1979). The hyperbolic relationship between the activity of the enzyme and AFB1 concentration is typical of enzymes obeying Michaelis-Menten equation. This could mean that at low concentrations, the initial velocity of the reaction and AFB1 concentrations are directly proportional while at higher concentration values, the initial velocity is maximal and independent of the actual concentration of AFB1 so long as it is "saturating". The degree of stimulation showed a marked dependence on the concentration of

TABLE 2: AFB1 EFFECT ON THE APPARENT KINETIC PARAMETERS, Km AND Vmax, OF GEM Ca- ATPase.

Enzyme treament	Double reciprocal data analysis		Direct linear plot analysis		
	y-intercept ± SEM	x-intercept± SEM	slope± SEM	Vmax± SEM	Km ± SEM
Control	0.134± 0.001	1.347± 0.005	0.099± .006	0.134± .001	1.60± .02
AFBI -treated	0.190± 0.008	1.311± 0.007	0.145± .004	0.196± .009	1.46± .03

The y-axis consists of the reciprocals of the activity values of the enzyme(1/V) while x-axis has the reciprocals of the corresponding ATP concentrations (1/[S]) . Each value represents the mean activity of two replicate experiments each assayed in duplicate.

AFB1. The slope and intercept effects suggest mixed activation of the Ca-ATPase by AFB1 (Fersht, 1977). The mixed activation implies that AFB1 can react with the enzyme at a site different from its substrate binding site and do not affect the concurrent combination of the enzyme with its substrate.

These observations imply a rapid rate of ATP hydrolysis due to increased ATPase activity. In terms of cell metabolism, the increased rate of ATP hydrolysis will result in the rapid depletion of the cell energy reserves as the cell strives to keep up with the increased ATP demand by the tissues. In the absence of exogenous supply of fuel molecules, for example, glucose, gluconeogenesis and consequently weight loss may result. In extreme cases, hypoglycaemia and its attendant consequences including impaired nervous function and eventual death of the animal may result (Penguin Medical Encyclopaedia, 1983).

The depletion of the ATP reserve and the probable reduction in the ATPase activity would lead to accumulation of the Ca ion within the cells. In terms of erythrocyte structure and function, the high intracellular Ca ion could cause polymerisation of spectrin in the red blood cell leading to the loss of the erythrocyte typical biconcave-disc shape and the extreme flexibility which facilitates the passage of erythrocytes through the capillaries may be affected (Swenson, 1970). This can affect the physiological role of erythrocytes in oxygen capture and delivery to the cells.

CONCLUSION

It is concluded that if industrial by-products would be used as non-conventional feed resources their susceptibility to aflatoxin contamination should be considered and measures taken to ensure that the maximum permitted amount of aflatoxin in feeds (0.005 mg/kg) is not exceeded. Also, the protection of the plant products from toxin-producing micro-organisms will reduce the cases of toxicity currently associated with contaminated livestock feed. Such a measure will also increase their potential for use in the food and allied industries.

REFERENCES

CASTILLO, L.S, GERPECIO, A.L, LUGOD, G.C, LAPITAN, J.E, MERCADO, C.I, BAUTISTA, J.G and GONZULEX, O.N (1986). Agro-industrial by-products and food waste materials as potential feed resource in the ASEAN region: The Philippine experience. Research-at-Los-Banos, 5(2): 6 - 9.

FIXON, M. and WEBB, E.C. (1979). Enzymes. 3rd ed pp. 281 - 390. Longman Group Ltd., London

DRICKAMER, L.K. (1975). The red cell membrane contains three different ATPase. J. Biol. Chem., 250: 1954 - 1957.

FERSHT, A. (1978). Enzyme Structure and Mechanism. pp. 85 - 102. W. H. Freeman and Co., Reading, United Kingdom.

GILLI, G., SCURSATONE, E., BONO, R., FEA, E., CARRARO, E. and PETITTI, G. (1989). Presence of aflatoxin in products intended for human consumption: the choice of methods and the results obtained. Ann. Ig., 1(1-2): 223 - 235.

GUPTA, B.S. (1988). Availability and utilization of non-conventional feed resources and their utilization by non-ruminants in South Asia. In: Non-conventional feed resources and fibrous agricultural residues: strategies for expanded utilization. Proceedings of consultation held in Hisar, India, 21 - 29 March, 1988 (C. Devendra, ed.), IDRC, Ottawa, Canada. pp. 62 - 75.

HAMLYN, J.M. and DUFFY, T. (1978). Direct stimulation of human erythrocyte membranne Na-K-Mg-ATPase activity in vitro by physiological

concentrations of d-aldosterone. Biochem. Biophys. Res. Commun., 84: 458 - 464.

HEATHCOTE, J.G. and HIBBERT, J.R. (1978). Aflatoxins: Chemical and Biological Aspects. pp. 112 - 129. Elsevier Scientific, Amsterdam.

HESKETH, J.E., LONDON, J.B., READING, H.W., and GLEN, A.J.M. (1978). The effect of lithium treatment on erythrocyte membrane ATPase activities and erythrocyte ion content. Brit. J. Clin. Pharmacol., 5: 323 - 329.

KELETI, G. and LEDERER, W.H. (1974). Handbook of Micro methods for the Biological Sciences. p. 83. Van Nostrand Reinhold Co., New York.

PLUMMER, D.T. (1971). An Introduction to Practical Biochemistry. pp. 142 - 145. McGraw-Hill, London.

PUNJ, M.L. (1988). Availability and utilisation of non-conventional feed resources and their utilisation by ruminants in South Asia. In: Non-conventional feed resources and fibrous agricultural residues: strategies for expanded utilisation. Proceedings of a consultation held in Hisar, India, 21 - 29 March, 1988 (C. Devendra, ed.), IDRC Ottawa, Canada. pp. 50 - 61.

RAVINDRAN, V. and BLAIR, R. 91991). Feed resources for poultry production in Asia and the Pacific region. 1. Energy sources. World's Poultry Sci., J. 43(3): 213 - 231.

REEDY, T.S. (1987). Use of non-conventional feed items in poultry rations, their effect on growth and egg production. Poultry Adviser, 20(1) 33 - 36.

SWENSON, M.J. (1970). Physiological properties, cellular and chemical constitutents of blood, In: Duke's Physiology of Domestic Animals (M.J. Swenson, ed.) 8th ed. -pp. 21 - 61. Cornstock Publishing Associates, London.

TIPTON, K.F. (1978). Enzyme assay and kinetic studies. In: Techniques in Protein and Enzyme Biochemistry. B112. pp. 1 - 56. Elsevier/North-Holland Biomedical Press, Netherlands.

WORLD HEALTH ORGANIZATION (1979). Environmental Health Criteria II: Mycotoxins. pp. 21 - 34. WHO, UK.