Science Focus **Vol. 19 (1) 2014 pp 81- 87**
Faculty of Pure and Applied Sciences, LAUTECH, Printed in Nigeria

INHIBITION STUDIES OF SOME KEY ENZYMES OF THE TERMITE *AMITERMES EVEUNCIFER* (SILVERSTRI) WORKERS: CLUE TO TERMITES SALT INTOLERANCE

[1]*Fagbohunka, B. S., [1]Ezima, E. N., [1]Adeyanju, M. M., [2]Alabi, M. A., [1]Oyedele D. E. and [1]Adeneye, A. A.

[1]Department of Biochemistry, Faculty of Basic Medical Sciences,
Olabisi Onabanjo University, Remo Campus, Ikenne, Ogun State.
[2]Bioresources Development Agency,
National Biotechnology Development Agency, Ogbomoso, Oyo State.

Abstract

Table salt which contains predominantly NaCl is used locally to control termites by pouring the salt on them or their habitat. This treatment is both toxic and lethal to the insects. Salts, containing cation and anion, will either inhibit or activate enzyme activity. It is suspected that either Na or Cl ion or both might be inhibiting or totally deactivating the activities of some crucial enzymes in termites thereby killing them. To provide a scientific explanation for this treatment and possibly design a pesticide for the destructive insect, we examined the effects of NaCl (table salt), some other chlorides and sodium salts on some important enzymes produced by termites. All the chloride salts tested inhibited all the enzymes even at a low concentration of 0.1mM. At this concentration, the effect was well pronounced on both the metabolic and detoxifying enzymes with the greatest effect on β-Glucosidase more than NaCl. However, it was observed that some chloride salts such as the chlorides of barium, calcium and magnesium were more potent on cellulase than NaCl (table salt), the conventional pesticide. Only CoCl inhibited 3-Mercaptopyruvate Sulphur Transferase (3 MST) activities more than NaCl but all other chlorides except NH_4Cl and $BaCl_2$ inhibited rhodanese more than NaCl. Also, NaCl inhibited all other enzymes more than cellulase. Finally, all the sodium salts tested except $NaCH_3COO^-$ and Na_2SO_4 inhibited cellulase more than NaCl.

Keywords*: termite, cellulase, cellulose, salt intolerance, enzymes*

Introduction

Termites' activities could sometimes be destructive and thus undesirable. In such cases they need to be controlled. Many methods have been designed locally and scientifically for termites' control. Traditionally, table salt which contains predominantly NaCI, both toxic and lethal to termites, is used to control the insect by pouring it on the insects or the habitat or both. To our knowledge, there has not been any scientific reason proposed to explain this phenomenon. Salts containing cation and anion will either inhibit or activate enzyme activity, it is suspected that either Na or Cl ion or both might be inhibiting or totally deactivating the activities of some crucial enzymes in termites thereby killing them. This prompted us to carrying out some tests on the effects of NaCl, some other chlorides and sodium salts on the major metabolism and detoxifying enzymes produced by termites.

Termites are a group of social insects. They live in a colony which is typically found underground, often near a tree stump, wood pile or other source of 'food' (Gay and Calaby, 1970; Smith, 2007). Termites feed essentially on wooden materials using cellulose as a food resource, and they play a key role in decomposing dead plant tissue in natural ecosystems. This habit of feeding requires many enzymes including metabolic and detoxifying enzymes. It can therefore be suggested that the toxicity of salts to termites stems from the effects of salts on the organism's enzymes of metabolism or detoxification or both. Nakashima and his colleagues had reported the discovery of termite genes that has led to the determination of the three-dimensional structure of cellulase enzymes, which will hopefully lead to the development of environmental-friendly inhibitors of these enzymes that can be impregnated into wood to inhibit termites' activity (Nakashima *et al*., 2002a; Nakashima *et al*., 2002b).

* *Corresponding author email: bsfagbohunka1963@gmail.com*

ISSN 1596-7026

Termites, such as *Amitermes eveuncifer* Silverstri, require cellulase, an enzyme complex comprising at least three major classes of enzymes including *endo*-1, 4-β-glucanases, *exo*-1, 4-β-glucanases, and β-glucosidase, to efficiently digest cellulose for survival (Fagbohunka *et al*., 2014; Ezima *et al*., 2011). Thus, inhibitors developed against any of these cellulase system enzymes would be a potential termite treatment avenue.
Termites also possess Rhodanese and 3-MST (Fagbohunka *et al*., 2014). Rhodanese (thiosulphate cyanide sulphur transferase, EC 2.8.1.1) catalyses the transfer of the sulphur atom of thiosulphate to cyanide to form thiocyanate and sulphide. 3-mercaptopyruvate sulphurtransferase (EC 2.8.1.2) catalyses the transfer of sulphur atom from mercaptopyruvate to sulphur acceptors such as cyanide. The sulphurtransferases are cyanide detoxifying enzymes that convert the toxic cyanide to thiocyanate which is easily excreted (Fagbohunka *et al*., 2004; Agboola *et al*., 2004; Agboola *et al*., 2006). Though the activities of these enzymes do not have direct bearing on the energy metabolism of animals, they also play a major role in detoxifying the termites of the toxic cellulolytic materials they feed on (Agboola *et al*., 2008). The presence of the detoxifying enzymes are required for the survival of the termites as they feed on wood, leafy and putrefying vegetables, many of which may be cyanogenic (Montgomery, 1980). It should be noted also that the detoxifying ability of these enzymes are secondary functions as they may possess other primary functions. For example, rhodanese main physiological function is the supply of sulphide for iron sulphur centres (White *et al*., 1981). These enzymes could also be target enzymes in termite control.

Materials and Methods

Materials

All the reagents used in this research were of analytical grades and were obtained from Bio – Rad Laboratories, Richmond, California, Pharmacia AB, Uppsala, Sweden, Sigma Chemical Company Limited, St. Luois, Mo, U.S.A., Pierce Chemical Company, Rockfold, Illinois, U.S.A., BDH Chemicals Limited, Poole, England., from Eastern Kodak, co; Rochester, N.Y., U.S.A., Glass distilled water was used for all preparations of solutions and all pH measurements were made at 25°C using the standard pH meter Radiometer, Copenhagen.
Apparatus used include, top load weighing balance (Mettler PN1210), pH meter (Mettler MP200) and UV/VIS Spectrophotometer (Cecil 2041), Ultracentrifuge (Beckman Optimal LE-80K Ultracentrifuge), mortar and pestle.

Enzyme Purification from Crude Extract

Termite soldiers were washed and rinsed well with distilled water. The termites were gently homogenized with a pre-frozen warring blender or mortar and pestle in an ice bath using acid washed sand. An aliquot of 10mM sodium acetate buffer, pH 5.0, containing 1mM EDTA, was added intermittently in the ratio of 5:1 (v/w); buffer/termites, while homogenizing.
The mixture collected was centrifuged at 15,000rpm for 15minutes with a bench centrifuge at room temperature. The supernatant was collected and stored in a refrigerator.
The supernatant was salted out by bringing the crude extract to 70% (w/v) saturation with Ammonium sulfate.

Enzyme Assays for Cellulase

Cellulase activity toward CM-cellulose was measured by the appearance of reducing end groups in solution of CM-cellulose. The assay mixture consisted of 0.5ml of 1% (w/v) CM-cellulose (in 10mM sodium acetate buffer, pH 5.0 containing 1mM EDTA) and 0.1ml of the enzyme solution. This was incubated at 40^0C for 30 minutes. After incubation, 0.4ml of water and 1.0ml of combined copper reagent containing alkaline solution of $CuSO_4$ buffered with a (carbonate-bicarbonate mixture containing Sodium potassium tartarate) were added according to the method of Hurst and his coworkers (Hurst *et al*., 1977). The above mixture was heated at 100^0C for 20 minutes and allowed to cool to room temperature. Arsenomolybdate reagent (containing concentrated H_2SO_4 and sodium arsenate) (1.0ml) was added and mixed immediately and thoroughly to dissolve all precipitated Cu_2O. For easy absorbance reading, 7.0ml of distilled water was added to dilute mixture which was thoroughly mixed by covering the tube with parafilm and inverting it 3 to 4 times. The absorbance was read at 540nm. A unit of cellulase activity in this work is defined as the amount of enzyme that produces a change of an absorbance at 540nm of 0.10 under the conditions defined (Hurst *et al*., 1977).

Assay for β-Glucosidase

β-Glucosidase activity was determined by the method of Umezurike (1976). The reaction mixture which was made up of 2.0ml of 1mM o-nitrophenyl-β-D-glucopyranoside (in 10mM sodium acetate buffer, pH 5.0 containing 1mM EDTA) and 0.1ml of enzyme solution was incubated for 30 minutes at 40^0C. After incubation, 10 ml of 0.1M sodium carbonate was added to 0.5ml of the assay mixture. The nitrophenol released was

measured by its absorbance at 400nm. One unit of β-glucosidase activity was defined as that amount of enzyme needed to liberate 1µmol of nitrophenyl per minute under the conditions of the assay.

Assay for 3-Mercaptopyruvate Sulphur transferase

3-MST was assayed according to the spectrophotometric method of Agboola *et al.* (2008). The 3-MST measurement was based on calorimetric determination of thiocyanate formed in the reaction. The reaction mixture contained 1.05ml of 0.38M Tris-HCl buffer, pH 9.0, and 0.15ml of 0.3M mercaptopyruvate, 0.15ml of 0.5M KCN and 0.03ml of enzyme solution in a final volume of 1.5ml. The reaction was initiated after the addition of the enzyme solution.

After 15 min, 0.1ml of 38% formaldehyde was added to terminate the reaction. The colour was developed by the addition of 0.5ml of ferric nitrate reagent. The absorbance was measured at 460nm. The control contained no salt. One enzyme unit is the amount of enzyme that catalyses the formation of 10µmol of thiocyanate under the assay conditions.

Assay for Rhodanese

The method of Fagbohunka *et al.* (7) was employed to measure rhodanese activity. The reaction mixture consisted of 0.5ml of 50mM Borate buffer, pH 9.4; 25mM KCN, 25mM $Na_2S_2O_3$, 0.09ml distilled water and 0.01ml of enzyme in a total volume of 10ml. The reaction was stopped by the addition of 0.5ml of 15% formaldehyde and 1.5ml of ferric nitrate solution (i.e. Sorbo reagent). The absorbance was then read at 460nm. The unit was expressed in rhodanese unit (RU). One rhodanese unit was taken as the amount of enzyme which under given the given conditions produced an O.D reading of 1.08 at O.D 460nm.

Termites Control Strategy

The effects of Chloride salts e.g. NaCl, $MnCl_2$, $SnCl_2$, $CoCl_2$, $NiCl_2$, NH_4Cl, $BaCl_2$, $ZnCl_2$ and sodium salts eg Na_2CO_3, NaH_2PO_4, Na_2HPO_4, $NaWO_4$, $NaHASO_4$, $C_4H_4O_6KNa$, $NaCH_3COO^-$, and Na_2SO_4 on the activities of the enzyme was determined with varied concentrations of 0.1mM, 0.2mM, 0.5mM and 1.0mM. The activities were measured based on the assay techniques for the enzymes as earlier described (Agboola *et al.*, 2008).

Results

Enzyme Purification Steps

The results of the purification procedure are summarized in Table I. The partial purification procedure yielded a cellulase with a specific activity of 493units/mg of protein.

Table I: Summary of the Purification Steps of Cellulase from the Termite (*Ametermes Eveuncifer* Silverstri) Workers

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	350	91520	260	352	100	1.0
Ammonium sulphate precipitation	110	88740	180	493	96.9	1.37

Termite Control

The results of the action of various chloride salts on various termite enzymes are presented in Tables II & VI. All the chloride salts tested inhibited all the enzymes even at a low concentration of 0.1mM. At this concentration, the effect was well pronounced on both the metabolic and detoxifying enzymes with the greatest effect on β-glucosidase. Only $BaCl_2$ inhibited β-Glucosidase more than NaCl. However, it was observed that some chloride salts were more potent on cellulase than sodium chloride (table salt), the conventional pesticide. The greatest inhibition was by the chlorides of barium, calcium and magnesium. Only CoCl inhibited 3-Mercaptopyruvate Sulphur Transferase activities more than NaCl. However, all other chlorides except NH4Cl and $BaCl_2$ inhibited rhodanese more than NaCl. All the sodium salts tested inhibited cellulase more than NaCl except $NaCH_3COO^-$ and sodium Na_2SO_4 (Table VII).

Table II: Percentage inhibition of all enzyme activities by NaCl salt

ENZYME	NaCl Salt Concentration			
	0.1mM	**0.2mM**	**0.5Mm**	**1.0mM**
B-Gucosidase	78.07	78.81	78.29	79.97
3-MST	56.59	57.25	57.69	58.62
Rhodanese	45.29	49.25	52.01	52.64
Cellulase	31.71	34.15	35.80	54.66

Table III: Percentage inhibition of β-Glucosidase activities by chloride salt

SALTS	Chloride Salt Concentration			
	0.1mM	**0.2mM**	**0.5mM**	**1.0mM**
$BaCl_2$	86.92	74.39	80.10	87.88
NaCl	78.07	78.81	78.99	79.97
$CoCl_2$	77.84	78.34	81.20	82.13
NH_4Cl_2	76.65	78.73	80.55	80.68
$MnCl_2$	76.14	67.91	69.74	70.49
$SnCl_2$	66.73	39.55	52.47	87.63
$ZnCl_2$	66.08	65.43	57.06	44.46
$NiCl_2$	30.72	38.54	53.61	61.39

Table VI: Percentage inhibition of cellulase activities by chloride salt

SALTS	Chloride Salt Concentration			
	0.1mM	**0.2mM**	**0.5mM**	**1.0mM**
$BaCl_2$	58.19	59.32	59.89	61.02
$CaCl_2$	46.81	53.19	55.32	61.70
$MgCl_2$	33.33	36.67	43.33	50.00
NaCl	31.71	34.15	37.80	41.46
NH_4Cl	28.20	30.77	33.33	35.50
$ZnCl_2$	27.47	33.33	38.11	39.20
KCl	18.49	19.18	24.66	26.71

Table V: Percentage inhibition of 3-Mercaptopyruvate Sulphur Transferase activities by chloride salt

SALTS	Chloride Salt Concentration			
	0.1mM	**0.2mM**	**0.5mM**	**1.0mM**
$CoCl_2$	57.46	57.44	53.03	43.40
NaCl	56.59	57.25	57.69	58.62
$BaCl_2$	46.89	50.74	59.42	64.02
$SnCl_2$	32.34	33.92	37.00	43.59
$NiCl_2$	32.25	49.84	56.42	64.02
$ZnCl_2$	28.33	34.17	36.20	40.86
NH4Cl	24.51	25.22	25.31	26.18
$MnCl_2$	14.52	14.62	20.92	28.00

Table VI: Percentage inhibition of Rhodanese activities by chloride salt

SALTS	Chloride Salt Concentration

	0.1mM	0.2mM	0.5mM	1.0mM
$NiCl_2$	56.26	52.18	52.07	50.69
$CoCl_2$	54.13	53.85	47.12	43.57
$MnCl_2$	50.69	47.59	47.01	42.71
$ZnCl_2$	48.68	47.30	46.50	39.95
$SnCl_2$	46.61	47.59	63.38	64.00
NaCl	45.29	49.25	52.01	52.64
NH4Cl	36.05	49.48	53.27	63.83
$BaCl_2$	10.68	32.66	43.40	51.72

Table VII: Effect of Sodium Salts on Cellulase from the Termite *Armetermes eveuncifer* Silverstri

SALTS	Sodium Salt Concentration					
	0.1mM	0.2mM	0.4mM	0.6mM	0.8mM	1.0mM
Na_2CO_3	96.60	96.80	97.30	98.20	98.30	98.40
NaH_2PO_4	87.40	95.80	96.80	96.80	97.90	97.90
Na_2HPO_4	69.60	78.30	87.00	91.30	100.00	100.00
$NaWO_4$	68.20	73.50	78.80	82.30	89.40	92.90
$NaHASO_4$	59.30	66.30	70.50	83.10	86.00	100.00
$C_4H_4O_6KNa$	45.30	51.20	54.70	58.10	74.00	72.10
$NaCH_3COO^-$	27.30	39.40	53.00	69.70	83.30	89.40
Na_2SO_4	1.60	4.00	8.80	12.80	13.60	16.80

Discussion

NaCl had appreciable inhibitory effects on all the enzymes tested even at low concentration as 0.1mM. However, its effect was highest on β-glucosidase followed by 3-MST, rhodanese and cellulase in that order (Table II). This result is a little bit different from what was obtained in our previous works on snail enzymes (Agboola *et al.,* 2008) where only the metabolic enzymes were seriously inhibited by the salts while the detoxifying enzymes were only slightly inhibited; and termite soldiers (Fagbohunka *et al*., 2014) where the metabolic enzymes were more inhibited by the salts than the detoxifying enzymes though both were appreciably inhibited. It had been reported that sodium chloride had an inhibitory effect on cellulolytic ability of 16 species of *Aspergillus* isolated from salt-affected areas of Punjab, Pakistan (Malik *et al*., 1980) and other enzymes such as alkaline protesase (Alabi and Daini, 2009). The feeding to termites of wood, antecedently soaked in aqueous solutions of various chemicals (NaCl, $CaC1_2$, $HgC1_2$, KCl, etc.) had been reported to result in killing both host and parasite (Cleveland, 1925).

β-glucosidase and cellulase are cellulolytic enzymes and are known to be involved in energy metabolism while 3-MST and rhodanese play secondary roles such as detoxification. It can be suggested that the toxicity of salts to termite stems from the effect of the salts on its key enzymes of both metabolism and detoxification. The inhibition of termite cellulase and β-glucosidase, the important enzymes in the overall metabolism of the termite (which feeds exclusively on wooden materials) which requires enzymes of the cellulase complex (of which β-glucosidase is a member) and Rhodanese and 3-MST which are involved in cyanide (CN) detoxication in animal system could be lethal to the organism. Though the activities of the latter enzymes do not have direct bearing on the energy metabolism of animals, they also play a major role in detoxifying the termites of the toxic cellulolytic materials they feed on.

Inhibition of the metabolic enzymes by NaCl indicates the organism may not be able to metabolise wood even if it feeds on it. Inhibition of the detoxifying enzymes by NaCl also suggests the organism may not be able to detoxify the toxic cellulolytic materials they feed on. These definitely will result in the death of the organism. To think of the fact that 0.1mM could be this harmful to the insects, then one can only imagine the degree of the harm the undiluted NaCl which is poured on termites will do on the organism.

To ascertain whether the enzymes can also be inhibited by other chloride salts; same concentrations of different chloride salts were tested on the enzymes (Tables III-VI). It was discovered that all the chloride salts tested inhibited all the enzymes even at a low concentration of 0.1mM. The effect was well pronounced on both the metabolic and detoxifying enzymes with the greatest effect on β-glucosidase with $BaCl_2$ inhibiting the enzyme most; followed by NaCl. However, it was observed that some chloride salts were more potent on cellulase than NaCl (table salt), the conventional pesticide. The greatest inhibition on cellulase was by the chlorides of

barium, calcium and magnesium. Only CoCl inhibited 3-Mercaptopyruvate Sulphur Transferase activities more than NaCl. However, all other chlorides except NH_4Cl and $BaCl_2$ inhibited rhodanese more than NaCl. This result is however different from what was earlier obtained for termite soldier cellulase (Fagbohunka *et al*., 2014).

Also, to find out whether the inhibition was due to the chloride or sodium ion, same concentrations of different sodium salts were tested on cellulase. It was clearly revealed that all the sodium salts tested inhibited cellulase drastically with most of them more potent than NaCl (Table VII). Thus both the sodium and chloride ions contributed to the inhibition. This result is also different from what was earlier obtained for snail cellulase in our previous work (Agboola *et al.,* 2008) but similar to the one obtained for termite soldier (Fagbohunka *et al*., 2014). However, though both the sodium and chloride ions contribute to the lethal action of Sodium Chloride on termites, it was observed that Sodium salts rather than Chloride salts were more potent as inhibitors unlike snail cellulase which was inhibited by chloride salts more than sodium.

Therefore, one of the chloride salts of barium or sodium or a combination of both can be used as a pesticide for termites. Also, almost all the sodium salts tested in this work or a combination of at least two can be used.

References

Agboola, F. K., Fagbohunka, B. S. and Adenuga, G. A. (2008). Activities of *Archactina (Calachatina) marginata* haemolymph enzymes: clue to terrestrial snails' salt intolerance. *Int. J. Biol. Chem. Sci*., 2(1): 66-71.

Agboola, F. K., Fagbohunka, B. S., and Adenuga, G. A. (2006). Activities of Thiosulphate and 3-Mercaptopyruvate-cyanide-sulphurtransferases in Poultry Birds and the Fruit Bat. *Journal of Biological Sciences* 6(5): 833-839.

Agboola, F. K., Kuku, A. Okonji, R. E. and Fagbohunka, B. S. (2004). Tissue distribution of thiosulphate and mercaptopyruvate sulphur transferases in the human. Science Focus 7: 81-84.

Alabi, M.A. and Daini, O.A. (2009). Enzymatic properties of purified serine protease from *Aspergillus fumigatus* grown on sabaraud dextrose agar. *Int. J. Biol. Sci.* 2(1): 51-59.

Cleveland, L. R. (1925): The ability of termites to live perhaps indefinitely on a diet of pure cellulose. *Biol. Bull.* 48: 289–293.

Ezima, E. N., Fagbohunka, B. S. and Adeyanju, M. M. (2011). The Effects of Partially purified Cellulase from Worker Termite (*Armetermes eveuncifer* Silvestri) on some wastes. International Journal of Biochemistry 3(1):15-20

Fagbohunka, B. S., Okonji, R. E., Adenuga, G. A., and Agboola, F. K. (2004) Properties of Rhodanese (thiosulphate sulphur transferase) from the hepatopancreas of giant snail, *Archachatina marginata*. Science Focus **7:** 76-80.

Fagbohunka, B. S., Edorh, S. E., Adeyanju, M. M., Ezima, E.N., Alabi, M. A. and Ogunlabi, O. O. (2014). Activities of a Cellulase of the Termite, Ametermes Eveuncifer (Silverstri) Soldier: Clue to Termites Salt Intolerance. IISTE-Journal of Natural Science Research. In Press

Gay and Calaby (1970). Termites of the Australian region. in; Krishna K Weesner FM eds. Biology of Termites, Vol. II Academic Press NY, Pp. 401.

Hurst, P. L., Nielson, J., Sullivan, P. A. and Shepherd, M. G. (1977). Purification and Properties of Cellulase from *Aspergillus niger. J. Biochem*.165, 33-41.

Malik, K. A., Kauser, F. And Azan, E. (1980). Effect of Sodium Chloride on the Cellulolytic Ability os some *Aspergilli. Mycologia*, 72(2), 322-328.

Montgomery, R. D. (1980) Cyanogens. In Toxic Constitution of Plants Food Stuff, Liener, I.E. (Ed) 2nd Edn., New York, Academic Press, pp: 143-161.

Nakashima, K., H. Watanabe, H. Saitoh, G. Tokuda and J. I. Azuma (2002a). Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem. Mol. Biol.*, 32, 777–784.

Nakashima, K; Watanabe, H; Azuma, J. (2002b). Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. *Cell. Mol. Life. Sci.*, 59**,** 1554–1560.

Smith, R. L. (2007). Termites. Arizona-Sonora Desert Museum. e*mail: info@desertmuseum.org*

Umezurike, G. M. (1976). The beta glucosidase in the gut content of the snail (*Archatina achatina*). *Biochem. J.* 157: 381-387.

White, A., Handle, P., Smith, E. L., Hill, R. L., and Lehman, I. R. (1981) Principles of Biochemistry (6th Edn). Mcgraw Hill: Kogakusha, Tokyo, Japan; pp: 391-734.