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ESTIMATION OF PROTEIN CONCENTRATION IN CELL LINES AFTER TREATED WITH TOXIN A EXTRACTED FROM *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Two cell lines REF (Primary Rat Embryo Fibroblast**)** and AMN3 (Ahmed-Mohammed-Nahi-2003) were used to investigated the role of toxin A extracted from *pseudomonas aeruginosa* in inhibition of protein synthesis in cell lines after exposure to purified toxin A. Results have shown that incubation of toxin A (purified toxin A alone in first group and purified toxin A activated with urea in the second group) at the concentration (5, 25, 50, 100) ng /ml with both REF and AMN3 cell lines for 48 hrs will cause significant decrease in protein concentration in both cell lines in comparison with untreated control when estimated by Bradford method.

KEYWORDS: *pseudomonas aeruginosa*, purified toxin.

INTRODUCTION

Pseudomonas aeruginosa are gram-negative bacilli. They are widely distributed in nature and seldom infect healthy people (Hoge *et al*., 2010). Yet, these bacteria are among the most problematic opportunistic pathogens. It's always infect immunodeficient individuals, such as patients suffering from burns, cystic fibrosis, or patients who take immunosuppressive drugs (Joseph *et al*., 2001).

Pseudomonas toxin A (ETA) is an ADP-ribosylating toxin that inhibits mammalian protein synthesis *in vivo* and *in vitro* by catalyzes the transfer of the adenosine diphosphate ribose moiety (ADP-ribose) from NAD to eukaryotic elongation factor 2, resulting (ADP-ribose-EF-2) complex (Yates and Merrill, 2004). The target residue in eEF2, diphthamide a modified histidine, this suggests that the diphthamide is involved in triggering NAD+ cleavage then

inhibition of protein synthesis and ultimately cell death (Jorgensen *et al*., 2005; Yates *et al*., 2006).

NAD + EF-2 = ADP- ribose - EF-2 + nicotinamide + H^+

ETA is a heat-labile, single polypeptide chain consist of 613 amino acid and four disulfide linkages with a molecular weight of 66 kilodalton (Susan *et al*., 2005). X ray crystallography studies and deletion mutation analysis of ETA revealed the overall tertiary structure of the molecule are consists of three functional domains in order to be toxic, namely binding to the receptor on the cell membrane, translocation across the membrane, and ADP ribosylation activities (Danielle, 2007).

The toxin bind to specific receptor receptor α 2-macroglobulin receptor (α 2-MR)/low-density lipoprotein receptor then internalized by receptor mediated endocytosis then shuttled to the endoplasmic reticulum (ER) to reducing and unfolding (Shafikhani *et al*., 2008). Subsequently, the 37 kDa carboxy-terminal fragment of *Pseudomonas* toxin A which includes the enzymatic domain is translocated in to the cytoplasm. In the cytoplasm, the enzymatic domain circumvents degradation by the proteasome long enough to enzymatically ADP-ribosylate eEF-2, thereby inhibiting protein synthesis (Parikh and Schramm, 2004).

Exotoxin A is a potent cytotoxin and is lethal for a variety of animals, including subhuman primates. During P. aeruginosa infections, exotoxin A apparently causes disease by inhibition of protein synthesis, direct cytopathic effects, and interference with cellular immune functions of the host (Armstrong and Merrill,2004). Antibodies to exotoxin A provide protection from some of the biochemical, pathologic, and lethal consequences of both experimental and clinical pseudomonas infections (*Xu et al*., 2006).

MATERIALS AND METHODS

Determination of Protein Concentration

Protein concentration was determined according to Bradford, (1976) and as follow:

A standard curve of bovine serum albumin was carried out by using different concentrations of BSA stock solution according to the following volumes:

BSA (µl)	Tris-OH buffer (µl)	Protein amount (µg)	Final volume (ml)
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

The portion of 2.5 ml of Coomassie brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.. The blank was prepared from 0.1 ml of Tris-OH buffer and 2.5 ml of the dye reagent then the absorbance at 595 nm was measured. A standard curve was plotted between BSA concentrations and the corresponding absorbance of the bovine serum albumin .The protein concentration of toxin sample was estimated by taking 0.1 ml of toxin solution, then subjected to the same previous addition and read the absorbance at 595 nm. The protein concentration was calculated from the standard curve.

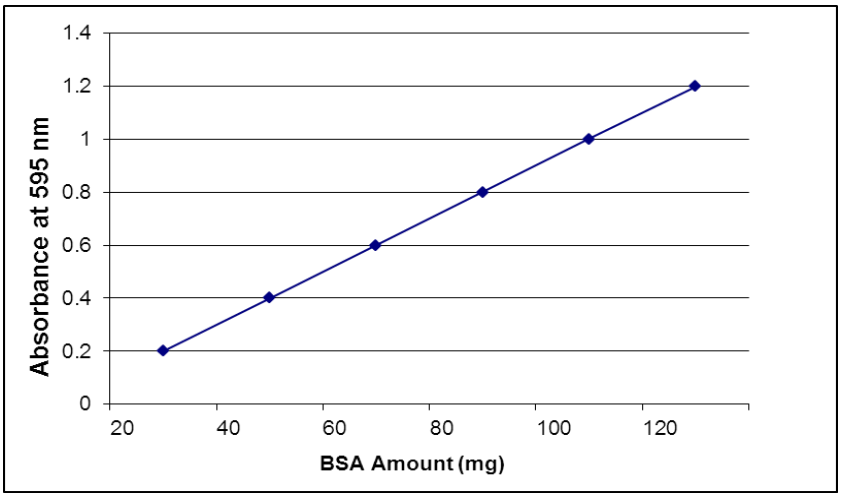

Figure. (1 -1): Standard Curve of Bovine Serum Albumin as described by Bradford Method, (1976).

For detection the ADP ribosylating activity of toxin A and its ability to inhibit protein synthesis in cell line .Two cell line (ReF and AMN3) was used for estimation of protein concentration. In each cell line RPMI medium supplemented with 15% fetal calf serum and containing 5 x10^4 Cell/ml were added to 24-well tissue culture plate that had been seeded for 24 hrs at 37°C. On each cell line two groups of purified toxin A were examined, one group contain the purified toxin A alone at concentration (5, 25, 50, 100) ng /ml, the second group contain purified toxin A that were activated with urea to reach to concentration (5, 25, 50, 100) ng /ml. In first group, 100 µl of serum diluted 1:10 were added to 2.5 ml of ETA alone at concentration (5, 25, 50, 100) ng /ml while 100 µl of serum diluted 1:10 were added to 2.5 ml of ETA activated with urea at the concentration (5, 25, 50, 100) ng /ml and incubated for 48 hrs at 37°C. After 48 hrs the monolayer were washed with a balanced salt solution to remove disattached cells. Remaining cells were solubilized by addition of 0.5 ml of 0.1 M NaoH, then this Portion were assayed for protein estimation by a automated Bradford procedure.

RESULTS AND DISCUSSION

For detection the effect of purified toxin A on protein synthesis in cell lines. Four concentration of purified toxin A (5, 25, 50, 100) ng/ml were tested on two cell line (ReF and AMN3) after exposure for 48 hrs. Results have shown that incubation of toxin A (purified toxin A alone in first group and purified toxin A activated with urea in the second group) at the concentration (5, 25, 50, 100) ng /ml with both REF and AMN3 cell line will cause significant decrease in protein concentration in both cell line in comparison with untreated control when estimated by Bradford method as shown in table (1.1). Also when the concentration of toxin A in both cases will increase, the protein concentration in both cell line (REF,AMN3) will decrease as shown in figure (1.2).

Also when compares the effects of toxin A in both cases on inhibition of protein synthesis, toxin A activated with urea cause much more decrease in protein concentration rather than the same concentration of toxin A alone (not activated) on both cell lines REF and AMN3 as shown in table (1.1). The concentration (50, 100) ng of toxin A activated with urea cause more inhibition of protein synthesis in both ReF and AMN3 cell lines rather than the same concentration of toxin in the first group (not activated). Also when both cell line (REF ,AMN3) were exposed to toxin A activated with urea at the same concentration. Activated toxin A caused more decrease in protein concentration and inhibition of protein synthesis in REF rather than the same concentration of toxin A activated with urea on AMN3 cell line. Also the decrease of protein concentration increased when the concentration of toxin A increased in both cases, Although the highest concentration (100) ng of toxin A in both cases caused higher reduction in protein synthesis, but toxin activated with urea have more higher effects than first group of toxin (not activated) and on REF cell line more than on AMN3 as shown in table (1.1). Also TCD50 of toxin A which is the amount of toxin which inhibits protein synthesis by 50% after a 48 hrs of incubation of toxin with AMN3 cell monolayer was (50) ng. while TCD50 of toxin A activated by urea after incubation of toxin for 48 hrs with AMN3 cell monolayer was (25) ng . TCD50 of toxin A and toxin A activated by urea which inhibits protein synthesis by 50% after a 48 hrs of incubation of toxin with REF cell monolayer was (25)ng in both cases.

When (REF, AMN3) cell line were treated with the same concentration of purified toxin A in both cases (purified toxin alone and purified toxin activated with urea), There is significant differences in protein concentration on cell line at concentration(5) ng of toxin in both cases

(P<0.05), also significant differences in protein concentration when exposed to other single concentration of purified toxin A in both cases as shown in table (1.2). Also when compared between each two concentration of toxin A in first group on inhibition of protein concentration in the same cell line (REF or AMN3) show there is significant differences in protein concentration between them (P<0.05) .Also when the same cell line(REF or AMN3) were exposed to purified toxin activated with urea at different concentration, there is significant differences in protein concentration between them (P<0.05) among all as shown in table (1.2).

Table (1.1): Estimation of protein concentration in REF and AMN3 cell lines after treated with toxin A alone and toxin A activated by urea.

Conc of toxin A	Protein conc in AMN3 cell line after add toxin A alone		Protein conc in AMN3 cell line after add toxin A activated with urea		Protein conc in REF cell line after add toxin A alone		Protein conc in REF cell line after Add toxin activated with urea	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	4.74	0.09	4.74	0.09	4.56	0.38	4.56	0.38
5ng	4.09	0.09	3.82	0.19	3.25	0.24	3.03	0.05
25ng	3.41	0.15	2.35	0.11	2.20	0.18	2.00	0.15
50ng	2.20	0.24	2.09	0.09	1.39	0.35	0.95	0.06
100ng	0.88	0.06	0.60	0.09	0.39	0.08	0.28	0.02
Total	3.14	1.39	2.86	1.48	2.46	1.52	2.21	1.58

Table (1.2). Comparison between various concentration of toxin A in inhibition protein concentration in REF and AMN3 cell line in P value.

	P value			
Toxin A concentration. (conc)	AMN3 exposed to toxin alone	AMN3 exposed to toxin activated with urea	REF exposed to toxin alone	REF after exposed to toxin activated with urea
Con. Vs 5ng	<0.0004	<0.0001	<0.0001	<0.0001
5ng vs 25 ng	<0.0002	<0.0001	0.030	<0.0002
25 ng vs 50 ng	<0.0001	<0.0001	<0.0001	<0.0001
50 ng vs 100 ng	<0.0001	<0.0001	0.001	0.001
Among all	<0.0001	<0.0001	<0.0001	<0.0001

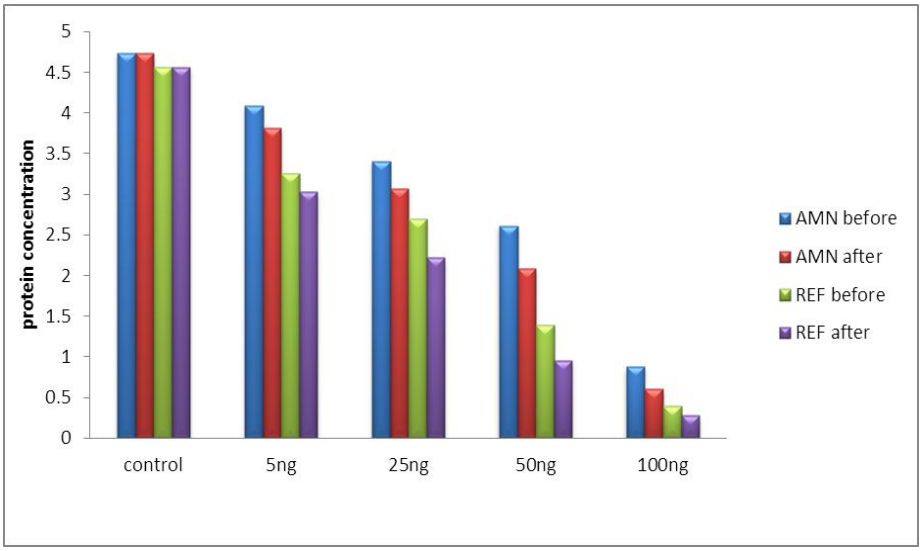

Figure (1.2): Effects of toxin A alone and toxin A after treated with urea on protein concentration in REF and AMN3 cell lines.

This results was in agreement with (Sundin, 2000) who used different concentration of toxin 100 and 50 ng per ml was used in the ADP ribosylation with the LM cells . The concentration of 50 and 100 ng cause significant decrease in of protein synthesis more than 60 % and 80% respectably.

Biological activity of toxin depends upon an intact toxin molecule, whereas enzyme activity requires only a functional A region. Since ADP ribosylation activity depends only on maintenance of an enzyme-active site within the toxin molecule and not on specific ligand interaction with an intact eukaryotic cell, enzyme activity may be retained after limited proteolysis, whereas biological activity is altered (Olgert and Francis, 2009). PA toxin is produced as a proenzyme, which may be spontaneously activated also may be activated by treatment with a combination of a denaturing and a reducing agent, or by freezing (-20°C) and thawing . However, the increase in enzyme activity of the treated purified preparations realated to that the purification methods that activated some of the PA toxin (Villis *et al*., 1999). The intact toxin can be activated 20- to 50- fold *in vitro* by treatment with urea and dithiothreitol (Verena *et al*., 2001).

The ADPR-transferase activity were produce during growth of *P. aeruginosa* and the activity is seen as early as 10 h, while the cells are still in the logarithmic phase. significant ADPR-transferase activity seen until 19 h, while it was in the stationary phase of growth. When samples are measured at 30 hrs, ADPR-transferase activity still to be increasing. Enzymatic

activity appears to be reached a peak level after 48hrs , while the cells was in the stationary phase of growth. This increase in enzyme activity may be a reflection of spontaneous activation or fragmentation of the toxin, resulting in an increase of ADPR-transferase activity (Anderson *et al*., 2004).

Experiments designed to elucidate cellular internalization of *Pseudomonas aeruginosa* exotoxin A. Inhibition of protein synthesis was used as an index of the biological activity of exotoxin A, and a biotinyl-toxin: avidin-gold system to follow its movement on the ultrastructural level. Addition of amantadine, methylamine and dansylcadaverine to cells enhanced the toxicity of exotoxin A at lower concentrations (Ghadir *et al*., 2013).

In addition, type (III) secretion system (TSS) of *P. aeruginosa* was highly sensitive to environmental conditions, such as temperature, pH, and Ca^{2+} level, as well as to metabolic balance Concerning temperature changes(Juliette *et al* ., 2009). Engel and Balachandran, (2009) reported that the highest cytotoxic effects and inhibition of protein synthesis were observed at 37 °C. This may be explained by finding the genes encoding TTSS are induced at a temperature of 37 °C. Moreover, the influence of incubation temperature will relay on effector toxin A protein observed that maximum effector protein synthesis occurs at 37 °C.

The enzyme activity of toxin also ability to inhibition of protein synthesis may be related to freezing and thawing and its agreement with (Michele *et al*., 1987) who noted that ADPR-transferase activity of PA toxin preparations stored at -20°C appeared to increase when compared with preparations that were stored at -70°C. There was approximately a sixfold increase in enzyme activity when a purified toxin was stored at -20°C instead of -70°C. Also repeated freezing and thawing of crude or partially purified PA toxin preparations resulted in an increase in the smaller ADPR-transferase, with a concomitant decrease in the larger-molecule-weight species (Canan *et al*., 2002. David *et al*., 2008 reported that *Pseudomonas* toxin pretreated with alkaline protease or elastase for 1 min inhibited protein synthesis in LM cells by 60%. a2M-protease regulates a number of proteolytic enzymes which participate in hemostatic and inflammatory events. A striking elevation of ADP ribosylation activity was observed after treatment of pseudomonas toxin with, a2M-protease .Exposure of toxin to thermolysin severely reduced the ability of toxin to block LM cell protein synthesis. Untreated toxin inhibited protein synthesis by 81%, whereas thermolysin-treated toxin caused a minimal reduction. Wiehlmann *et al*., 2007 noted that culture filterate from P. aeruginosa strain PA103 (protease deficient) and strain M2 (protease producing) that incubated with

toxin for up to 90 min reduced ADP ribosylation activity of pseudomonas toxin When toxin was incubated for 5 min with culture filtrates derived from both strain, ADP ribosylation activity was reduced to 59 and 34% of normal, respectively.

It is believed that enzymatic activity requires only a functional A region, whereas toxicity to mammalian cells requires an intact toxin molecule. It has been recently reported that the mouse L-cell toxicity and the mouse toxicity of PA toxin may be reduced or destroyed by thermal denaturation without significantly reducing ADPR-transferase activity. A purified toxin preparation treated with urea showed a slight increase in enzyme activity. In contrast, the same toxin preparation had a greater than 10-fold reduction in mouse lethality. In each instance where there was an increase in enzymatic activity, there was a decrease in mouse lethality (lee *et al*., 2010).

Both toxin A and diphtheria toxin have ADP- ribosylating activity but there is similarities and differences in inhibition of protein synthesis. The enzymatic activity is related to the structure of both toxin (Mellisa *et al*., 2000). In both toxins maximal expression of activity in ADP-ribosylating EF-2 occurs only after covalent alterations involving fragmentation, reduction, or both. However, there are differences in the expression of NAD+-glycohydrolase activity (Morimoto and Bonavida, 1992).

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