

Stress Proteins as a Suitable Biomarker of Environmental Pollution

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ABSTRACT: Soils bacteria are frequently faced with various adverse environmental conditions and have developed a complex regulatory network to respond rapidly to environmental changes. In this study usefulness and applicability of stress proteins of bacteria which are produced after stress conditions were examined. The adaptation of soil bacteria involves the induction of stress proteins provide a nonspecific protective function regardless of stress types. Thus, biomarkers like bacteria can be used as an early warning system for pollutions. These proteins were extracted from soil's bacteria of three different locations by usage of various kinds of buffers. Between these buffers, acidic sodium phosphate buffer gave highest yield of proteins, also Sodium dodecyl sulphate (SDS) was added to soil buffers as a powerful anionic detergent to denature more proteins by binding them. Then comparison between soil bacterial stress proteins and level of pollution according to distance from congested road were investigated by a quantitative comparison of total protein concentration which measured by Bradford's protein test. The result of this assay indicated a direct relation between increase of pollution and the level of stress protein, also it was specified that the concentration of stress proteins have adverse relation to distance from xenobiotic induced stressors like traffic pollution. As a result stress proteins have high sensitivity to changes in the environment and determination of their amounts can be suggested as a specific biomarker of exposure for biomonitoring of pollution within an ecosystem and also could be useful point in ecotoxicological studies.

Key words: Stress protein, Environmental pollution, Biomarker, Bio-monitoring, Ecotoxicology

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INTRODUCTION

There are many different types of pollutants released to environment in every moment by the human activity which classified in five categories: inorganic and organic pollutants, organo-metalic compounds, radioactive isotopes and gases (Walker, 2003). These toxic elements enter different ecosystems easily and their effects remain for a long period of time. Pollutants enter to the soil ecosystem from different ways, like industrial wastes, human disposal, toxic chemicals, sewages, radio nuclides, organic pollutants, air, and trafficked pollutants, so they have affected not only all living organisms in the soil, but also have

main influence directly on plants, animals, and indirectly on human lives.

Also, the activity of the soil microbial community can change very rapidly due to changes in environmental variables like pollutants, or due to sudden stresses like changes in pH and metal concentrations. These stresses can make change in the bacterial populations and eventually cause perturbation of the structure and function of bacterial communities. For instance, proximity to the highway increased abundance of bacteria and fungi (Fig. 1a) while the number of actinomycetes tended to decrease (Fig. 1b).

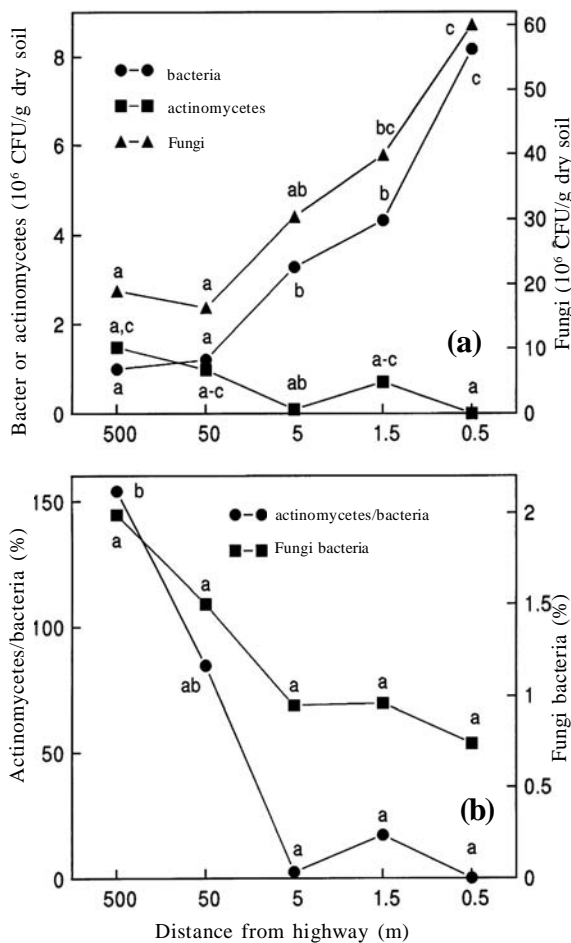


Fig.1. (a) Abundance of the principal groups of microorganisms in relation to the distance from the highway. (b) Occurrence of Actinomycetes and fungi related to the number of bacteria (Tuhackova, 2001)

Occurrence of actinomycetes and fungi related to the number of bacteria (Tuhackova, 2001). Bio-monitoring which is a sub-branch of environmental monitoring is important because biological responses may be elicited at chemical concentrations below analytical detection limits or after chemical exposure has ceased (Rand *et al.*, 1995). Also the term biomarker is referring to the use of physiological, biochemical, and histological changes as indicators of xenobiotic's effects, at the organismal or suborganismal level (Hugget *et al.*, 1992). Therefore biomarkers which are based on biomonitoring program have important information and biomonitoring programmer should ideally be able to demonstrate the effect of pollutants to individuals and preferably, through to a population, community and ecosystem level. Thus, they can be used as an early warning

system and protect the whole ecosystem from toxic chemicals which released to it in different ways (Depledge, 1993). And in practical terms, biomarkers are the endpoints of ecotoxicological tests that register an effect on living organisms (Lam, 2003).

By using suitable biomarker, if it appears that the environment was polluted through various toxic elements, then there are several ways to measure the stressor response. These variations include of specific enzyme activities, protein levels (Kille, 1999) and heat shock proteins (Sanders, 1993).

Heat shock protein was first reported by Ritossa (1962), who observed them in *Drosophila* in response to heat shock by accident. But now the term stress protein, instead of heat shock protein, is more commonly used because the induction of these proteins occurs in response to many other types of environmental stressors (Sanders, 1993).

Any modification of environmental parameters leading to a response by organisms may be considered as a stress. Therefore high levels of stress proteins are triggered by exposure to different kinds of environmental stress conditions like infection, inflammation, exposure of the cell to toxins like ethanol, arsenic, heavy metals (Sanders, 1991; Bauman, 1993; Williams, 1996), ultra violet light (Nepple and Bachofen, 1997) and organic pollutants (Sanders, 1990).

In general, heat and harmful substances denatured the proteins and causing them unfolded lose their original conformation, and are no longer able to do their function properly. When the denatured proteins are detected by the cell, stress proteins are produced and molecular chaperones which are substances inside the cells, binds and stabilize proteins at intermediate stage of folding, assembly and movement across membranes. But under adverse conditions stress proteins counter with proteotoxic effects by preventing the denaturation of proteins and holding them in the state of folding or assembly to facilitate repair, by promoting the degradation of abnormal proteins. Therefore proteotoxicity (misfolding and protein aggregation) analogous to genotoxicity is used to describe the primary mechanism of cell toxicity (Hightower, 1991) and increased in rate of synthesis of some kind of proteins under stress conditions.

Generally stress protein has special characteristics which make them as preferable biomarkers of pollution:

-This kind of protein is presented in all cells and in all life forms from bacteria to human (Lindquist, 1986; Schlesinger, 1986). All cells produce a common set of stress proteins in response to a variety of stresses, including changing the temperature, pH, exposure to toxic compounds and other conditions that cell normally do not experience.

-The induction of stress proteins are generally slow, but persist for a long time, specially during exposure to chemical stresses rather than thermal stress.

-There is specific increase on its activity within a biological system which can be used as a biomarker of exposure in environmental pollution and it may be used as an indicator of changes in environmental quality or conditions.

-They are part of cellular defense response.

-Stress proteins are strong candidates for biomarker of environmental pollution and they are activated very early in the cascade of cellular events that follow toxic exposure and at concentrations below the lethal dose.

-The most important aim for using stress proteins as biomarker in environmental risk assessment is to prove that they can give initial information on the effects of pollutants in shortest period of time.

In this study the objective of monitoring was to collect sufficient data to assess the quality of soil in the environment and the importance of stress protein in bacteria as a biomarker for biomonitoring pollutants were investigated. Also because of the ability of bacterial cells to survive and adapt to various stressful conditions, many of the stress proteins are induced in bacteria by various environmental changes. Therefore they used to find if there was any change in the amount of stress proteins in bacterial from the soils with different distance from trafficked road which was considered as a source of stress inducer.

MATERIALS & METHODS

For sample collection the upper 30 mm of soil was discarded, large roots and stones were moved

from remainder soil sample and about 0.5 Kg soil were taken aseptically from the depth of 5-10 cm and sieved to get soft and unique soil.

Extraction and release of bacteria from soil was done according to Steffan (1988) method. General scheme of soil protein extraction used in this study was shown in Fig 2. About 30 grams dry weight soil suspended in 100 ml of 0.1 M of different buffers. The sample homogenized by blending at low speed (Kitchen blender) for 2 min (This speed helps the smallest particles like bacteria stayed in suspension and most macromolecules would be in sediment or pellet rather than suspension), then 0.7 ml of 20% sodium dodecyl phosphate (SDS) was added to the different buffers and blended for 5 seconds.

For removal of non-bacterial fractions, soil buffers centrifuged at 1000g for 10 min at 10°C. Supernatant pooled or cooled and held on ice. The bacterial fractions were concentrated and supernatant divided in Eppendorf tubes and span at 13000g for 20 min and supernatants discarded.

Then for reduction of particulate and organic contaminants, pellets were suspended in 70 ml of 0.1 % sodium hexametaphosphate and 0.1 % sodium pyrophosphate, shake for 1 min on ice. After that they were centrifuged at 1000g for 30 min at 10°C (Repeated twice) and 50 ml of Chrombach buffer (0.33 M Tris hydrochloride with 0.001 M EDTA at pH 8) were added. After span at 3000 g for 15 min at 10°C supernatants discarded. In next stage 25 ml of chrombach buffer added and span at 10,000 g for 30 min at 10°C. Supernatants discarded and pellets used for protein extraction.

In the first step pellets suspended with 100 µl of the solution contains: 150 mM sodium chloride, 50 mM Tris buffer, 5 mM calcium chloride, 1 mM PMSF and 1 µg per ml of pepstatin A. (PMSF or phenyl-methyl-sulfonyl florid is a protease inhibitor which inactivate serine proteases and some cysteine proteases, also Tris buffer consist of 0.15 M Tris pH 8.8, 2 % SDS, 6 M urea and 20 % glycerol).

Then bacterial cell membranes were disrupted by freezing the vials containing the cell suspension in liquid nitrogen and throwing them in warm water repeatedly (3 times each for 1 min). Bacterial cells suspension centrifuged at 10,000 g for 20 min and 80 µL of the -20°C, acetone was added.

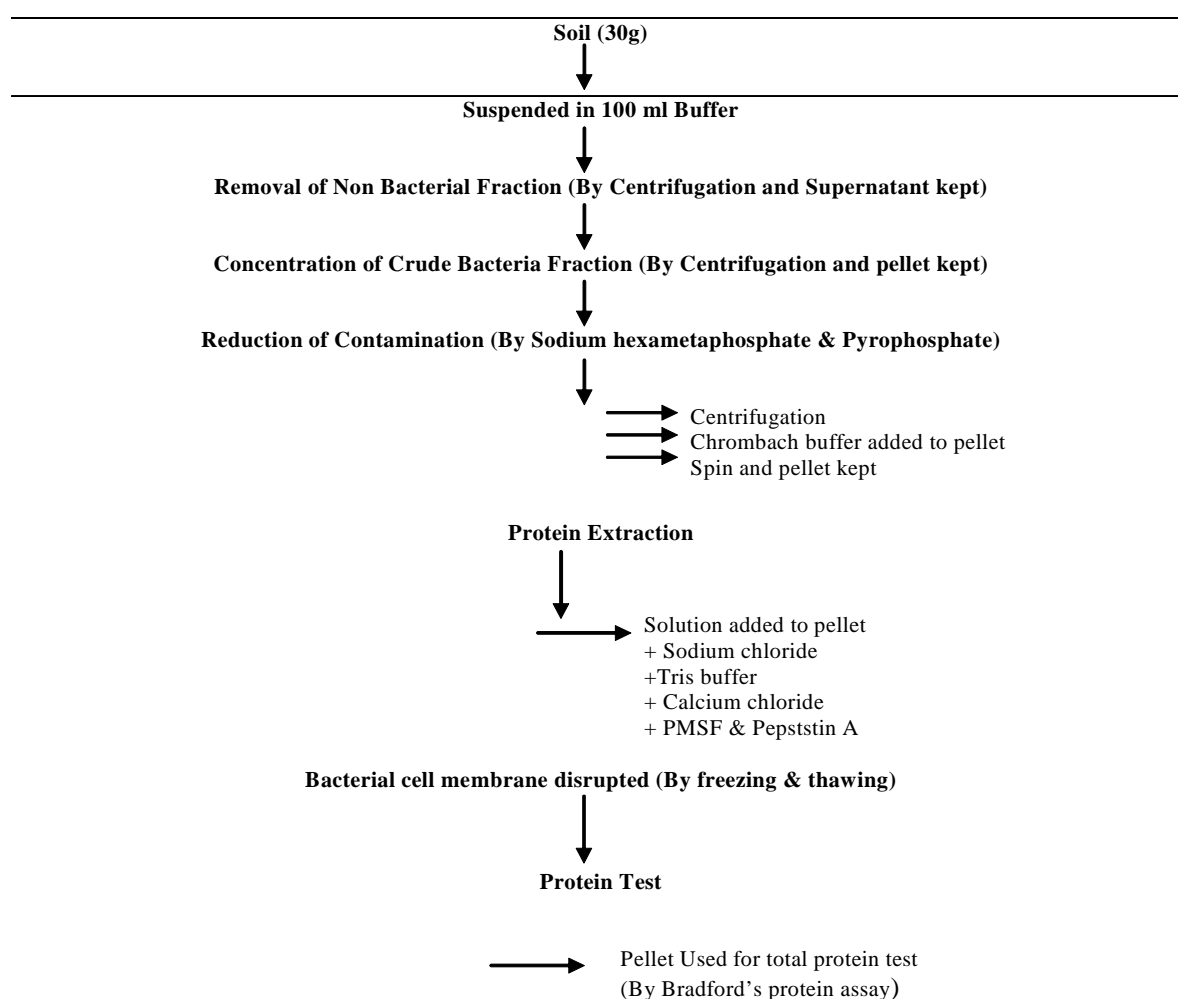


Fig. 2. Basic features of bacteria and protein extraction from soil

The pellets were collected for total protein measurement by the Bradford (Bio-Rad) assay with bovine serum albumin as a standard.

Bradford's Protein Assay

- 1- For protein standard curve, eight dilutions of bovine serum albumin were prepared as: 0, 5, 10, 15, 20, 25, 30 and 50 µg/ml.
- 2- 100 µl of distilled water was pipette to each of them to get dilutions: 0, 5%, 10%, 15%, 20%, 25%, 30% and 50% µg/ml.
- 3- Then 100 µl from sample solutions were put in microtiter plate.
- 4- 100 µl of diluted Bio-Rad Protein assay dye reagent was added (one part dye: one part distilled water).
- 5- After 5 minutes the absorbance of standard and unknown solutions were measured at 595 nm by Multiskan spectrum.
- 6- The standard curve of absorbance versus micrograms protein was drawn.

7- From the standard curves, the concentrations of protein in samples were determined. Data obtained from three independent soil samples analyzed by One-Way ANOVA and Tukey's multiple comparison tests were done as a post test.

RESULTS & DISCUSSION

Soil samples were collected in July & August (2005) from three different distances from congested road (Ramford Road - Stratford) in central part of London:

- First one was more than ten meters far from congested road. The color of this soil was light brown.
- Second one was less than one meter distance from trafficked road. It has almost black color.
- Third one was more than five meters far from congested road. It has dark brown color.

Generally, in comparison between three kinds of buffers with SDS (Fig. 3), Acidic sodium

phosphate buffer release higher amount of bacteria from soil, consequently higher level of protein were extracted (approximately 3-4 times more), after that, PBS buffer with SDS showed higher level of protein followed by more release of bacteria rather than Distilled water buffer.

The impact of pollution generated by congested traffic on soil bacterial population and also finding accurate and rapid method for assessing was the main objective of this study. Stress proteins have high sensitivity to changes in the environment; therefore, they could be suggested as possible biomarkers of exposure in ecotoxicology studies. Experiments with bacteria have shown that increased in production of stress proteins can protect organism against stress induced damage, and function of these proteins which are part of a cell's own repair and defense system, and cellular stress response can be used as an early warning of pollution.

In first step, it was needed to use rapid method for release of bacteria from soil and then measurement of their proteins in different types of solvents was examined:

- Distilled water.
- PBS buffer which is more physiological than the other buffers, and it is found in living

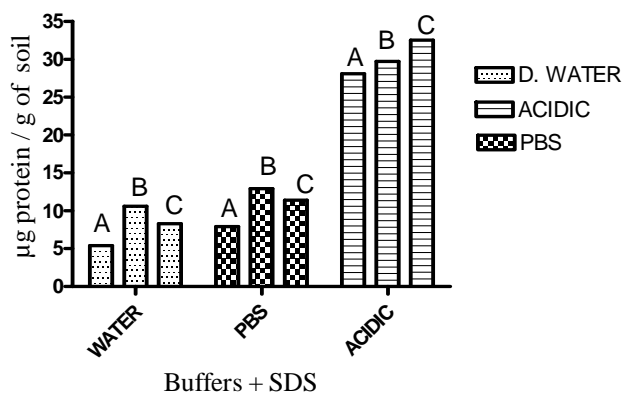


Fig. 3. Protein concentration released from bacteria in different soil buffers

By using Bradford's protein assay for soil samples with different distances from congested road: (A) soil > 10 m, (B) soil < 1 m and (C) soil > 5 m, protein concentration were examined in three kinds of soil buffers; Distilled water buffer, Acidic sodium phosphate buffer and PBS buffer with SDS. The graphs showed that with 95% confidence Interval, differences between means of protein concentrations when acidic sodium phosphate buffer used in compare with the others, was significant and the amount of p-value was less than 0.05 ($p < 0.001$).

systems in the form of inorganic phosphates and it is non-toxic to cells.

-Acidic sodium phosphate buffer pH 4.5 is normally used in soil investigations to release more bacteria and therefore high level of extracted proteins, made it more useable than the other buffers.

Also Sodium dodecyl sulphate (SDS) is a detergent with the importance of adding this solution to buffers is due to its effectiveness on the release of bacteria from soil, as a result increase in level of extracted stress protein. Thus after bacterial cells suspended with SDS, their membrane dissolved and proteins would be solubilized by the detergent.

Although all these buffers by adding SDS solution were more suitable for this purpose but acidic sodium phosphate buffer gave better recoveries of bacterial cells from soil rather than the other buffers and also higher level of proteins would be extracted (Fig. 3). On the other hand, soil samples were taken from natural polluted area from three different points of congested road, and the soil which was taken more than 10m distance from trafficked road was considered as a control.

There is an increase in total numbers of bacteria and fungi with increasing proximity to automobile traffic. This correlation suggests that the extent of hydrocarbon deposition alongside a frequently trafficked highway is sufficient to affect the microbial populations. A feasible reason of the raised in the total number of soil micro-organisms could be a supply of hydrocarbons in significant amounts as an energetic input. Therefore traffic pollution not only made increase in the soil bacterial population close to congested road but also increased the rate of stress proteins.

Bradford's protein assay was used for a quantitative comparison of bacterial total proteins. The acquired results for these different soils were accurately compatible with the expected theory (Kohan and Morgan 2005); it suggested that as the taken samples are closer to the trafficked road, the total amount of proteins showed an increase (Fig. 3). Therefore total proteins which determined by Bradford's protein assay could be used as a valuable parameter in environmental pollution investigations.

Also it seems that most part of the natural soils have different level of pollutants, because soils received substantial inputs of natural and anthropogenic solutes as a result of different human activities and have affected on micro-organisms which can be used as key role in soil assessment. Thus they were the best biomarker for biomonitoring of soil pollution that caused by air pollutants, pesticides, and acidic rain, polluted water, sewages, industrial wastes and radioactive activity. Pollutants are constant challenge for soil micro-organisms, and their ability to sense, respond and adapt to pollutant stress, are vital to their metabolic functions, growth and survival.

As a result increase in total protein of bacterial cell had approximately direct relation with distance from trafficked road which is a xenobiotic stressor source. These changes can be used as a specific biomarker of exposure for within an ecosystem. Also, there were many problems with removing of debris and organic pollutants from the soil before the extraction of proteins were done, therefore the use of appropriate methods which reduce this debris as less as possible were suggested. By detection of the bacterial stress protein which was highly sensitive to changes in the environment it appears that they can be used as early biomarker for biomonitoring of ecotoxicological studies.

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