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EFFECT OF SURFACTANTS ON PROTEIN THREONINE PHOSPHATASE (PTHPASE) ACTIVITY IN PEANUT (*ARACHIS HYPOGEA* L.) SEEDLINGS

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ABSTRACT

Protein threonine phosphatase (PThpase) activity has been linked with several pathological states, including diabetes, cardiovascular disorder, cancer and Alzheimer's disease. Therefore the pathological manipulation of phosphatase activity is an attractive strategy for the treatment of various diseases. It is well established that surfactants interact with proteins in diverse ways depending on surfactant concentration and structure. These proteins also play a key role in the cellular signaling process. The effect of surfactant on PThpase was carried out by immersing the eight days old growing peanut seedlings using different concentration of sodium dodecyl benzene sulphonate (NaDBS) (1-10%) for 2 h. The specific activity of PThPase was found to decrease as the concentration of NaDBS increases. A 2 h treatment of 6% NaDBS decreased the specific activity of up to 72%. Among different parts of plant, epicotyls showed the minimum relative specific activity hence are highly stressed by applying the same treatment. The decreased specific activity can play a very salient role in curing many pathological diseases. The decrease in specific activity might be due to electrostatic and hydrophobic interactions between enzyme and surfactants.

Key Words **: Protein threonine phosphatase (PThPase), Protein tyrosine phosphatase (PTPase), Protein phosphatase (PPase) and Peanut**

A phosphatase (Pase) is an enzyme that removes a phosphate group from its substrate(O-phospho-L-threonine $C_4H_{10}NO_6P$) by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group (1). Protein phosphatases (PPases) catalyze the dephosphosrylation of post translational modified serine/threonine and tyrosine residues in phosphoproteins. Protein kinase (PKase) catalyzes the phosphorylation in which the addition of a phosphate group takes place from energetic molecules such as

ATP, to a protein usually at a serine/threonine, tyrosine or histidine residue (2). PPases, in coordination with PKases, play crucial roles in regulation of signaling pathways. Unlike most enzymes, the serine/threonine specific PPases show broad and overlapping substrate specificities *in vitro* and their classification requires the use of specific inhibitors and activators. By using these criteria, four major classes of PPase catalytic subunits have been identified in eukaryotic cells (Type1 phosphatases (PP1) highly conserved among all eukaryotes and Type 2 phosphatase comprising of three enzymes (PP2A, PP2B, and PP2C) (3).

Surfactants are widely used in everyday personal care and household products as well as in a variety of industrial applications (13). In fact, many surfactants and their degradation products have been found worldwide in waste water discharges, sewage treatment, plant effluents, natural water and sediments (14). Since many surfactants are ubiquitous, the potential toxic effects of these chemicals and a very little work on the interaction between surfactant and PThPase, have attracted much research attention in the past several decades (15,16, 17,19). There is a very little work on the interaction between surfactant and PThPase Specifically PThPas dephosphorylates are the beta-subunits of phosphorylase kinase and, each functional PP1 enzyme consists of a catalytic subunit and a regulatory subunit. The catalytic subunit of PP1 is with approximately 70% or greater protein sequence identity in any pair wise alignment. At least 100 putative PP1 binding regulatory subunits have been identified with many more expected to be found (4).

Dephosphorylates, the alpha subunits of phosphorylase kinase preferentially and are unaffected by the inhibitors. Type 2 phosphatases are comprised of three enzymes (PP2A, PP2B, and PP2C) that can be distinguished by their requirement for cations (5, 6). These type 2 enzymes play an important role in biological functions such as development, cell proliferation, dynamics of death cell mobility, muscle contractility, cell cycle control, gene transcription, cholesterol, protein biosynthesis and metabolism and the regulation of numerous signaling pathways (5, 7-11).

The phosphorylation status of a protein or more specifically, the pattern of phosphorylation on a given protein can determine its activity. The presence or absence of a phosphate group can change the conformation of target protein. The balanced transfer of phosphoryl groups from one entity to another as catalysed by phosphatase and kinases is the basal mechanism by which cellular function is controlled (12).

Hence, the aim of this study is to visualize the effect of surfactants on protein threonine phosphatase (PThpase) activity in peanut (*Arachis hypogea* L.) seedlings.

MATERIALS AND METHODS

Surfactants :

Following anionic surfactants were used in this study including stannous laurate, stannous palmitate, stannous stearate AgDS (silver dodecyl sulphate), MgDBS (magnesium dodecyl benzene sulphonate), NaDS (sodium dodecyl sulphate) and NaDBS (sodium dodecyl benzene sulphonate). The cationic surfactants include HTAB (hexa-tetraethyl ammonium bromide, whereas Tween-80 (polyoxyethylene sorbiton monolaurate), Criton X-100 (isooctyl phenoxy polyethoxy ethanol) and Brij-35 (polyoxyethylene 23 lauryl ether). Whereas TEMED (N, N, N, N -tetra methyl ethylene diamine), Tris (tris-(hydroxymethyl) amino methane), O-Phospho L- threonine, EDTA (ethylene diamine tetra acetic acid), and â-mercaptoethanol were used as nonionic surfactants. All the chemicals were purchased from reputed commercial firms and were of high purity grade.

Enzyme extraction and assay :

The peanut seeds were purchased from an authorized seed store and washed with double distilled water. The seeds surface was sterilized with 1% $HgCl_2$ and the seeds were allowed to germinate under aseptic conditions for 2-14 days on autoclaved whatman filter paper (20). The maximum specific activity of PThPase was found in 8 days old growing seedlings. The 8 days old growing seedlings (whole seedlings) and different parts of plant like root, hypocotyl, epicotyl and cotyledon

were crushed manually and the crude enzyme extract was prepared separately by homogenizing the plant tissue with extraction buffer (100 mM-Tris pH 7.5 + 50 mM NaCl + 10 mM-EDTA + 0.04% beta-mercaptoethanol) in 1:3 ratio at 0-4°C.

The homogenate was filtered through four layered cheese cloth. The filtrate was centrifuged (Remi-CPR24) at 10,000 rpm for 30 min. The supernatant obtained from centrifugation was used for enzyme assay. Protein phosphatase (PPase) activity was assayed by using casein (SIGMA) and PThPase activity was assayed by using O-Phospho L- threonine (SIGMA, Aldrich, MO, USA).

The reaction mixture was composed of 10 µl substrate, 10 µl enzyme, 180 µl Tris-HCl (100 mM) (total volume 200 µl). The reaction mixture was incubated for 30 min at 30°C. A 10% TCA solution was added to stop the reaction and the reaction mixture was kept in ice for 5 min and centrifuged again at 10,000 rpm for 5 min in a refrigerated centrifuge (0-5°C), so that the protein is completely precipitated out. A 200 µl of supernatant of the reaction mixture was assayed for inorganic phosphate (Pi) release by the malachite green (BDH) method (18). Briefly, 100 µl supernatant was mixed with 1 ml of malachite green solution (45 mg malachite green in 100 ml distilled water, 1.4 g ammonium molybdate in 12 ml HCl and 25 ml distilled water) and the absorbance was measured spectrophotometrically (mini-1240-UV-visible (Simazdu, Japan). The similar method was also used for the (PThPase), protein tyrosine phosphatase (PTPase), protein serine phosphatase (PSPase), by using O-phospho-L-threonine, O-phospho-L-tyrosine, O-phospho-L-serine as substrates, respectively. One unit was defined as the amount of protein in mg that liberated one nano mole of inorganic phosphate (Pi) per minute under assay condition. The protein concentration was measured as per Lowry method (21) using bovine serum albumin as the standard substance.

Surfactant stress :

The surfactant stress was carried out by treating the germinated seedlings as well as different parts of seedlings through the following steps: (1) treatment of whole seedlings with 2% solution of different surfactants for 2 h; (2) treatment of whole seedlings with 1-10% solution of NaDBS for 2 h and (3) treatment of different parts of germinated seedlings with 6% solution of NaDBS for 2 h.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (NaDS- PAGE) :

Gel electrophoresis was carried out in presence of NaDS by Laemmli (1) method using gel slabs (18×18×1cm) with 13 wells of about 1.0 mm width and 3.0 mm spacing in between two consecutive wells. All reagents were prepared in double distilled water. Enzyme sample (100-200µg protein) from different steps of purification were dialysed against distilled water, concentrated using sucrose and dissolved in 100 µl of the sample buffer (100 mM Tris-HCl). This was followed by heating for 5 min in a boiling water bath. Molecular mass markers (14.3 kD-97.4 kD) were also subjected to the same treatment. These proteins were then loaded in the wells and electrophoresis was carried out at constant current of 20 mA for stacking gel and 30mA for the separation gel. When the tracking dye reached close to the base (1 cm from bottom) of the gel, the gel was removed and immersed in fixing solution (30% ethanol) for 30 min. Now, it was stained by immersing in Coomassie brilliant blue R-250 (CBBR-250) staining dye overnight at room temperature. Gel was destained by washing with destaining solution (40% methanol) till color of staining dye is completely removed from gel. Mobilities of different protein bands were determined, relative to bromophenol dye (Glaxo) using the following equation:

Relative mobility = [(Gel length before staining/gel length after staining)/Distance travelled by protein band/ Distance travelled by bromophenol]]

RESULTS AND DISCUSSION

Effect of surfactants on specific activity of PThPase in 8 day old germinating peanut seedlings:

The effect of anionic, cationic and non-ionic

surfactants on activity of phosphatase has been extensively studied. PThPase, a key regulatory enzyme involved in many different processes in the cell and distinctly affected by anionic, cationic and non-ionic surfactants. The decreasing specific activity values of PThPase in various surfactants were in following order: anionic: stannous stearate> stannous palmitate> stannous laurate>MgDBS>NaDS> AgDS>NaDBS; non-ionic: Tween-80>Brij-35>Criton X-100; whereas HTAB was found only the cationic surfactant which increased the specific activity of PThPase (Table 1).

Among anionic surfactants, the stannous surfactants (laurate, palmitate and stearate) showed slightly stimulating effect while AgDS, MgDBS, NaDS, NaDBS showed inhibitory effect on PThPase activity. The activity of PThPase was highly inhibited (up to 62%) by 2% NaDBS solution for 2 h treatment (Table 2). The slightly enhanced activity by stannous surfactants might be due to different nature of alkyl groups; therefore, the hydrophobic interaction varied between enzyme and surfactant.

The other anionic surfactants (AgDS, MgDBS, NaDS, and NaDBS) showed the inhibitory effect due to different ionic nature of metals. Under the same conditions, the cationic surfactant HTAB also decreased the specific activity of PThPase (up to 17%) due to hydrophobic interaction of the surfactant.

The nonionic surfactants showed dual behavior towards PThPase activity. Brij-35 and CritonX-100 decreased the activity of PThPase whereas Tween-80 had little stimulatory effect. These results suggest that the stimulatory and inhibitory effect shown by surfactants on PThPase activity, involved both electrostatic and hydrophobic interactions. The enzyme-surfactant interaction probably occurred at or near the active site of PThPase. It was also observed that the specific activity of PThPase was sharply reduced upto 4% and afterwards decreases slowly from 5-10% when the seedlings were immersed in 1-10% of NaDBS solution (Fig. 1). These results obtained did not reveal that which part of the seedlings was most affected. Therefore, the specific activity level of PThPase after 3 h treatment of peanut seedlings with 6% NaDBS was then measured in various parts viz roots, hypocotyls, epicotyls and cotyledons. The specific activity of PThPases is highly reduced in (62%) epicotyls, while under the same treatment in hypocotyls, roots and cotyledons, the specific activity of PThPase decreased approximately 60-13% (Fig. 2). These results suggest that among the developing parts, the epicotyls and hypocotyls appeared

Table 1: Effect of different surfactants on the specific activity of PThPase in 8 day old germinating peanut seedlings

Surfactants	PThPase	
	Specific activity, unit/mg-protein	Relative activity (%)
*Control	24.4	100
Anionic:	25.9	106
St. Laurate		
St. Palmitate	27.9	114.3
St. Stearate	29.1	119.2
AgDS	12.4	50.8
MgDBS	19.2	78.6
NaDS	14.6	59.8
NaDBS	9.4	3 8.5
Cationic:	20.4	83.6
HTAB		
Nonionic:	27.8	114
Tween-80		
CritonX-100	14.8	6 0.6
Brij-35	18.2	74.5

as the best tissue of choice for study of PThPases activity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (NaDS-PAGE) :

NaDS-PAGE profile of protein after or before immersing different parts of germinating peanut seedlings in 6% NaDBS for 2 h revealed a change in different protein bands using 12% polyacrylamide gel (Fig. 3). A high molecular weight protein band was visible between 97.4 kDa to 120 kDa marker range in stressed hypocotyls and epicotyls, whereas the same protein band was found absent in unstressed hypocotyls and epicotyls. A new strong protein band was over expressed between 66.0 kDa to 97.4 kDa marker range in stressed cotyledon, while the same protein band was found missing in unstressed cotyledon of germinating peanut seedlings. Another protein band was found, although of a low molecular weight which was over expressed between 14.3 kDa to 20.1 kDa marker range in stressed hypocotyls, while the same band disappeared

Table 2 :Effect of surfactants (stress) on PThPase applied by immersing the seedlings in 2% solution of different surfactants for 2h

Sample	Protein(mg/ml)	Specific activity
Anionic		
Stannous laurate	11.2	25.9
Stannous palmitate	10.2	27.9
Stannous stereate	11.2	29.1
AgDS	19.6	12.4
MgDBS	11.2	19.2
NaDS	11.4	14.6
NaDBS	14	9.4
Cationic		
HTAB	14	20.4
Nonionic		
Tween-80	13	27.8
Critonx-100	16.4	14.8
Brij-35	17.2	18.2
Different % of NaDBS		
1%	20	23.2
2%	19.8	16
4%	19.2	9.4
6%	13.6	20.4
8%	14.4	13.4
10%	19.8	9.4
4% NaDBS for different parts		
Root-gen	4.8	52
NaDBS	8.2	17.7
Hyp-gen	5.8	82.5
NaDBS	8.6	28.2
Epi-gen	10.4	28.6
NaDBS	13.2	8.4
Coty-gen	19.6	11.6
NaDBS	22.4	7.7

AgDS= silver dodecyl sulphate; MgDBS= magnesium dodecyl benzene sulphonate; NaDS= sodium dodecyl sulphate; NaDBS= sodium Dodecyl benzene sulphonate; HTAB= hexadecyl trimethyl ammonium bromide. *Control (unstressed) show 100% relative activity of PThPase.

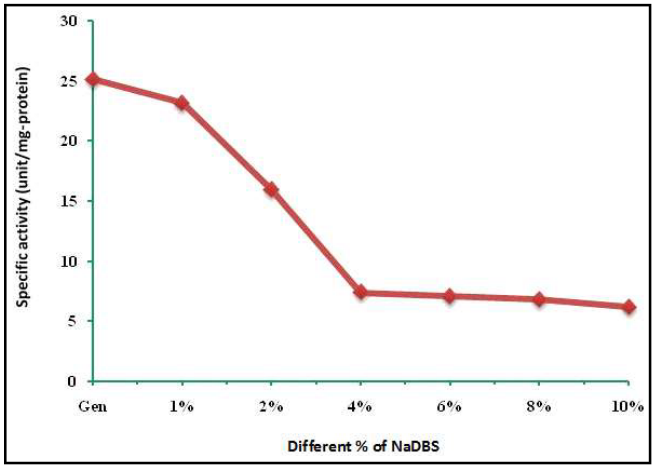

Fig. 1: Effect of different %NaDBS on the specific activity of PThPase in peanut seedlings for 2h treatment.

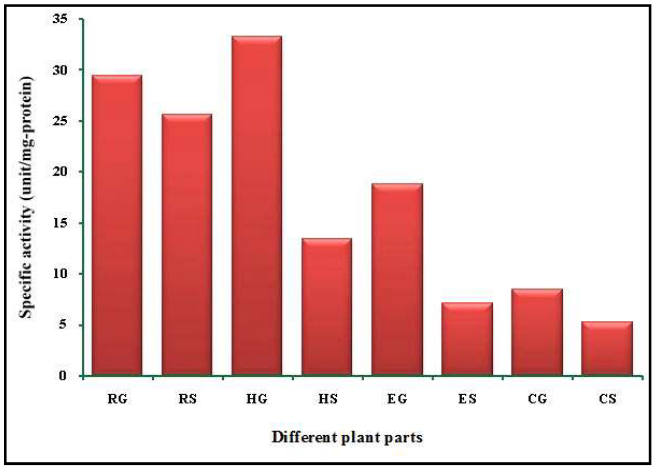

Fig. 2: Effect of 6% NaDBS on the specific activity of PThPase in different parts of the peanut seedlings for 2h treatment. Rg= root general, Rs = root stress; Hg= hypocotyl general, Hs= hypocotyl stress; Eg= epicotyl general, Es = epicotyl stress; Cg = cotyledon general, Cs = cotyledon stress.

in unstressed hypocotyls germinating peanut seedlings. So, therefore, there are two stressed proteins expressed in hypocotyls, one each in epicotyls and cotyledon while such activity was not visualized in roots under stress or growth conditions. However, different molecular mass of protein bands in NaDBS gel electrophoresis was found to be reduced after stress clearly stating that surfactants have profound effect on PThPases which might be due to the synthesis and inhibition of different

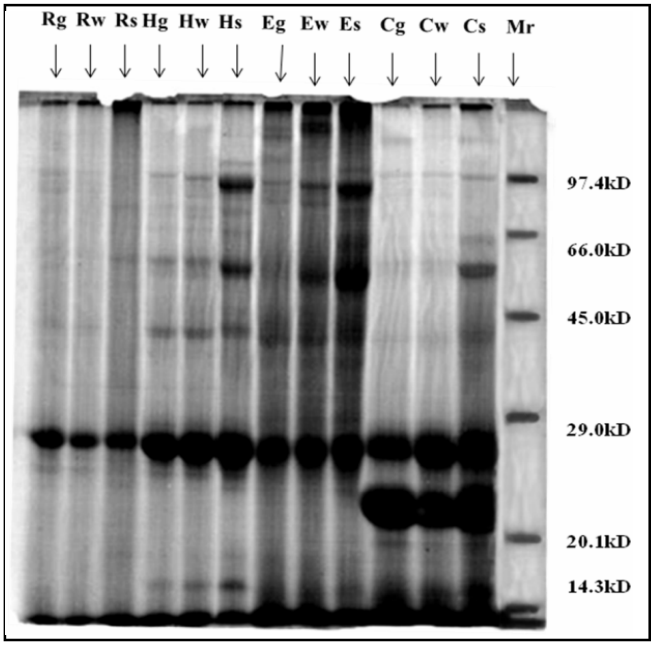

Fig. 3: A comparison of protein bands profile by NaDS-PAGE in different parts of 8 day old germinating peanut (*Arachis hypogea* L.) seedlings immersing in 6% NaDBS. Soluble proteins were extracted and analyzed by NaDS-PAGE using 12% polyacrylamide gel. Protein were stained with coomassie brilliant blue R-250. The difference in the intensity of protein bands with reference of standard molecular marker range of 14.3-97.4 kDa. Rg= root general, Rw= root water, Rs= root stress; Hg= hypocotyls general, Hw= hypocotyls water, Hs= hypocotyls stress; Eg= epicotyls general, Ew= epicotyls water, Es= epicotyls stress; Cg= cotyledon general, Cw = cotyledon water, Cs = cotyledon stress.

isoenzymes during stress conditions provided using different surfactants

Discussion :

The stimulatory effect shown by anionic and non-ionic surfactants, the later provides better stability in extracting peanut seedlings acid phosphatases. Triton X-100 is an activator for the three phosphatase enzymes (PSThPase, PTPase and DSPase). Shekhar *et al.* (24) have also reported the same effect of Triton X-100 on the lysophosphotidic acid phosphatase activity from developing peanut cotyledons. The acid phosphatase enzyme appeared to be stable in the presence of non-ionic surfactants such as Triton X-100 (25). Anionic

surfactants such as NaDS exert toxic and harmful effects on cell membranes and can solubilize proteins causing their denaturation. They can also modify the activity of an enzyme by binding to it (26).

The specific activities of PThPase among different parts, epicotyls showed the minimum specific activity during highly stressed while it was less decreased in hypocotyls, roots and cotyledons by applying the same treatment. Shekar *et al.* (2002)**27** have also reported the same effect of Triton X-100 on the lysophosphotidic acid phosphatase activity from developing peanut cotyledons. Different protein bands were over expressed and some others reduced during the stressed. The acid phosphatase enzyme appeared to be stable in the presence of non-ionic detergents such as Triton X-100 (28). It is due to the many different mechanisms of toxicities exist for different types of surfactants and one single surfactant can produce its toxicity through more than one mechanism.

Cationic surfactants are more effective than nonionic surfactants in increasing, glyphosate phytotoxicity (Wyrill and Burnside, 1977; Riec'hers, 1992) (29, 30). Our initial hypothesis suggested that cationic arnine surfactants form a neutra1 complex with the negatively charged glyphosate molecule that increases membrane permeability.

The results of this study demonstrate that both nonionic and cationic surfactants possess similar **a** bilities to enhance the plasma membran's permeability to glyphosate. Yet, in the field these surfactants do not exhibit comparable levels of efficacy in promoting glyphosate phytotoxicity. de Ruiter *et al.* (1988)31 reported that surfactants do not possess equal abilities to penetrate the leaf and enhance glyphosate phytotoxicity in winter wheat.

In addition, the percentage of a given PhTPase that is inactivated varies widely between studies. Although much of this may arise from the varied experimental protocols, we have yet to define the necessary levels of PhTPase inactivation that would be required for the sustained activation of RPTKase signaling. This is not a trivial undertaking, but it has far-reaching consequences for our understanding of PhTPase regulation.

Conclusion :

These results indicate that the PhTPases are closely involved in many levels of cell biological control. Furthermore, the enzymes can be controlled and modulated in diverse and novel ways, including through oxidation, dimerisation, cleavage, differential localization, and also appears to be cellular and developmental tolerance to their individual loss of function in many cases, suggesting that highly conserved redundancy or degeneracy is in place in order to maintain control of PTK signaling pathways. These results also indicate that the inactivation and activation of the PThPase by anionic, cationic and nonionic surfactants involved both electrostatic and hydrophobic interactions and the enzyme-surfactants interactions probably occurred at or near the active site. This observation suggests that effective surfactants are also involved in increasing the permeability of the cuticle, plasma membrane, or both in increasing foliar uptake of glyphosate and promoting phytotoxicity.

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