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Protein Tyrosine Phosphatases Extracted from Peanut Seedlings Express Differential Activity on Exposer to Surfactants

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The surfactants interact with proteins in multifarious ways depending on surfactant concentration and structure. Proteins like PTPases play a key role in the cellular signaling process. The effect of surfactant on PTPase was carried out by immersing peanut (8 day old germinating) seedlings using different concentrations of NaDBS (1-10%) for 2 h. The specific activity of PTPase was found to decrease with increase in surfactant concentration. 8-fold decrease in specific activity of PTPase was noticed at 6% NaDBS. However at high concentration of NaDBS it becomes constant. However the different parts of the peanut seedlings treated with 6% NaDBS for 2 h at room temperature shown that the specific activity (unit/mg-protein) of PTPase was lowered to about 5.0 fold in root and 3.0 fold in cotyledon. This might be due to the inactivation and activation of the PTPase by anionic, cationic and nonionic surfactants involved in both electrostatic and hydrophobic interactions. From our observations it was found that the enzyme-surfactant interaction probably occurred near the active site and the role of PTPases in plants is a stress-related cellular process.

Key words: *Arachis hypogaea* L, Peanut, Protein tyrosine phosphatases (PTPases), Anionic surfactant, Cationic surfactant and Non-ionic surfactant.

Protein tyrosine phosphatases (PTPases: EC 2.7.1.112) are hydrolytic enzymes that remove phosphate from the phosphorylated tyrosine residues. The sequence context surrounding the target tyrosine plays a key role in determining its recognition by PTKs and PTPases. PTPases are most essential regulators of a variety of fundamental cellular processes such as cell growth, mitogenesis, metabolism, gene transcription, cell cycle control and the immune response (Vander Geer *et al*., 1994). They are also key participants in kinase dependent signal transduction pathways where they can play both positive and negative regulation roles (Walton and Dixon, 1993). Maintenance of specific levels of protein tyrosine phosphorylation and dephosphorylation are vital for normal cell proliferation, differentiation and metabolism (Tonks *et al*., 1990). Furthermore, PTPase activity has also been associated with diabetes (Ahmad *et al*., 1995). The function of many proteins, particularly those involved in signal transduction pathways, are dependent upon their tyrosine phosphorylation status, which is finely regulated by the action of PTKases and PTPases. While the roles and mechanisms of protein tyrosine kinases are well documented, our present understanding of protein tyrosine phosphatases is very limited (Alonso *et al*., 2004). In this regard we still have much more to learn about PTPs.

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These PTPases with important cellular functions are stimulated by the action of surfactants. Anionic and nonionic surfactants are known to cause stimulatory action on phosphatases of plants including peanut seedlings (Cserhati *et al*., 2002). Surfactants might alter properties such as the conformational stability of the protein structure, the hydrophobicity of the protein surface (Miller *et al*., 2000) or the catalytic activity of enzymes (Green *et al*., 2000). At solid surfaces surfactants are known to displace adsorbed proteins either by replacement or by inducing solubilization of the protein if the surfactants are able to bind to the protein structure (Antipova *et al*., 2001). Under competitive adsorption, surfactants might compete with protein for adsorption site at various interfaces.

Many surfactants and their degradation products have been found worldwide in waste water discharge, sewage treatment, plant effluents, natural water and sediments (Cserhati *et al*., 2002). Stress responses in plants cause changes in the structure and activity of one or more proteins. Therefore, characterization of these proteins and understanding their function with respect to surfactant stress is an important method for studying responses of the plants. In addition, the surfactant might specifically interact with hydrophobic sites of the protein surface, where protein aggregation could potentially originate (Bam *et al*., 1995). Furthermore, the chaperon-like action of surfactants, adding refolding of proteins, is discussed (Bam *et al*., 1996). Interaction of ionic surfactants with oppositely charged proteins has been investigated for long time. The interaction of sodium dodecyl sulphate (SDS) as anionic surfactants with lysozyme and other proteins has been widely investigated from viewpoints of binding isotherms, energetic and phase behavior. It has been shown that, at the beginning of the interaction and at low concentration of SDS, the trend is specific and electrostatic in which negative SDS binds to positive residues, neutralizes the protein surface charges and leads to precipitation and turbidity of the solution to reducing the net charge of the protein. One of the important applications of the surfactants is the breakdown of protein structure.

This study was carried out to observe the effect of different type of surfactant such as cationic, anionic and non-ionic on the level of the specific activity of protein tyrosine phosphatases in 8 days old germinated seedling of peanut. These surfactants could be very helpful in significantly improving the current understandings about the roles and mechanisms of the PTPs.

MATERIALS AND METHODS

Germination of Peanut seeds and Application of Stress

Peanut seeds were surface sterilized with 1% (w/v) $HgCl_2$ solution for 20-30 min and allowed to germinate under aseptic conditions (moistened whatmann filter paper) for 0-8 days. Stress was applied by immersing the plant seedlings in 2% of anionic (Stannous laurate, Stannous Palmitate, Stannous stearate, Silver dodecyl sulphate (AgDS), magnissium dodecyl benzene sulphonate (MgDBS), Sodium dodecyl benzene sulphonate (NaDBS), dodecyl sulphate (NaDS,), cationic (HTAB) and nonionic (Tween-80, CritonX-100, Brij-35) surfactants for 2 h.

Stress on PTPases using NaDBS

Sodium dodecyl benzene sulphonate (NaDBS) shows maximum stress on PTPases in above studies was further used with different concentrations (0-10%) for 0-6 h at room temperature and observed for stress on germinated peanut seedlings. Further, the different parts (root, hypocotyl, epicotyl and cotyledon) of peanut seedlings were immersed in 6% NaDBS for 2 h at room temperature.

Extraction of Crude PTPases

For extraction of crude enzyme after stress application, the seedlings were excised manually and the crude enzyme extract was prepared by homogenizing the different tissues of plants (l:3) in the homogenizing buffer (100 mm Tris-HCl pH 7.6, containing 10 mM EDTA and 0.04% β-mercaptoethanol) at 0-4°C. The homogenate was filtered through 4 layers of autoclaved cheesecloth. The filtrate was centrifuged at 9000 rpm for 30 min at 0-4°C. The supernatant (crude enzymes extract) were collected and used for enzyme assay for protein tyrosine phosphatase (PTPase) by using O-phospho-L-tyrosine (Sigma) as a substrate.

Assay for PTPases

The reaction mixture contained the

following components in a total volume of 200 µl: enzyme (20-200 µg protein), 100 mm Tris-HCl (pH 7.6), 0.5 mM EDTA and 0.5 mM O-phospho-L-tyrosine as substrate. After 30 min incubation at 30°C, the reaction was terminated by adding 250 µl 10 % TCA (Trichloro acetic acid) and the assay was kept in ice bath for 30 min to allow complete precipitation of protein. The insoluble matter was removed by centrifugation at 9000 rpm for 10 min in refrigerated centrifuge at 0-4°C. An aliquot (100 µl) of the clear supernatant of the reaction mixture was assayed for inorganic phosphate (Pi) by the malachite green method which was described by Lenzetta et. al. (1979) and the determination of PTPase and carried out in similar way using, O-phospho-L-tyrosine as substrates. One unit was defined as "the amount of protein (mg) that liberated one nmole of inorganic phosphate (Pi) per minute under assay condition". Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

SDS-PAGE of Crude Enzyme

Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis was carried out according to the method of Laemmli (1970) using 10% polyacrylamide gel. Coomassie brilliant blue R-250 staining was carried out to visualize protein bands on the gels. The molecular weight of the protein was estimated by comparing the relative mobility of proteins of different molecular size using standard molecular weight marker (97.4-14.3kDa). All the experiments were replicated atleast thrice with three replicates each and the data was pooled to mean of the values obtained individually.

Table 1. Effect of surfactants (stress) on PTPase was applied by immersing the seedlings in 2% solution of different surfactants for 2 h

Surfactants	PTPase	
	Specific activity, unit/mg-protein	Relative activity (%)
*Control	38.6	100
Anionic: St. laurate	48.0	125
St. palmitate	41.3	107
St. stearate	40.8	106
AgDS	31.1	80
MgDBS	28.6	72
NaDS	22.6	58.5
NaDBS	15.6	37.8
Cationic: HTAB	55.7	145
Nonionic: Tween-80	41.9	108
CritonX-100	29.6	76
Brij-35	28.4	73

St. Laurate = Stannous laurate; St. Palmitate = Stannous palmitate; St. Stearate = Stannous stearate; 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) shows 100% relative activity of PTPase

RESULTS AND DISCUSSION

This study showed the effect of various surfactants such as anionic (stannous laurate, stannous palmitate, stannous stearate AgDS, MgDBS, NaDS and NaDBS), cationic (HTAB,) and nonionic (tween-80, criton X-100 and brij-35) on the specific activity of PTPase extracted from germinated peanut seedlings (Fig. 1). Decreased specific activity values of PTPase in various surfactants are shown in Table 1 in their respective orders; anionic: stannous laurate > stannous Palmitate > stannous stearate > AgDS > MgDBS > NaDS > NaDBS; nonionic: tween-80 > criton X-100 > brij-35; HTAB is only the cationic surfactant which increases the maximum activity of PTPase. The effect of anionic, cationic and nonionic surfactants on specific activity of PTPase has been extensively studied. PTPase, a key regulatory enzyme involved in many different processes in the cell and distinctly affected by anionic and cationic (Hexadecyl trimethyl ammonium bromide) and nonionic surfactants. Among anionic surfactants, the stannous surfactants (laurate, palmitate and stearate) show slightly stimulating effect while AgDS, MgDBS, NaDS, NaDBS show inhibitory effect on PTPase activity. The activity of PTPase highly inhibited (upto 65%) in presence of NaDBS. The slightly enhanced activity by stannous surfactants is due to different nature of alkyl groups therefore the hydrophobic interaction varies between enzyme and surfactant. HTAB is only the cationic surfactant which increases the maximum specific activity of PTPase (up to 145%) due to hydrophobic interaction of the cationic surfactants.

The nonionic surfactants show dual behavior towards PTPase activity. Brij-35 and CritonX-100 decreased the activity of PTPase

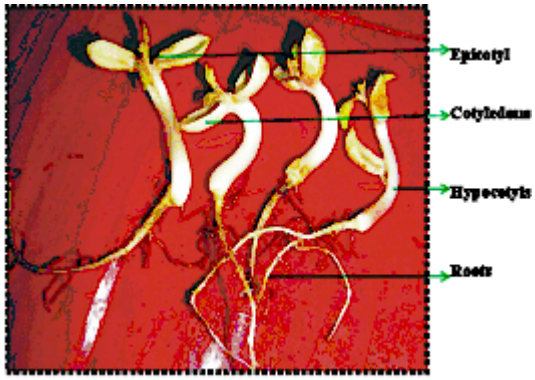

Fig. 1. A population of 0-14 days old germinated seedlings. Peanut (*Arachis hypogeal* L.) were germinated on whatman (3 mm) filter paper moistened with sterile double distilled water in dark at 28 ± 2ºC under aseptic conditions in a seed germinated chamber. The different anatomical parts which are clearly visible in the seedlings only healthy seedling were used

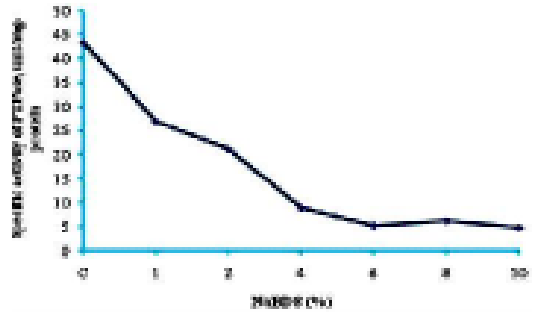

Fig. 2. Effect of surfactant on the specific activity of PTPase was applied by immersing the seedlings in different percentage of NaDBS for 2 h

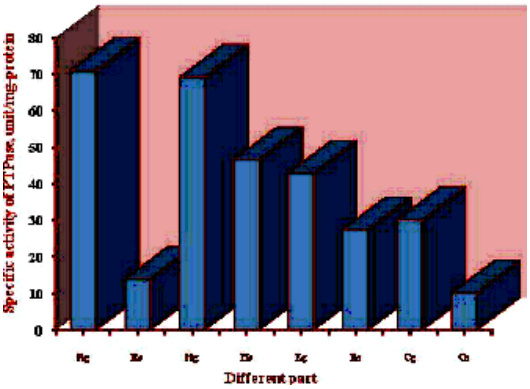

Fig. 3. Effect of 6% NaDBS on the specific activity of PTPase in different parts of the peanut seedlings for 2 h treatment. Rg = Root (general), Rs = Root (stressed); Hg = Hypocotyl (general), Hs = hypocotyl (stressed); Eg = Epicotyl (general), Es = Epicotyl (stressed); Cg = Cotyledon (general), Cs = Cotyledon (stressed)

whereas Tween-80 had little stimulatory effect on PTPase. These results suggest that the stimulatory and inhibitory effect by surfactants on PTPase involved both electrostatic and hydrophobic interactions. The interaction of enzyme with surfactants probably occurred near the active site of PTPase. From it was observed that the specific activity of PTPase was strongly inhibited (approximately 90%) by 6% NaDBS when immersing the seedlings in 1-10% of NaDBS solution for 2h treatment (Fig. 2). In this case the specific activity of PTPase strongly affected by immersing the seedlings in 0-6% NaDBS solution for 2 h after that the activity of PTPase remained almost constant by increasing the concentration of NaDBS from 6-10%. These results were obtained using the whole seedling of peanut. Hence, it was not possible to observe that which part of the seedlings was most affected. The specific activity level of PTPase after 3 h treatment of peanut seedlings with 6% NaDBS was therefore measured in various parts viz roots, hypocotyls, epicotyls and cotyledons. The specific activity of PTPase highly reduced (85%) in roots, while under the same

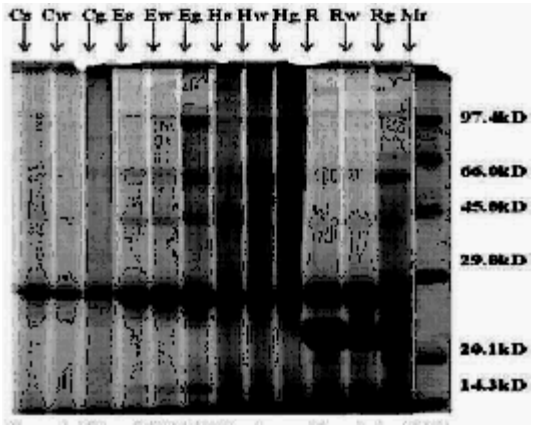

Fig. 4. A comparison of protein bands profile in different parts of seedlings immersing in 6% NaDBS. Soluble proteins were extracted and analyzed by NaDS-PAGE using 12% polyacrylamide gel. Proteins were stained with coomassie brilliant blue R-250 using standard molecular marker (14.3 - 97.4kDa). Rg = Root (general), Rw = Root (water), Rs = Root (stressed); Hg = Hypocotyl (general), Hw = Hypocotyl (water), Hs = Hypocotyl (stressed); Eg = Epicotyl (general), Ew = Epicotyl (water), Es = Epicotyl (stressed); Cg = Cotyledon (general), Cw = Cotyledon (water), Cs = Cotyledon (stressed), Mr = Marker

treatment in cotyledons, epicotyls and hypocotyls the specific activity of PTPase decreased cotyledons appeared as the best tissue of choice for study of PTPases activity (Fig. 3). SDS-PAGE profile of protein after or before immersing different part of germinating peanut seedlings in 6% NaDBS for 3h revealed a change in different protein band using 12% polyacrylamide gel (Fig. 4). A high molecular weight protein band was visible between 97.4kDa to 120kDa marker range in stressed hypocotyls and epicotyls, whereas the same protein band was found absent in unstressed hypocotyls and epicotyls seedlings. A new strong protein band was over expressed between 66.0kDa to 97.4kDa marker range in stressed cotyledon, while the same protein band was found missing in unstressed cotyledon of germinating peanut seedlings. Low molecular weight protein band was over expressed between 14.3kDa to 20.1kDa marker ranges in stressed hypocotyls, while the same band disappeared in unstressed hypocotyls germinating peanut seedlings. Since, the stress effect on protein in hypocotyls in the region of Mr 66-96kDa appeared more pronounced these tissues were used for further studies.

In addition, 2.0% anionic surfactant solution, reduced the specific activity of PTPase approximately 2.8 fold. While under the same conditions, the effect of cationic surfactants on the PTPase enzyme activity was increased approximately 1.5 fold. In contrast to cationic surfactants the effect of anionic surfactants on the enzyme activity was irreversible. The treatment of peanut seedlings with 6% NaDBS solution (anionic surfactant) for 2 h, decrease the specific activity of PTPase approximately 8.0 folds. It means that PTPase activity was completely inactivated in the presence of anionic surfactants. Inorganic phosphate, a known competitive inhibitor of PTP, protected the enzyme against inactivation by the surfactants. Our results suggest that the inactivation of the low marker PTPase by anionic and cationic surfactants involved both electrostatic and hydrophobic interactions, and that the interactions of enzyme-surfactants probably occurred at near the active site (Granjeiro *et al*., 2004). The effect of anionic and cationic surfactants on acid phosphatase denaturating has been extensively studied. Low molecular mass (LMr) protein tyrosine phosphatase (PTP), a key regulatory enzyme involved in many different processes in the cell, was distinctly affected by anionic (homologous series of *n*-alkyl sulfates (C8-C14)) and cationic (*n*-alkyl trimethyl ammonium bromides (C12-C16)) surfactants (Granjeiro *et al*., 2004).The enzyme activity increased significantly with addition of non-ionic surfactants Triton X-100 and reducing agent DTT. Keratinase activity decreases with anionic NaDS addition. The effect of anionic surfactants on the cell membranes in the activity of enzymes, with the binding to various proteins and to other cell components and on their human toxicity is presented and the possible mode of action is elucidated (Tibor *et al*., 2002).

Protein- surfactant mixtures are complex non-ideal systems due to potential interactions (Bam *et al*., 1995), concurrent surface activity of both components and dynamics of protein structure at interfaces (Miller *et al*., 2000).Various features of PTPs chemistry and regulation await rapid investigation, such as: the type and origin of ROS that inactivates PTPs during growth factor signaling (Shen *et al*., 2009), the principal mechanisms and the molecular details of the transcriptional regulation of PTPs (Kappert *et al*., 2007), the identification of PTPs physiological substrates that may bear the key cellular tasks necessary for signaling, allowing the use of PTPs as therapeutic targets (Liang *et al*., 2007).

It is interesting to find out to what extent PTP induction by RTK ligands represents a general mechanism for feedback inhibition of RTK (Kappert *et al*., 2007). Then, an urgent imperative remains optimization of PTPs inhibitory activity and selectivity (Forghieri *et al*., 2009). It is especially important when diluting PTP stock solutions to low-micromolar or nanomolar concentrations. The use of non-ionic surfactants is a double-edged sword of sorts. While they prevent loss of enzyme activity from non-specific adsorption, the presence of non-ionic surfactants can also lead to destruction of PTP catalytic activity. Triton X-100 has been reported to activate several enzymes, including alkaline pyro phosphatase (Tetlow *et al*., 1993), glucose-6-phosphatase (Behyl, 1986) and ATPase active P-glycoprotein (Doige and Sharom, 1993). Surfactants are frequently used at concentrations that greatly exceed the amount required for a maximum reduction of surface tension and with

little consideration of the fact that they are chemicals of wide diversity with the potential of exerting biochemical effects upon growth and metabolic processes of living organisms (Parr and Norman, 1965). Ahmad *et al*. (1995) have also reported the stimulatory 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 (Cserhati *et al*., 2002). Anionic surfactants such as NaDS exert toxic and harmful effects on cell membranes and can solubilize proteins causing their denaturating (Ahmad *et al*., 1995; Cserhati *et al*., 2002); they can also modify the activity of an enzyme by binding to it.

Interaction of a protein with a surfactant plays an important role in many biological processes. Many proteins possess specific binding sites for the surfactants. Protein-surfactant interaction is an important area of research, which has many practical applications including washing surfactants and stabilizing emulsions

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