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Research Paper

Partial Purification and structure prediction of salinity induced NADP-dependent Sorbitol-6-phosphate dehydrogenase from ground nut

Biotechnology

KEYWORDS : sorbitol-6-phosphate dehydrogenase, oxidoreductases, NADP, ground nut

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ABSTRACT

To study the role of sorbitol-6-phosphate dehydrogenase in sorbitol synthesis in leaves of groundnut, properties of the enzyme were investigated. The activity of the enzyme, which catalyzes an NADP-dependent oxidation of the substrate to glucose-6-phosphate, was detected. The enzyme was purified about 88 fold from leaves of ground nut (salinity induced) using gel filtration and ion exchange chromatography with DEAE-cellulose and Blue Sepharose. The enzyme showed its maximum activity within a broad pH range between 7 and 9 for glucose- 6- phosphate reduction. The structure was predicted based on target-template similarity and alignments, which showed a monomer structure. The enzyme has an aldo-keto reductase motif and a parallel 8 beta/8 alpha barrel motif and establishes a new motif for NADP binding oxidoreductases. The structure of the enzyme should allow for the rational design of inhibitors that might provide molecular understanding of the catalytic mechanism, as well as potential therapeutic agents.

Introduction:

Sorbitol is known to be a product of photosynthesis and is a translocated sugar in some plants of the Rosaceae or Fabaceae family i.e. Ground nut. At least two enzymes which metabolize sorbitol have been reported in higher plant tissue, i.e. sorbitol dehydrogenase and sorbitol-6-phosphate dehydrogenase. Sorbitol-6-phosphate dehydrogenase could participate in the synthesis of sorbitol during photosynthesis. For the fruit enzyme, the optimal pH range is narrow and at near pH 9, it is in the direction of glucose-6-phosphate reduction. In contrast, the leaf enzyme showed its maximum activity over a broad pH range from 7 to 9. [5,16-19]

S-6-P dehydrogenase + $NADP^+$ = Glucose-6-phosphate + NADPH

Effect of pH shows that the enzyme is more effective for G6P reduction than for S6P oxidation at neutral pH. Increase in S6P dehydrogenase activity coincided with sorbitol accumulation in fruit and seedlings. On the other hand, sorbitol may be oxidized to neutral sugar for carbon or energy sources in these plant tissues.

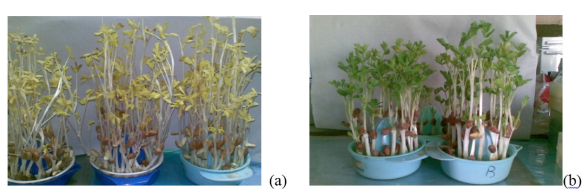
S-6-P dehydrogenase plays an important role in abiotic stress [1,2,3,4] especially in salt stress by synthesizing sorbitol.[6,7,8] In plants, accumulation of which helps in generating a desired level of osmotic balance. It also maintains the energy state of the plant which gets hampered due to stress. Since, ground nut which is a good source of protein and fatty acids and can better grow in saline soils because of its higher resistivity against saline condition, the study of salinity induced NADP-dependent Sorbitol-6-phosphate dehydrogenase in ground nut is of a significant value. According to the previous studies on sorbitol-6-phospahate dehydrogenase in ground nut, it has become clear that the gene of sorbitol-6-phosphate dehydrogenase can be controlled at transcription level, so its biochemical and molecular study in ground nut has become indispensable.[21]

Methods:

Pretreatment of seeds and growth:

Sterilization of seeds was done as per method described [20]. Seeds were surface sterilized by treatment of disinfectant 0.1% $HgCl_2$, for 5 min. with continuous stirring, washed thoroughly and imbibed for 12 hrs in sterile water. Imbibed seeds were placed on moist stack of blotting sheets for sprouting. After that, well sprouted seeds with uniformity were selected and transferred to plastic trays for growing hydroponically in Hoagland's medium under controlled temperature (28 °C) at salinity 200m M NaCl in a plant growth chamber in dark at 80% relative humidity. Some seeds were grown in light also just to see the difference in growth pattern and the expression of S6P dehydrogenase. Medium was changed after every 48 hrs to avoid nutrient depletion. Seedlings were harvested after seven days for experimental work. Seedlings were harvested also at different stages of their growth as the expression of S6P dehydrogenase has been found to be season and time dependent. Expression of S6P dehydrogenase is found to be more in younger leaves than that of mature leaves. Regarding salinity, most of the evaluations reported so far have been carried out at seedling stage, although this type of evaluation has been reported to have little correspondence, if any, with how plants will later perform under salt stress. [6,7,8]

Results:



(a) (b)

Fig.1. (a) Groundnut seedlings grown in dark condition, under controlled temp. (28°C) and 80% relative humidity. (b) Ground nut seedlings grown under light at room temp.

Extraction of total protein:

Seedlings were washed with double distilled water, cut into small pieces and powdered in liquid nitrogen using mortar pestle. The powder was homogenized in (1:1 w/v) extraction buffer [Tris-HCl buffer(50 mM, pH 8), 1mM PMSF, 5mM β-mercaptoethanol, 1mM EDTA) at 4 °C and centrifuged at 7000xg for 40min. at 4 °C. Supernatant was collected for further experiments. [19]

Enzyme Extraction:

5g of tissue was homogenized in 5 ml of extraction buffer (0.1 M Tris-HCl pH 8, 1OmM 2-β-mercaptoethanol and 0.2g polyvinylpolypyrollidone). The homogenate was centrifuged at 15000xg for 20min. , the supernatant was collected and enzyme activity of crude preparation was assayed. The concentration of protein was measured according to Bradford's method using Bovine Serum Albumin (BSA) as standard. [19]

NADP- Dependent Sorbitol-6-phosphate dehydrogenase (S6PDH) Assay:

The enzyme assay mixture contained in a total vol. of 550µl,

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45mM Tris-HCl (p H 9.3) with 0.91mM $NADP^+$, 18mM (6mM) of sorbitol-6-phosphate and 100µl of enzyme extract. Enzyme activity was assayed in a recording spectrophotometer by measuring increase in absorbance at 340nm as $NADP^+$ was reduced to NADPH. An enzyme unit of NADP-dependent sorbitol-6-phospahte dehydrogenase is defined as 1µmol $NADP^+$ reduced per min. [19]

Crude Extract:

Table 1: Conc. and Activity of S6P dehydrogenase in crude extract

Vol. (cm^3)	Conc. ($\mu gUcm^{-3}$)	Total Prot. (µg)	Activity ($\mu gUcm^{-3}$)	Sp. Activity ($U\mu g^{-1}$)
5000 (E, a)	57µg	285	2081.5	36.5
5000 (E,a)	30µg	150	1427	47.5
3000 (E,b)	13µg	39	642	49.3
4000 (P,c)	13µg	52	660	52.6
4000 (E,c)	13µg	52	684	50.7
2000 (E,d)	13µg	26	156.4	12.7
2000 (P,d)	13µg	26	250	19.2

[Leaves, grown in light (a); Leaves, grown in dark (b); Seedlings, grown in light (c); Seedlings, grown in dark (d); Enzyme (E), Total Protein (P); Total Prot. in µg (Conc. X total vol. of extract); Activity in µg U/cm; Total activity= activity X total vol. of extract; Sp. activity(Units/ µg) = Total activity/ Total vol.]

The S6P dehydrogenase expression was more in leaves in comparison to the seedlings grown in light or dark as shown in Fig. 1 and Table 1. Thus, S6P was purified from ground nut leaves.

Purification of S6P Dehydrogenase from ground nut leaves:

Crude extract (Table 1. shows activity) was applied to a column of DEAE cellulose (2.5x7cm) equilibrated with 20mM Tris-HCl buffer (pH 8.7) containing 1mM DTT (medium A). The active fraction was not retained on the resin but passed through it. The fraction was collected adjusted to pH 8.0 with 0.1 N HCl, and then loaded on a column of Blue Sepharose CL-6B (2x5cm) equilibrated with 20mM Tris-HCl buffer(pH 8.0) containing 1mM DTT (medium B). The column was washed with medium B, containing 0.15 M KCl and then the enzyme was eluted with medium B containing 0.25 M KCl. The initial fraction was discarded and the subsequent active fraction was collected and purified further by column chromatography with DEAE cellulose column. The enzyme was purified about 88 fold. Purity of the final enzyme preparation was checked electrophoretically.

Table 2: Purification of S6P dehydrogenase from groundnut leaves

Fraction	Volume (cm3)	Conc. (µgUcm-3)	Total Prot. (µg)	Activity (µgUcm-3)	Tot. activity (U)	Sp. activity (Uµg-1)	Yield (%)
Crude Extract	5000	57µg/ml	285000	2081.5	10407500	36.5	100
First DEAE	10000	15µg/ml	150000	1095	10950000	73	105.2
Blue Sepharose	7000	13µg/ml	91000	819	5733000	63	55
Sec. DEAE	4500	9µg/ml	49500	4955	2450250	49.5	23.5

The last steps decrease the Sp. Activity and also Yield of the purified enzyme (Table 2), thus can be eliminated in the purification procedure as suggested in other reports. [19] Although the enzyme preparation from groundnut leaves grown in saline conditions was not pure enough and also not crystallizable and it utilized S6P as substrate at a low rate. The SDS PAGE result showed a peptide of 33KD.

Sequence similarity and alignment studies:

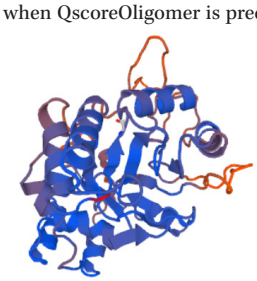
The Sorbitol-6-phosphate dehydrogenase sequence from Apple was retrieved from the NCBI databases and aligned with sequences from Arachis sp. . We found three best hits in the protein and Psi BLASTs (Both Non-redundant proteins and specific protein groups). These are sequences acetyl-CoA carboxylase carboxyltransferase beta subunit [Arachis *hypogaea*] Sequence ID: gb|ACX69849.1| , beta-carboxyltransferase 1 [Arachis *hypogaea*] Sequence ID: gb|ACO53622.1|, resveratrol synthase [Arachis *hypogaea*] Sequence ID: gb|ADJ17766.1|.

Based on sequence similarity and conserved domain searches we identified Sorbitol-6-phosphate dehydrogenase as an enzyme of Aldo-Keto- reductase superfamily. Then we align these sequences for both pair wise and global levels and also scoring scaffold methods. The multiple sequence alignment was processed through Clustal methods and HHBlits [10], which generates HMM models.

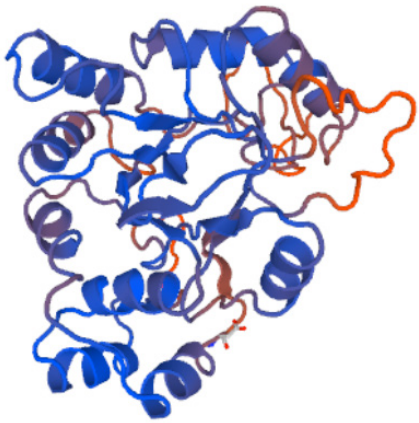
Template selection and Model building:

The Swiss Model [11] template library was searched with Blast [9] and HHBlits [10] for evolutionary related structures matching the target sequence. For, each identified template, the template's quality was predicted from features of the target-template alignment. The template (3q67.1 A, Human Aldose Reductase C298S mutant in Complex with NADP+ in Space Group P212121 with sequence identity 42.21 %) was selected for model building. The models were built based on target-template alignment with ProMod Version 3.70. The models have GMQE value 0.72 and Seq. similarity 0.41. Coordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodeled using a fragment library. Side chains are then rebuilt. Finally the geometry of the resulting models is regularized by using a force field. In case loop modeling with ProMod Version 3.70 does not give satisfactory results, an alternative model was built with MODELLER [12]. The global and per- residue model quality was assessed using the QMEAN scoring function. [13]

The model has an oligomeric state of Monomer. Homo-oligomeric structure of the target protein is predicted based on the analysis of pairwise interfaces of the identified template structures. For each relevant between polypeptide chains (interfaces with more than 10 residues-residues interactions), the QscoreOligomer [14] is predicted from features such as similarity to target and frequency of observing the interface in the identified templates [14] . The prediction is performed with a random forest regressor using these features as input parameters to predict the probability of conservation for each interface. The oligomeric state of the target is predicted to be the same as in the template when QscoreOligomer is predicted to be higher or equal to 0.5.



(a)



(B)

Fig 2. S6P dehydrogenase structure: (a) S6P dehydrogenase (b) the characteristic parallel 8alpha/8beta conformation of aldose reductase (S6P dehydrogenase)

The enzyme, which has been identified as aldose reductase, catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of a wide variety of aliphatic and aromatic carbonyl compounds is implicated in the synthesis of sorbitol during photosynthesis. The enzyme remains active over a broad pH range from 7 to 9. The structure prediction reveals that the enzyme contains a parallel 8 beta/8 alpha –barrel motif (Fig. 2b) and establishes a new motif for NADP-binding oxidoreductases.[15] The substrate binding site seems to be located in a large deep elliptical pocket at the COOH- terminal end of the beta barrel with a bound NADPH in an extended conformation. Since, the active site pocket is highly hydrophobic, it favors aromatic and apolar substrates over highly polar monosaccharides. [15] The structure of the enzyme should allow for the rational design of inhibitors that might provide molecular understanding of the catalytic mechanism, as well as potential therapeutic agents.

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