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Δ^1-Pyrroline-5-carboxylate synthetase(P5CS) - Isolation, partial purification, and its characterization.

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Abstract

Δ^1-Pyrroline-5-carboxylate synthetase, the bi functional enzyme participating in proline biosynthetic pathway was isolated and partially purified from groundnut leaves. Following ammonium sulphate fractionation, purification was performed by several chromatographic methods: Sephadex G-75 and DEAE cellulose. The enzyme was resolved by anion exchange chromatography. The γ -GK activity was detected by the hydroxamate assay.The subunit molecular mass of P5CS was estimated to be 70KDa.Proline accumulation has been correlated with tolerance to drought stress in plants. P5CS is able to catalyze the conversion of glutamate to pyrrroline-5-carboxylate, which is then reduced to proline by pyrroline-5-reductase.

Keywords: *Arachis hypogaea*, Drought stress, Proline,Δ^1-Pyrroline-5-carboxylate synthetase

INTRODUCTION

Among all the abiotic stresses, drought is a major factor that limits the agricultural crop production. Higher plants accumulate free proline in response to drought stress, a phenomenon first observed by Kemble and McPherson (1954) in rye grass. Measurement of proline accumulation is an important criteria for determination of plant tolerance to drought stress and widely advocated for use as parameter of selection for drought stress parameter (sairam etal., 2002). Proline synthesis is catalyzed by two enzymes, Pyrroline -5-Carboxylate- synthetase (P5CS) and Pyrroline -5-Carboxylate reducatase, both using NADPH as a cofactor. Genes encoding the two enzymes for proline anabolism have been identified in several plant species and reported to be unregulated in response to water deprivation and high salinity(Hare etal.,1999) . Different genes involved in osmoprotectant biosynthesis are upregulated under- salt and drought stress (Zhu.,2002) . Proline has been reported to accumulate in higher plants up to 80% of total aminoacid pool under drought and salt stress as compared to mere 5% under normal conditions in various crop species including groundnut (Kavi Kishore etal.,2003). Literature survey indicated that the function of proline in stressed plants is often explained by its property as osmolyte, able to balance water stress. In addition, other possible roles of proline has been proposed with greater of lesser convictions, which include stabilization of proteins, membranes and subcellular structures (Vanrensburz etal.,1993), buffering of cellular redox potential (Chinnuwamy etal.,2005) . Proline biosynthetic pathway starts in bacteria with the phosphorylation of glutamate, which is converted to r-glutamyl phosphate and then to glutamine-r-semi aldehyde (GSA) by the enzymes -glutamyl kinase and glutamic-r-semialdehyde dehydrogenase respectively. GSA gets converted to pyroline-5-carboxylate(PSC) by spontaneous cyclisation. However, glutamate is directly catalyzed to GSA by pyroline-5-caboxylatesynthetase (P5CS)in plants and other eukaryotes (HW etal.,1992). P5CS gene is a 2417-basepair sequence, which contains a single major open reading frame, encoding a polypeptide of 73.2 KDa.

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MATERIALS AND METHODS

Plants material- Groundnut (*Arachis hypogaea*) cultivars namely K-1375(drought tolerant) and R-9251 (and drought susceptible) were selected for this study . Certified seeds were obtained from Andhra Pradesh Agricultural Experiment Station, Anantpur,India and Agriculture Research Station, Raichur,India.

Growth conditions and drought stress treatement

Seeds of the two cultivars were surface sterilized with 0.1%(w/v) sodium hypochlorite solution for 5 minutes and thoroughly rinsed with distilled water, germinated in plastic pots in soil : sand (1:2) mixture and allowed to grow for 30 days. Potted seedlings were divided into two sets, one set of plants (control) maintained at 100% field capacity (FC) by watering daily, another set of plants (stress) subjected to drought stress, by withholding water and stress was continued till wilting symptoms were observed.

Biochemical analysis
Determination of proline content

Proline content was determined in shoots of control and stressed cultivars(drought tolerant and susceptible varieties) following by the method of Bates et al(1973). Samples (500mg) were homogenized in 5 ml of aqueous sulfosalicylic acid (3%) using mortar and pestle. Two ml aliquot of supernatant was mixed with equal volume of glacial acetic acid ninhydrin. The reaction mixture was boiled in waterbath at 100`C for 30 min.The reaction was terminated in an ice bath following by the addition of 4ml toluene and then transferring to a separate funnel. After thorough mixing, the

chromophore containing toluene was separated and absorbance was recorded at 520nm in spectrophotometer against toluene blank. Concentration of proline was estimated by referring to a standard curve of proline.

Extraction and assay of P5CS enzyme

The control and treated plant leaves were crushed in potassium buffer (150mm,pH 7.0) and centrifuged at 10,000rpm for 10min.The supernatant was used for the assay of P5CS The P5CS activity was assayed by hydroxamate to detect the γ -GKL (Glutamyl Kinase) activity as described by the Hayzer and Leisinger (1980). The reaction mixture contained the following in a final volume of 0. 1ml at pH 7.0, 50mm -glutamate-HCL, 50 mm Tris and Enzyme. The reaction was started by the addition of the enzyme. After 5 min at 37° C, the reaction was terminated by addition of 0.2 ml of stop buffer(2.5g of FeCl and 6.0g of Trichloroaceticacid in a final volume of 100ml of 2.5N HCl. Precipitated proteins were resolved by centrifugation and the absorbance at 534 nm was recorded against the blank, identical to the above but lacking ATP.The amount of -glutamyl hydroxamate was determined by the A534 by comparison with a standard curve of glutamyl hydroxamate. One unit of - γ GK activity was defined as the amount of the enzyme required to produce 1µM of γ-glutamyl hydroxamate in 1 min. This assay was used during all steps of purification.

Partial purification of P5CS
Ammonium sulfate precipitation

The 500 ml crude enzyme extract was saturated to30% (w/v) with ammonium sulfate. The saturation was carried by adding ammonium sulpate slowly into the crude extract, with constant stirring. The stirring was continued for 2 more hours The precipated protein was separated by centrifuging at 10,000g for 10min. The protein pellet was dissolved in minimal volume of extraction buffer and dialyzed against dialysis buffer (5mM) potassium buffer (pH 7.0) for 8 hrs with three changes in the buffer.

Column purification

For the purification of P5CS, two column matrices were used. The first approach was carried with gel filtration column followed by Ion-Exchange chromatography

Gel filtration chromatography

The sephadex G-75 column material was used for purification. The sephadex G-75 column material was activated by heating the matrix at 50°C for 30 min with 50 MM potassium phosphate buffer, followed by overnight incubation at 4°C. The pooled active fractions from column were saturated with 80% ammonium sulphate, to precipitate the proteins. The precipitate was separated by centrifuging at 12,000g for 20 min at 4°C and dissolved in 3ml of extraction buffer. This enzyme was loaded onto sephadex G-75 column followed by elution with 3 bed volumes of extraction buffer with simultaneous fraction collection.

Ion exchange chromatography

The prepared ion-exchange was packed into the column without forming any air bubble .The column was allowed to settle and washed with 4 bed volume of 50mm potassium phosphate buffer (pH 7.0). The dialyzed enzyme was loaded onto a DEAE- cellulose column pre-equilibrated with extraction buffer. The column was run with 3 bed volumes of the same buffer and the bound enzyme was eluted with linear gradient of 50-500 mM potassium phosphate buffer .The 50 fractions of 1ml were collected. The fractions were analyzed for the eluted protein by reading the absorbance of 280nm. The fractions were screened for P5CS activity and the active fractions were pooled. The protein concentration of the pooled fractions was determined by Bradford (1976) method. The specific activity of the enzyme eluted at every step of purification was measured and the total fold was further analyzed for determination of its molecular mass by polyacrylamide gel electrophoresis.

Biochemical characteristics of P5CS
Determination of optimum pH

The pH for the P5CS was optimized by assaying at different pH using different buffer systems. 50mm acetate buffer was used to determine the activity between pH 3.0-5.8, 50mm potassium phosphate buffer was used for pH 6.0-7.8 and 50mm Tris –cl buffer was used for 8.0-9.0 and 50mm glycine-NaOH buffer used for pH 10-11.

Determination of optimum temperature

The reaction mixture was incubated at different temperatures, from 37-95°C and read for activity in 50m potassium phosphate buffer.

Molecular mass determination

The subunit molecular mass of the P5CS was determined by SDS-Polyacrylamide gel electrophoresis.

Analytical method

Protein concentration was determined by the method of Bradford (1976) with BSA as standard.

RESULTS
Proline content

Amongst the two groundnut cultivars ,comparably high proline content was observed in K-1375 than the other one cultivar,R-9251. Drought stress induced higher amount of proline contents in all cultivars, irrespective of their drought tolerance,as shown in table1.1

Purification of pyrroline-5-carboxylate synthetase

The enzyme was purified as summarized in Table 1.Gel fitration chromatograpy using Sephadex G-75 resulted in 68.11% fold purification of enzyme over crude extract,followed by 271.09%fold purity,but this step also lost a significant amount of enzyme.The bound enzyme was eluted from the column using linear gradient of 50-500Mm potassium phosphate buffer. The unusually high ammonium sulphate concentration (80% saturation) necessary to precipitate the enzyme and used of gel filtration along

with ion exchange chromatography led to a three step purification procedure., that resulted in a homogenous enzyme purification,as judged by SDS-PAGE.The optimum temperature and pH of P5CS was found to be 40°C and 6.7

Table 1.1 Effect of drought stress on free proline accumulation

Sl.No	Variety	Treatment	Proline content µg $^{-1}$g fresh weight
1	k-1375	Control	34.96 ^{a} ±4.11
2	k-1375	Test	108.01 ^{d} ±6.70
3	R-9251	Control	28.04^{b}±3.22
4	R-9251	Test	65.56^{c}±5.23

All the above mentioned results are mean of replicates standard error (S.E).Mean within columns followed by letters in superscripts were significantly different from each other according to Turkey's one way Anova test.

Table 1.2 Purification of P5CS from groundnut leaves

Purification step	Protein Concentration (mg/ml)	Activity (µmoles/ml/min)	Specific Activity (U/g)	Yield (%)	Fold purity (%)
Crude	1.62	0.081	50.3	100	1
Dialysis	4.64	0.028	5.95	286.4	11.83
Gelfiltration	1.08	0.037	34.26	66.7	68.11
Ion-exchange	0.11	0.015	136.36	6.8	271.09

Table 1.3 Effect of temperature on P5CS activity

Temperature (^{O}C)	Activity(µmoles/ml/min)
0	0
37	0.492
45	0.160
55	0.03
65	0
75	0
85	0

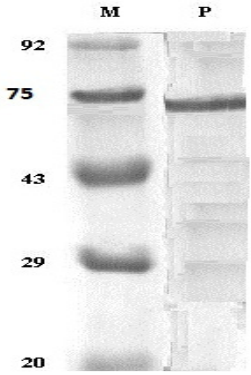

Fig 1.SDS-PAGE showing the purification of groundnut P5CS.
Lane-1 (M)Protein markers in KDa
Lane2(P)Active fractions(2µgms)from Ion exchange Chromatography

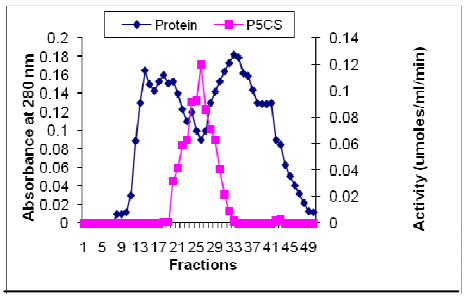

Fig 2. Gel Filtration chromatography data-The protein fractions from Sephadex G-75 column were drawn and assayed for P5CS activity.

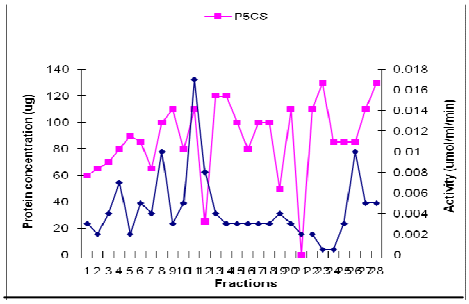

Fig 3. Ion exchange chromatography data-The fraction from Sepahadex G-75 column was applied to DEAE-Sephadex column,and the proteins were eluted by linear gradient of 50-500 mM potassium phosphate buffer

DISCUSSION

We described a purification procedure for bifunctional enzyme,P5CS,catalyzing the first step of proline biosynthesis in plants.The γ -GK activity of the purified P5CS can be detected by the hydroxamate assay.The molecular mass of P5CS subunit is likely to be 70KDa. The native molecular mass of P5C is about 450KDa(Chun-Sheng zhang. *et al.,*). Proline is one of the most common compatible osmolytes in water-stressed plants. The accumulation of Proline in dehydrated plants is caused both by activation of the biosynthesis of Proline and by inactivation of the degradation of Proline. In plants, L-Proline is synthesized from L-glutamic acid (L-G1U) via Δ^1-pyrroline-S-carboxylate (P5C) by two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR).

Proline is an important osmoprotectant in plants and useful for osmotic balancing under a range of abiotic stresses, including drought stress. Plants respond to a variety of stresses by accumulating certain specific metabolites, the most conspicuous aminoacid in general, and proline in particular(Asinall and Paleg,1981).Proline continues to be the most studied molecule under abiotic stresses in plants positive correlation between magnitude of free proline accumulation and drought tolerance has been suggested as an index for determining drought tolerance potentials between cultivars(Sivaramkrishnan *et al*.,1998: Ramanjulu and Sudhakar2000,:Thimmanaik,2002).Similarly in this study a greater pool of free proline was observed in the drought stressed leaves of groundnut genotypes than control. But the accumalation of free proline was more in K-175(drought resistant variety),than in R-9251(drought sensitive variety),under drought stress, and established the drought tolerance nature of K-1375.

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