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Leghaemoglobin sub-fractional components in chickpea root nodules during extended darkness

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Abstract: The aim of study was to investigate qualitative behavior of leghaemoglobin sub-fractional components during dark induced nodular senescence. A conventional protein purification method using ion exchange chromatography (HPLC) readily resolved ferric Lb into eight sub-fractional components namely a_1, a_2; b; c_1, c_2 and d_1, d_2 d_3 in the unstressed chickpea nodules. Lb complexes behave differently during growth phases of the nodules. Lb *'a'* complex is directly related to the growth and developmental of nodules wherein proportion of Lb a_2 content increases with age of nodule accompanying concurrent decrease Lb a_1. Early appearance of senescence related isoprotein Lb a_2 at vegetative phase of chickpea cultivar correlates its stress-susceptible nature. Further, the turnover rates of Lb a_1 to a_2 and Lb b were insensitive to reduced supply of photosynthesis during dark stress and even re-illumination. The relative proportion of c_2 to c_1 inversion increases during darkness. Further, Lb *'d'* complex is affected the most during prolonged darkness. Thus, ratio between individual sub-fractional components of Lbs' can be correlated with the development phase, longevity and supply of carbohydrates to nodules.

Keywords: Components, Dark Stress, Ion-Exchange, Sucrose

1. Introduction

Leghaemoglobin, a haemoprotein in the N_2-fixing legume root nodules facilitate diffusion of oxygen to endosymbiotic bacteroids like *Rhizobium* and *Bradyrhizobium*. According to O'Brian *et al*. (1987) haemoprotein is a product of both plant (apoprotein) and the bacterium (haeme). Newer findings however, indicate that the haeme moiety is also produced by plant (Santana *et al*. 1998). Several components of Lbs can be isolated chromatographically based upon amino acid sequencing, oxygen affinities and spectroscopic properties (Becana and Sprent 1989, Dakora *et al*. 1991, Appleby 1992, Singh 1994, Mendonca *et al*. 1999, Shleev *et al*. 2001). The presence of four major species Lb *a,* c_1, c_2 and c_3 as translational products of separate genes and four minor species as Lb *b,* d_1, d_2 and d_3 as post-translational acetylation products of the major species were reported in soybean (Fuchsman 1992).

The relative rate of biosynthesis of Lb fractionated components change during nodule development (Verma *et al*. 1979, Sżybiak-Stróżycka *et al*. 1987, Rao 1991). Such changes are a mechanism to retain maximal oxygenation (Fuchsman and Appleby 1979a). It is yet to be determined if these distinct forms have specific functions during root nodule development (Wittenberg *et al*. 1972, Uheda and Syono 1982a,b). The stress induced decline in biological nitrogen fixation is a control exerted through leghaemoglobin/oxygen availability affecting nitrogenase function (Marino *et al*. 2013). Hence, nodular senescence, if delayed by maintaining the optimum Lb content, could be of considerable importance in improving the capacity of nitrogen fixation. Prolonged periods of darkness diminish the supply of photosynthesis to nodules which lessens nitrogen fixing efficacy within 24 h. It establishes a good co-relation between N_2-fixation and deprived assimilates. An attempt was made to study the effect of exogenously sprayed sucrose in concert with the dark treatments to understand qualitative behavior of Lb sub-fractional components and the dark stressed induced nodular senescence.

2. Material and Methods

Chickpea (*Cicer arietinum* L. var. C-235) plants growing (50 DAS) under natural daylight conditions (C) were

exposed to extended periods of darkness (T_1) of 24, 48, 72 and 90 h in dark room at room temperature (25±3 °C). Half of the potted plants during dark treatment were sprayed exogenously 1% sucrose 5 times a day (T_2). After assigned periods of darkness, half of the pots (with and without sucrose) were shifted to natural day light for re-illumination studies.

The samples were prepared, purified and isolated following the method of Sarath *et al*. (1986) with some modifications. Freshly harvested root nodules (0.3 g) macerated in 3 ml of cold 10 mM Tris-HCl buffer (pH 9.2) in the presence of $K_3Fe(CN)_6$ crystals to ensure oxidation of Lb Fe(II) to Lb Fe(III) were followed by centrifugation at 20,000g (20 min). All purification steps were performed at 4°C.

Sephadex G-25 column equilibrated with chilled 10 mM Tris-HCl buffer (pH 9.2) was used for filtration and removal of the oxidants and endogenous nicotinic acid strongly bound to Lbs. Samples were passed through 0.45 µm membrane filters (Millipore) before injecting. Ion exchange chromatography at room temperature with DEAE-5PW Protein-PAK column (7.5 mm x 7.5 cm stainless steel) using Waters (Millipore) HPLC system, M-510 pumps, U6K injector, M-481 UV detector attached to 545 (Millipore) data integrator.

The column was equilibrated with 20 mM Tris-HCl buffer pH 8.0 (Buffer A) and eluted with a gradient of increasing NaCl concentrations generated by Waters automated gradient controller. The limit buffer was 20 mM Tris-HCl buffer containing 0.6 M NaCl at pH 8.0 (Buffer B). The gradient program was 0-12 min 0-5% B (convex); 12-25 min 5% B (isocratic); 25-26 min 5-20% B (linear); 26-30 min 20% B (isocratic); 30-31 min 20-0% B (linear). A constant flow rate of 1.0 ml min^{-1} was maintained during all the separations. No guard column was used by ensuring careful centrifugation and microfiltration. Back pressure was between 300-500 psi.

3. Results and Discussion

The multiple components of chickpea ferric Lb were readily resolved into eight types of sub-fractions. Six major and two minor peaks appeared in the unstressed nodules (Control). P_1 and P_2 peaks were marked as components representing Lb '*a*' complex, P_3 as Lb '*b*', P_4 and P_5 as Lb '*c*' and P_6, P_7, P_8 as Lb '*d*' complex on the basis of earlier reports. Both peaks of Lb '*a*' complex appeared in almost equal proportions (Fig. 1).

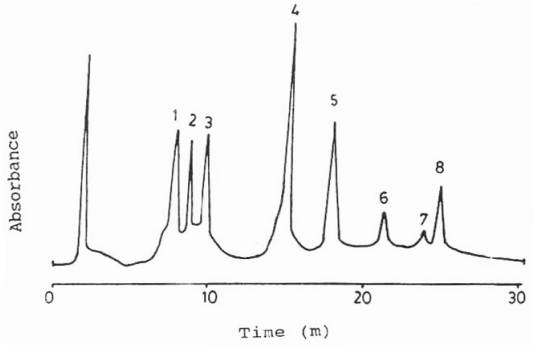

Fig. 1. *HPLC profile of Lb subcomponents in chickpea nodules at 50 DAS (Control).*

Extended darkness (T_1) of 24-90 h had no effect on Lb a_1, c_1, c_2 and d_1 components. Other components like Lb a_2, and b were marked by their reduced peak area and height. Further, Lb d_2 and d_3 components could not be eluted in any of the dark treatment (24-90 h). Thus, a differential sensitivity of Lb *'d'* complex to extended darkness is an important observation (Fig. 2).

Sucrose application in dark (T_2) showed that components a_1, c_1 and d_1 were not much affected but, the decline in relative content of Lb a_2, b, c_2, d_2 and d_3 was more significant (Fig. 3). The exogenous treatment was able to prevent the possible degradation of Lb d_2 and d_3 sub-components (24 h).

Re-illumination (3d) of dark stressed T_1 plants was unable to restore the reduced levels of Lb components. In none of the analysis Lb *'d'* complex could be detected (Fig. 4). On the other hand, re-illumination was able to restore the declined levels of all eight types of Lb sub-fractions in the sucrose provided plants (Fig. 5). The relative proportion of Lb c_2 to c_1 also increased in both T_1 and T_2 set of plant nodules.

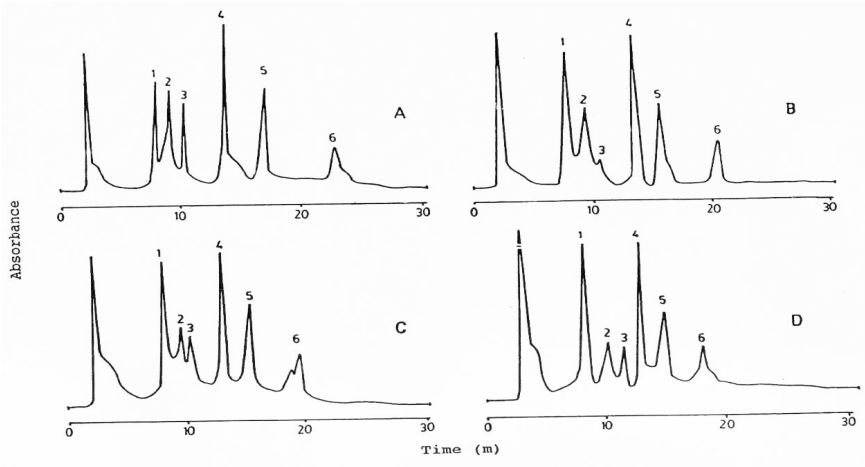

Fig. 2. *HPLC profile of Lb subcomponents in dark stressed (24, 48, 72, 90 h) chickpea nodules (T_1).*

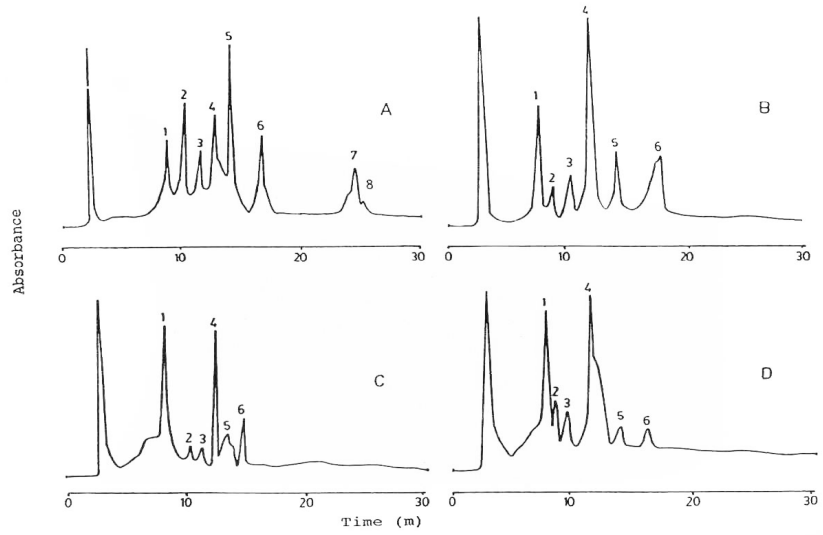

Fig. 3. *HPLC profile of Lb subcomponents in sucrose treated dark stressed (24, 48, 72, 90 h) chickpea nodules (T_2).*

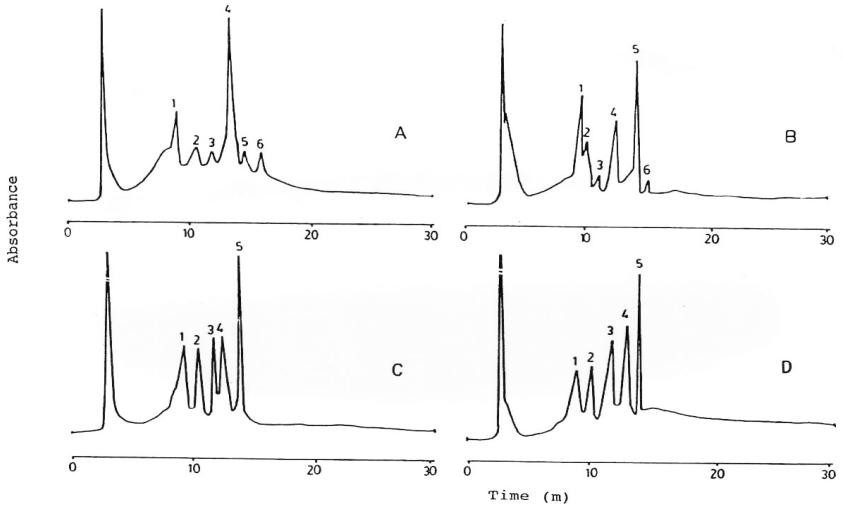

Fig. 4. *HPLC profile of Lb subcomponents in dark (24, 48, 72, 90 h) stressed chickpea nodules (T_1) after re-illumination.*

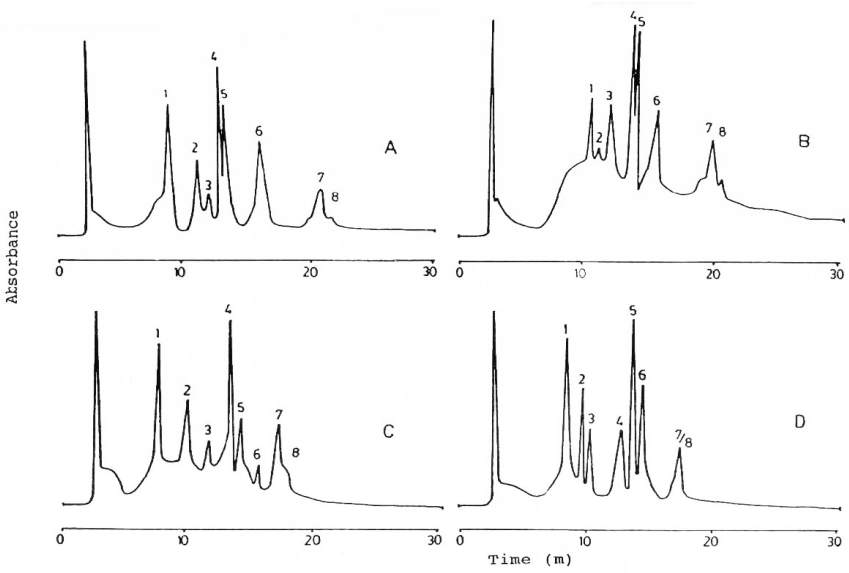

Fig. 5. *HPLC profile of Lb subcomponents in sucrose treated dark stressed (24, 48, 72, 90 h) chickpea nodules (T_2) after re-illumination.*

Dark induced stress did not appear to have any adverse effect on Lb *'a'* and *'b'* complexes. However, the relative proportion of Lb c_2 to c_1 increased during darkness possibly due to inversion. Further, Lb *'d'* complex is affected the most, perhaps due to depleted supply of carbohydrates in chickpea nodules.

Leghaemoglobins in the effective nodules are considered to be an index to nitrogen fixing efficiency (Roponen 1970, Swaraj and Garg 1977, Bisseling *et al*. 1978) and their total content is known to decrease under different stress conditions (Sprent 1976, Becana *et al*. 1986, Muneer *et al*. 2012). The presence of both Lb *'a'* complex components in equal proportions at vegetative phase is indicative of active phase of nodular metabolism. Lb *'a'* complex is directly related to the growth and developmental of nodules wherein Lb a_2 content increased with age of nodule and Lb a_1 decreased concurrently. This variation in Lb *'a'* complex can be considered as an index to nodule maturation (Singh 1994). Earlier, Sarath *et al*. (1986) have also suggested such a behavior of the isoprotein. Interestingly, occurrence of senescence related isoprotein Lb a_2 at vegetative phase of chickpea *cv.* C-235confirms its stress-susceptible nature. Further, the turnover rates of Lb a_1 to a_2 remain unaffected during prolonged periods of darkness. The relative rate of biosynthesis of Lb fractionated components change during nodule development (Verma *et al*. 1979, Szybiak-Stróżycka *et al*. 1987, Rao 1991). Age dependent changes in the relative concentrations of Lb I and Lb V were shown to be common in *Pisum sativum* and such variations were independent of breeding lines and cultivars (Ulrich *et al*. 1997).

Lb *'b'* protein was insensitive towards reduced supply of photosynthesis during extended darkness or re-illumination. The relative proportion of c_2 to c_1 increased during darkness possibly due to inversion. Whether a new heme-protein (Lb c_3) is synthesized or the same c_2 component got eluted along with c_1 due to changed surface charge specificity, cannot be said at this stage. Further, Lb *'d'* complex is affected the most during prolonged darkness, probably because of diminished carbohydrate supply. The recovery of all the three sub-fractions of Lb *'d'* in sucrose applied plants upon re-illumination confirms its dependence upon photosynthate supply or C/N ratio in the nodules. Earlier, Fuchsman and Appleby (1979a) have postulated the ratio of Lb *'b'* to Lb *'a'* and Lb *'d'* to Lb *'c'* has a variable pattern during development of the nodule. The occurrence of inversion in the relative abundance of Lb I and II under the influence of nitrate stress was reported by Becana and Sprent (1989). Such changes are a mechanism to retain maximal oxygenation (Fuchsman and Appleby 1979b). Changes in oxygen binding affinities of Lb I, II and IV have been reported in Glycine during nodule development (Wittenberg *et al*. 1972, Uheda and Syono 1982) however, Saari *et al*. (1988) have questioned the physiological significance of these structural changes in soybean nodules. Thus, Lb heterogeneity and their physiological functioning offer an insight into the relationship between individual sub-fractional components with the development phase, longevity and supply of carbohydrates to nodules.

4. Conclusions

Lb readily resolved into eight sub-fractional components namely a_1, a_2; b; c_1, c_2 and d_1, d_2 d_3 using ion exchange chromatography (HPLC). Lb complexes a, b, c and d behave differently during growth phases of the nodules. Lb *'a'* complex is directly related to the growth and developmental of nodules wherein proportion of Lb a_2 content increases with age of nodule. The turnover rate of Lb a_1 to a_2 and Lb b were insensitive to reduced supply of photosynthesis during dark stress and even re-illumination. The relative proportion of c_2 to c_1 inversion increases during darkness. Lb *'d'* complex is affected the most during prolonged darkness. Thus, ratio between individual sub-fractional components of Lbs' can be correlated physiologically with the development phase, longevity and supply of carbohydrates to nodules.

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