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

Kamal Jit Singh

Department of Botany, Panjab University, Chandigarh 160 014, India

Email address: kamal@pu.ac.in

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 $c_1$ 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, Szybiak-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 (T₁) 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 (T₂). 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 K₃Fe(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). P₁ and P₂ peaks were marked as components representing Lb ‘a’ complex, P₃ as Lb ‘b’, P₄ and P₅ as Lb ‘c’ and P₆, P₇, P₈ as Lb ‘d’ complex on the basis of earlier reports. Both peaks of Lb ‘a’ complex appeared in almost equal proportions (Fig. 1).

Extended darkness (T₁) of 24-90 h had no effect on Lb a₁, c₁, c₂ and d₁ components. Other components like Lb a₂, and b were marked by their reduced peak area and height. Further, Lb d₂ and d₃ 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 (T₂) showed that components a₁, c₁ and d₁ were not much affected but, the decline in relative content of Lb a₂, b, c₂, d₂ and d₃ was more significant (Fig. 3). The exogenous treatment was able to prevent the possible degradation of Lb d₂ and d₃ sub-components (24 h).

Re-illumination (3d) of dark stressed T₁ 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 c₂ to c₁ also increased in both T₁ and T₂ set of plant nodules.

Fig. 1. HPLC profile of Lb subcomponents in chickpea nodules at 50 DAS (Control).
Fig. 3. HPLC profile of Lb subcomponents in sucrose treated dark stressed (24, 48, 72, 90 h) chickpea nodules (T₂).

Fig. 4. HPLC profile of Lb subcomponents in dark (24, 48, 72, 90 h) stressed chickpea nodules (T₁) after re-illumination.

Fig. 5. HPLC profile of Lb subcomponents in sucrose treated dark stressed (24, 48, 72, 90 h) chickpea nodules (T₂) 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 c2 to c1 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 a1 content increased with age of nodule and Lb a2 decreased concurrently. This variation in Lb ‘a’ complex can be considered as an index to nodule maturation (Singh 1994). Earlier, Sarah et al. (1986) have also suggested such a behavior of the isoprotein. Interestingly, occurrence of senescence related isoprotein Lb a2 at vegetative phase of chickpea cv. C-235 confirms its stress-susceptible nature. Further, the turnover rates of Lb a1 to a2 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-Strzofycka 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 c2 to c1 increased during darkness possibly due to inversion. Whether a new heme-protein (Lb c1) is synthesized or the same c2 component got eluted along with c1 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 photosynthesize supply or C/N ratio in the nodules. Earlier, Fuchsan 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 (Fuchsan 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, Ulheada 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 a1, a2; b; c1, c2 and d1, d2, d3 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 a2 content increases with age of nodule. The turnover rate of Lb a1 to a2 and Lb b were insensitive to reduced supply of photosynthesis during dark stress and even re-illumination. The relative proportion of c2 to c1 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.

References


