

RESEARCH ARTICLE

Separation and Properties of Rubisco and its Inhibitor Ca-1P from *Solanum xanthocarpum* Leaves

Ashwini A. Pawar and Anita S. Goswami-Giri*

Chemistry Research Laboratory, Department of Chemistry, B. N. Bandodkar College of Science, Thane- 400601 (Maharashtra), India.

*Corresponding Author E-mail: anitagoswami@yahoo.com, ashwini_pawar22@yahoo.com

ABSTRACT:

Separation of Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) with its naturally tightly bound inhibitor 2-Carboxyarabinitol-1-phosphate (Ca-1P) from leaves of *Solanum xanthocarpum* was carried out using polyethylene glycol precipitation method. The inhibitor was purified by ion exchange chromatography. The potential of Rubisco activity was determined by the relative rate of biosynthesis and degradation. Rubisco had a Mass of 55 KDa with two subunits, LSU (55 KD) and SSU (14 KD) showing single peak on HPLC. Properties of isolated CA1-P were analyzed by TLC, HPLC, UV, IR and LC-MS. Rubisco efficiency may potentially lead to a faster plant growth, quicker sequestration of CO₂ from the air and more efficient of plant removal of green house gases from the atmosphere.

KEYWORDS: Photosynthesis, Rubisco, Ca-1P, *Solanum xanthocarpum* L., enzyme regulation, enzyme inhibitor.

INTRODUCTION:

Rubisco (Ribulose 1, 5-bisphosphate carboxylase/oxygenase) [EC 4.1.1.39], is the key enzyme in photosynthesis, which incorporates CO₂ and O₂ into substrate RuBP (Ribulose-1, 5-bisphosphate) to initiate photosynthesis and photorespiration processes. Compared to other enzymes of the Calvin cycle, Rubisco has a low turnover number meaning that relatively large amount of it must be present to sustain sufficient rates of photosynthesis¹. Rubisco is a major store of nitrogen in actively photosynthesizing mature leaves; the regulation of the breakdown it enzyme has been subject to considerable study². 2-Carboxyarabinitol-1-phosphate (Ca-1P) is a naturally occurring analogue of the transition-state of the carboxylase reaction that binds tightly to the active site of carbamylated Rubisco and thus inhibits catalytic activity³. Ca-1P accumulates in varying concentrations in many species under low light and dark conditions with the highest amounts. Interaction of Ca1P with the amino acids in the active sites of the Rubisco using Hyperchem7.5 and GOLD software has also been reported recently with the aid /availability of Bioinformatics tool⁴ still the biochemical pathway of Ca-1P metabolism remain largely unknown.

According to literature data, Rubisco bound Ca-1P has been purified from wheat and *Rhamnus alaternus*², *Spinacia oleracea*⁵, *Nicotiana tabacum*⁶, Red Kidney Bean L.⁷, *Phaseolus vulgaris* L.⁸. The Calvin cycle enzyme has also been characterized from the thermophilic and obligatory purple sulphur bacterium, *Chromatium tepidum* in active form at room temperature by indicating no evidence for second form of enzyme; lacking small subunit⁹. *Porphyra yezoensis* thylakoid membrane has PSII with high photo reduction activity having 102 KDa extrinsic proteins which may be present in the active site of PS II particles of sporophyte¹⁰. Therefore, Rubisco attracts much attention of genetic engineers and plant phylogeneticists. The Rubisco has also been studied extensively by biochemist by exploring its secondary and tertiary structures and mechanism but modification of it have not yet availed. Wild plants have still not been considered by many researchers yet even though it being a rich source of remedial products. Present paper thus emphasis on *Solanum xanthocarpum* L.; an Indian night shade, wasteland weed found throughout India. The plant is known to have multiple medicinal properties¹¹⁻¹³. The extract of various parts of it have been used against agricultural pests as repellent¹⁴, contact poison¹⁵ and as molluscicide¹⁶ in public health. The plant extract is also used in the treatment of diseases like fever, asthma, tuberculosis, kidney disorders, cough, constipation, tooth-ache, sore-throat, rheumatism and gonorrhoea¹¹. Phytoactive compounds extracted from this plant include solanocarpine, solanocarpidine, disgenin, carpestrol, sitosterol and steroids¹⁷. It has ability to tolerate poor

compacted soil and drought which indicates presence of Rubisco. Most species contains Ca-IP which may accumulate in dark leaves, to concentration approaching that of Rubisco active site¹⁸. Because of its high affinity towards the carbamylated active site of Rubisco, responsible for the complete inhibition of Rubisco in leaves. Reversible inhibition of carbamylated Rubisco by Ca-IP shows light dependent and dephosphorylation of Ca-IP. Because the world's food supply depends almost entirely on carbon fixation by Rubisco in a small number of crop plants, even slight improvements in CO₂ affinity could lead to substantial increases in photosynthetic ability and ultimately crop productivity^{1,19}. With this view, separation and properties of Rubisco and its inhibitor Ca-IP from *Solanum xanthocarpum* L were evaluated for its possible application to improve crop quality, its productivity and quicker utilization of CO₂ (greenhouse gasses) from atmosphere for pollution free environment²⁰.

MATERIALS AND METHODS:

Tris buffer, NaHCO₃, MgCl₂, Mercaptoethanol, EDTA, Ascorbic Acid, Polyethylene glycol (P-4000), HClO₄, Potassium hydroxide, Coomassie brilliant blue, Acrylamide, N,N'-bismethylene-acrylamide, Ammonium persulphate, Glycine, Bromophenol Blue, Methanol, N,N,N,N'-Tetramethylethylenediamine (TEMED), Sodium dodecyl sulphate (SDS), Glycerol were procured from SD Fine Chemicals, Mumbai and ATP, NADPH, DTT, Ribulose 1,5-bisphosphate (RuBP), GPDH, PGK from Sigma Aldrich. All other chemicals were of analytical reagent grade.

Source:

The *Solanum xanthocarpum* leaves were collected from Ambarnath, District-Thane, Maharashtra state, India in April 2010.

Extraction of Rubisco with inhibitor:

The method of Berry et. al.⁵ was used to isolate Rubisco with inhibitor with slight modification and compared with the Rubisco was purified according to the procedure of Edmondson et al.²¹. *Solanum xanthocarpum* leaves (25gm) kept in darkness overnight was frozen in liquid nitrogen and pulverized in a mortar and pestle. The powder was mixed with 25 ml of 50mM Tris, pH8.0/20 mM NaHCO₃/60 mM MgCl₂/10mM Mercaptoethanol/5 mM ascorbic acid/1 mM EDTA and then homogenized using homogenizer; the homogenate was filtered and centrifuged. Rubisco was precipitated by adding 60% (wt/vol) polyethylene glycol to yield a final concentration of 18%. This solution was kept at 0°C for 1 hr with stirring and centrifuged at 12,000 rpm for 30 min. The precipitated Rubisco with bound inhibitor was dissolved in minimum quantity of water. HClO₄ (0.45 M) was added to precipitate Rubisco and to release the bound inhibitor (Ca-IP). The suspension was centrifuged at 15,000 rpm for 10 min, and ClO₄⁻ ions were removed from the supernatant by adding KOH to pH 7 followed by centrifugation at 5000 rpm for 10 min. The supernatant was passed through a column of Amberlite IR-120 (Cation

exchange resin) and the column was washed with 2-column volumes of H₂O. The effluent was then applied to a column Amberlite IR-400 (Anion exchange resin) of that bound the inhibitor and later was eluted with a linear gradient of Formic acid (0-8 M). These fractions were assayed, combined and dried in a rotary evaporator under vacuum and used for the further study.

The Ca-IP content was determined using the method²².

Determination of Protein:

Protein Concentration was determined by the Lowry method²³. TLC was performed on 20cm x 20cm x 250µm cellulose plates with the solvent mixture of ethyl acetate/chloroform/methanol (6:3:1 by v/v).

Polyacrylamide gel electrophoresis:

Denaturing SDS-PAGE was performed following the Laemmli method²⁴ on 12.5% gels, using low molecular weight protein markers. Samples were denatured with a sample buffer containing 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue and 2% SDS in 0.125M Tris, pH 6.8 for 2-5 min at 100°C. The SDS gel (8cm x 10cm, and 0.8mm thick) had a 4.5% stacking gel and 12.5% separating gel. The sample was subjected to electrophoresis for 2 h at 220V. After electrophoresis, proteins were stained with 0.05% coomassie brilliant blue R-250.

Thin Layer Chromatography:

TLC was performed on 20cm x 20cm cellulose plates with the solvent n-propanol: ammonia: water (6 :3:1, by vol.).

HPLC:

Rubisco were separated by anion exchange chromatography and eluted with gradient sodium acetate at 1ml min⁻¹ and the eluate was monitored for A₁₉₀ nm.

MS:

The Ca-IP plant sample was collected after HPLC and the mobile phase was removed on anion exchanger chromatography. Electron impact mass spectra of Ca-IP plant sample were obtained with liquid chromatograph mass spectrometer used in a 70eV.

RESULTS AND DISCUSSION:

Solanum xanthocarpum leaves Rubisco activity is light independent²⁵. Specifically understanding; the relative importance of carbamylation and inhibition in regulating Rubisco activity by binding metabolites and possible role of Ca-IP, in binding of these metabolites to Rubisco needs to be addressed. Hence, it leads to understand multiple mechanisms that regulate Rubisco activity. It is very difficult to activate the Rubisco and interpretation in relation to it. The Ca-IP phase was not visible in the wild type plants during activation from darkness, which is not surprising in view of the speed of process even though Ca-IP was present in plants. Rubisco content in wild type plants is similar to those in the antisense plants when darken for the same period. The reason may be, due to

activation of Ca-IP or inhibition of Rubisco before complete CO₂ assimilation becomes limited.

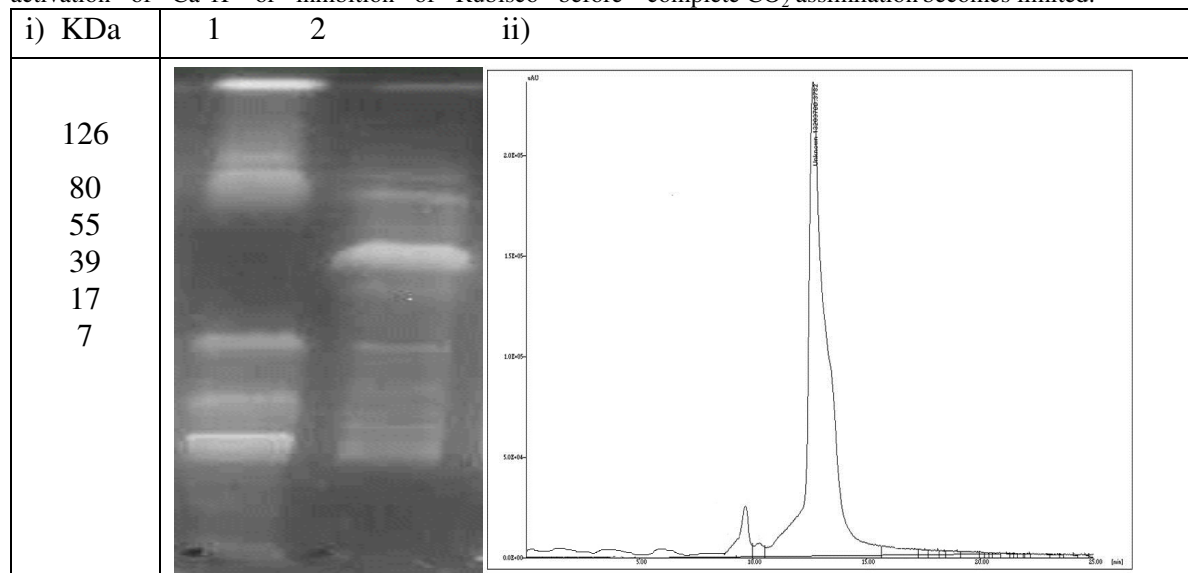


Fig 1: (i) SDS-PAGE of Rubisco : polypeptides present at various stages of the purification separated on the basis of its mobility in SDS-PAGE. Lane 1 Mw marker lane 2 *Solanum xanthocarpum* L. Rubisco sample. (ii) HPLC of Rubisco: Elution profile of Rubisco *Solanum xanthocarpum* L after passing through anion exchange.

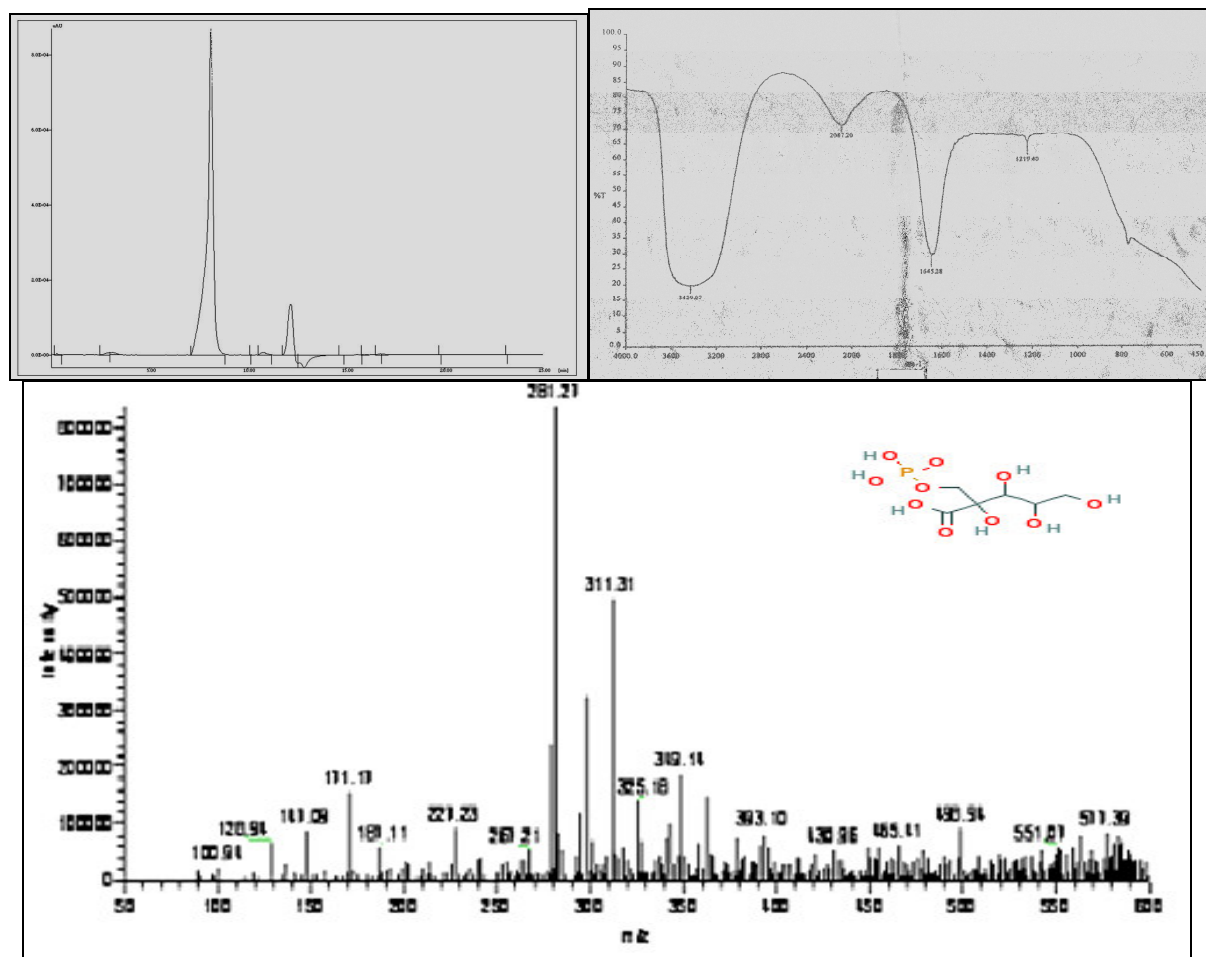


Figure 2 (i) HPLC chromatogram of Ca-IP after passing through anion exchanger, (ii) IR (iii) LC-MS of Ca-IP purified from *Solanum Xanthocarpum* L.

To optimize the Rubisco efficiency, NaHCO_3 , MgCl_2 , SDS, EDTA, Mercaptoethanol, polyethylene glycol were used in extraction and it was purified on cation and anion exchanger chromatography. During purification, low specific activity of it remains unchanged⁶. But when high concentration of sulphate was used for the precipitation of enzyme, loss of phosphate ester and increasing specific activity of Rubisco was observed. Therefore sulphate was replaced with formic acid/perchloric acid for the segregation of inhibitor (Ca-1P). Interestingly isolated RuBP in absence of CO_2 or O_2 are capable to accumulate the substrates. It may require additional carbon source and probably reduces the sugar phosphates which deactivates the enzyme (due to in absence of CO_2). The presence of O_2 did not stabilized activation and there is no coordination between rate of RuBP carboxylase inactivation and the internal concentration of RuBP. Therefore, proper understanding of the metabolites system is required because both enzymes (carboxylase/oxygenase) are activated by CO_2 and Mg^{2+} ions²¹. Although no stable complexes of Rubisco have yet been isolated, enzyme must interact directly to alter its structure, making the active site accessible for carbamylation²⁶.

SDS-PAGE and HPLC of Rubisco: After purification, Rubisco was loaded on HPLC (C_{18} column) which eluted homogeneous single sharp peak indicating the purity of the Rubisco [Figure 1(ii)]. HPLC eluted sample was loaded on SDS-PAGE to confirm its molecular weight. Separated polypeptides present at various stages of the Rubisco embraced by staining with Coomassie Blue. On the basis of its mobility in separation of units was observed. The enzyme had a Mass of 55 KDa [Figure 1(i)]. The potential Rubisco activity was determined by the relative rate of biosynthesis and degradation.

Purification of Inhibitor/Ca-1P:

Specific binding of inhibitor Ca-1P to activated Rubisco eluted with the strongly acidic solution and that was injected to HPLC which shows high purity by showing sharp peak. LC-MS is frequently used in drug development. The sample was first separated on an SDS-PAGE gel or HPLC. MS is used for this due to high sensitivity and exceptional specificity compared to UV confirming Ca-1P structure (Figure 2(i)). These activities were restored by adjusting pH between 7 and 8.

The naturally occurring inhibitor of Rubisco has been concurrently identified with TLC, UV, IR, HPLC, LC-MS. Spectroscopic properties of inhibitor from *Solanum xanthocarpum* leave enforce to elucidate the compound. This compound closely resembles to Ca-1P which plays important role in the regulation of C_3 cycle in stroma of chloroplast.

Colorless sample: Volume: 10ml, Rf value: 0.64, UV: 194nm, IR: 3429.07cm^{-1} (H bonding -OH) 1645.28cm^{-1} (C=O stretching), 2087.20cm^{-1} (monophosphate ester), 1219.40cm^{-1} (C-O stretching), 787cm^{-1} (out of plane bend),

CONCLUSION:

Structural elucidation of *Solanum xanthocarpum* CA1P; inhibitor of Rubisco was carried out by spectroscopic and chromatographic methods. Purified Rubisco may be used for its catalytic efficiency evaluation and for its biotechnological approaches against global warming.

Abbreviations:

Rubisco- Ribulose 1, 5-bisphosphate carboxylase/oxygenase; Ca-1P- 2-Carboxyarabinitol-1-phosphate, SDS PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis, ATP- Adenosine-5'-triphosphate, NADPH- nicotinamide adenine dinucleotide phosphate-oxidase, DTT- Dithiothreitol, GPDH- Glycerol-3-phosphate dehydrogenase, PGK- 3-Phosphoglyceric Phosphokinase.

ACKNOWLEDGEMENT:

The authors are greatly acknowledged to IIT, Powai for providing Instruments facility.

REFERENCES:

- Martin A J Parry, Alfred J Keys, Pippa J Madgwick, Ana E Carmo-Silva and John Andralojc (2008) Rubisco regulation: a role for inhibitors. J Experimental Botany, 59(7), 1569-1580.
- Shahnaz Khan, P. John Andralojc, Peter J. Lea and Martin A. J. Parry (1999), 2-Carboxy-D-arabinitol 1-phosphate protects ribulose 1,5-bisphosphate carboxylase/oxygenase against proteolytic breakdown. Eur. J. Biochem. 266, 840-847.
- P John andralojc, Alfred J keys, Jens Kossmann and Martin A J Parry (2002) Elucidating the biosynthesis of 2-carboxyarabinitol through reduced expression of chloroplastic fructose 1,6-bisphosphate phosphatase and radiotracer studies with $^{14}\text{CO}_2$ PNAS, 99(7), 4742-4747.
- Ounissi Mourad, Kameli Abdelkrim, Boudjeniba Messaoud, Kherraz Khaled and Redouane Nouredin (2009), Binding of 2Ca-1P (nocturnal inhibitor) to the active site of Rubisco using genetic algorithm(GA). Bioinformation 4(5): 206-209.
- Berry J.A, Lorimer G.H, Piece J, Seemann J.R, Meek J, Freas S (1987) isolation, identification and synthesis of 2-carboxyarabinitol 1-phosphate, a diurnal regulator of ribulose-bisphosphate carboxylase activity, Proc Nalt Acad Sci USA 84:734-738.
- Servaites JC (1985) Binding of phosphorylated inhibitor to Ribulose bisphosphate carboxylase/Oxygenase during night¹. Plant Physiol 78: 839-843.
- Gary C Harris and Arthur I Stern (1977) Isolation and some properties of Ribulose-1,5-bisphosphate carboxylase-oxygenase from Red kidney bean primary leaves¹. Plant Physiol. 60, 697-702.
- Alison H Kingston-Smith, Iain Major, Martin A J Parry and Alfred J Keys (1992) purification and properties of a phosphate in French bean (*Phaseolus vulgaris* L.) leaves that hydrolyses 2'-carboxy-D-arabinitol-1-phosphate. Biochem.J. (287) 821-825.
- Ghanshyam D. Heda and Michael T. Madigan (1989), Purification and characterization of the thermostable Ribulose-1,5-bisphosphate carboxylase/oxygenase from the thermophilic purple bacterium Chromatium tepidum. Eur. J. Biochem., 184, 313-319.
- Z Q Gao, C X Meng, G C Wang and N H Ye (2008), Isolation and characterization of photosystem II from the filamentous sporophyte of *Porphyrta yezoensis*. Indian J.of Biochem. and Biophysics, 45,244-249.
- S. Govindan, S. Vishwanathan, V. Vijayasekaranand and R. Alagappan (1999), A pilot study on the clinical efficacy of

- Solanum xanthocarpum and Solanum trilobatum in bronchial asthma. *J. Ethnopharmacol.*, 66(2), 205-210.
12. N. P. Bector and A. S. Puri (1971), Solanum xanthocarpum (Kantakari) in chronic bronchitis, bronchial asthma and non-specific unproductive cough (an experimental and clinical correlation). *J. Assoc. Physic. Ind.*, 19, 741-744.
 13. S. S. Gupta, S. C. Verma, C. Singh and P. Khandelwal (1966), Chemical and pharmacological studies on Solanum xanthocarpum (Kantakari). *Indian J. Med. Sci.*, 20(8), 554-559.
 14. M. M. Hussain (1995), Repellent effect of Katabegun (Solanum xanthocarpum Sch.) leaf on *Triboleum castaneum* Herbst. *Pak. J. Zool.*, 27, 279-280.
 15. U. K. Pandey, G. S. Verma and M. Pandey (1980), Efficacy of some plant origin insecticide on *Bagrada crucifera* Kiak. *Indian J. Entomol.*, 42(4), 775-777.
 16. F. H. Wei, X. J. Xu, J. B. Liu, Y. H. Dai, G. Dussart and J. Trigwell (2002), Toxicology of a potential molluscicide derived from the plant Solanum xanthocarpum: a preliminary study. *Ann. Trop. Med. Parasitol.*, 96(3), 325-331.
 17. P. C. Josekutty (1998), Selection and characterization of Solanum xanthocarpum cell line with augmented steroid metabolism. *Phyton Buenos aires*, 62, 125-130.
 18. Edward T Hammond, T. John Andrews and Ian E. Woodrow (1998), Regulation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase by Carbamylation and 2-Carboxyarabinitol 1-Phosphate in Tobacco: Insights from Studies of Antisense Plants Containing Reduced Amounts of Rubisco Activase. *Plant physiol*, 118(4): 1463-1471.
 19. Carmo-Silva AE, Barta C and Salvucci ME (2011) Isolation of ribulose-1,5-bisphosphate carboxylase/oxygenase from leaves. *Methods Mol. Biol.*, 684, 339-347.
 20. M. R. Parikh (2006), Directed evolution of Rubisco hypermorphs through genetic selection in engineered E. coli. *Protein Engineering, Design and selection*, 19(3), 113-119.
 21. Edmondson DL, Badger MR, Andrews TJ (1990), A kinetic characterization of slow inactivation of Ribulose bisphosphate carboxylase during catalysis. *Plant Physiol* 93, 1376-1382.
 22. Brandon D. Moore, Thomas D. Sharkey, John Kobza and Jeffrey R. Seemann (1992), Identification and levels of 2'-Carboxyarabinitol in leaves. 99, 1546-1550.
 23. O H Lowry, N J Rosebrough, A L Farr and R J Randall (1951), Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem.* 193, 265-275.
 24. Laemmli U K (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
 25. Miziorko HM, GH Lorimer (1983), Ribulose-1,5-bisphosphate carboxylase-oxygenase. *Annu Rev Biochem* 52:507-535.
 26. Portis AR, Li C, Wang D, Salvucci ME (2008), Regulation of Rubisco activase and its interaction with Rubisco. *J. of Exp. Botany*, 59(7), 1597-1604.