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Kinetic study of Rubisco from *Solanum xanthocarpum* leaves

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Abstract

Rubisco, the most abundant protein serving as the primary engine generating organic biomass on Earth, low specificity for CO_2 leading to photorespiration. The high concentration of Rubisco exceeding the concentration of its substrate CO_2 makes application of Michaelis–Menten kinetics invalid and requires alternative kinetic approaches to describe photosynthetic CO_2 assimilation. Double-reciprocal plots of Rubisco activity at several concentrations of Substrate (RuBP) and inhibitor (Ca-1P) were consistent with Michaelis-Menten kinetics. The apparent Km and Vmax (0.03µM and 1×10^{-4} µmol/lit/min) values are critical to attempt for the understanding the working of Rubisco to control the metabolism. Other factors may also influence the rate of activation in vitro. *Solanum xanthocarpum* is acts as traditional medicine. Hence, to understand the *Solanum xanthocarpum* plant performance in photosynthetic field, it is necessary to study spectroscopic analysis of Rubisco in relation to kinetic factors.

Keywords: Rubisco, Kinetic study, *Solanum xanthocarpum*, Inhibition.

Abbreviation

SXLR	: *Solanum xanthocarpum* leaves Rubisco
EDTA	: Ethylenediaminetetraacetic acid
ATP	: Adenosine-5'-triphosphate
NADPH	: Nicotinamide adenine dinucleotide phosphate-oxidase
GPDH	: Glyceraldehyde 3-phosphate dehydrogenase
PGK	: Phosphoglycerate kinase

Introduction

Photosynthesis is the origin of oxygenic life on the planet. Inside plant cells, the enzyme Ribulose1, 5-bisphosphate carboxylase/oxygenase (Rubisco [EC 4.1.1.39]) forms the bridge between life and the lifeless, creating organic carbon from the inorganic carbon dioxide in the air. However, barely three carbon dioxide molecules are fixed by Rubisco per second. In chloroplasts, Rubisco comprises half of the protein which contributes upto 50 % soluble protein and 30 % of total leaf nitrogen in leaves of C_3 plants. This makes Rubisco the most plentiful single enzyme on the earth that is the major catalyst for production of fixed carbon in the biosphere. Rubisco catalyzes the carboxylation and oxygenation of RuBP, which initiates the reciprocally divergent plant processes of photosynthetic CO_2 fixation and photorespiration due to Rubisco which is an inefficient enzyme with a low turnover number[1, 2]. The oxygenation reaction leads to the photorespiratory pathway, resulting losses of fixed carbon is between 25% and 30%. Environmental variables, such as high temperature and drought, can result in an increase in the oxygenase reaction. Therefore net photosynthetic CO_2 fixation would be increased if Rubisco could work faster with greater specificity for CO_2[3, 4].

2-Carboxyarabitinol 1-phosphate (Ca-1P) is a naturally occurring analogue of the transition-state of the carboxylase reaction, which binds tightly to the active site of carbamylated Rubisco and thus it inhibits catalytic activity of Rubisco [5, 6]. It has been the subject of much interest as a means of understanding and improving agricultural crop and green biomass productivities. Recently, attention has turned to the possibility of replacing native Rubisco in agriculturally important plants species where the introduced protein displays superior carboxylation kinetics under physiological conditions[7, 8, 9].

Kinetic properties of Rubisco were studied from *Triticum thyasiris*[10], Tobacco[11], Rice[12, 13], Soybean and peanut[13], Wheat[14] and Spinach[15].

Solanum xanthocarpum is nontoxic, abundantly available in arid areas and procured phytoactive compounds having versatile medicinal properties[16-19]. Extraction of Rubisco from *Solanum xanthocarpum* leaves without further enzyme activation, and subsequent rapid assay of activity in the extract, is believed to provide a good estimate of the enzyme activation in vitro. Measurements of Rubisco activity have been performed to study the regulation of the rate of leaf photosynthesis. In present evaluation, whether temperature dependences of Rubisco activation state and in vitro Rubisco kinetics which are responsible for net photosynthetic rate and Rubisco activation state in *Solanum xanthocarpum* leaves were mentioned.

Therefore attempts was made on *Solanum xanthocarpum* leaves Rubisco (SXLR), was studies in relation to Km, Vmax, pH, Temperature and thermal stability. The effects of the differences in these parameters on the photosynthetic performance of *Solanum xanthocarpum* leaves were quantitatively evaluated.

Material and Methods

Material

Tris buffer, $NaHCO_3$, Magnesium chloride, Mercaptoethanol, EDTA, Ascorbic Acid,

Polyethylene glycol (P-4000), $HClO_4$, Potassium hydroxide Sodium dodecyl sulphate (SDS), Glycerol were procured from SD Fine Chemicals, Mumbai and ATP, NADPH, Dithiothreitol, Ribulose 1,5- bisphosphate (RuBP), GPDH, PGK from Sigma Aldrich. All chemicals and reagents were used of analytical grade.

Source:

The *Solanum xanthocarpum* leaves for isolation of Rubisco and Ca-1P were collected from Ambernath, District-Thane, Maharashtra state, India in April 2011.

Methods:

Partial purification of Ribulose-1, 5-bisphosphute carboxylase/oxygenase from *Solanum xanthocarpum* leaves:

Solanum xanthocarpum leaves (25 gms) 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 $NaHCO_3$/ 60 mM $MgCl_2$/ 10mM Mercaptoethanol / 5 mM ascorbic acid/ 1 mM EDTA. In a blender, blend in short bursts until all the leaves are fragmented. Then blend 5-8 s on high. Filter through 8 layers muslin, followed by filtration through 2 layers miracloth (or muslin). Rubisco was then precipitated by adding 60% (wt/vol) polyethylene glycol to yield a final concentration of 18%. This solution was kept at 0^oC for 1 hr with stirring and spin at 10-12,000 rpm for 30 min. The precipitated Rubisco with bound inhibitor was dissolved in minimum quantity of water. $HClO_4$ (0.45 M) was added to the precipitated of Rubisco and to release the inhibitor (Ca-1P).

Co-factor specificity:

Salts of the divalent metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}) were prepared in 100mM Tris buffer, pH 8.2. Rubisco free of metal ions was prepared by extensive dialysis against 10mM Tris (pH8.2) containing 0.5 mM EDTA-Na, and 14.2mM-2-mercaptoethanol. RuBP carboxylase activity was 49.84 IU before dialysis, and was not detectable after dialysis.

Enzyme free of metal ions was incubated in assay mixture (not containing $MgC1_2$) containing a final concentration of 20 mM metal ions listed, for 30 min. Assays were initiated with RuBP, terminated after 5 min, and the protein concentration in the assay was 0.922 mg/ml.

Assay for Rubisco activity:

Rubisco activity was measured essentially as described by Edmondson et al.[20] with slight modification. This is a coupled or linked assay, in which the immediate product of Rubisco, 3-phosphoglycerate, is converted primarily into 1,3-bisphosphoglycerate, and then to glyceraldehyde 3-phosphate using ATP, NADPH, and glycolytic enzymes such as phosphoglycerokinase and glyceraldehyde 3-phosphate dehydrogenase. The disappearance of NADPH was monitored by the $A_{340.}$ There are several components to the reaction mixture are:-

Buffer: 0.2 M Tris, 10 mM $MgCl_2$, 0.132 M $KHCO_3$, 10 mM DTT, pH 7.8 (set the pH before adding DTT)Add 13.8 mg ATP to 2.5 ml buffer, mix well and kept at 0^oC.

NADPH: 2 mM (1.4 mg/ml)
Coupling enzymes: 500 units GPDH and 1000 units PGK mixed and dialyzed overnight against 50 mM bicine, pH 8, 0.1 mM EDTA, 20% (v/v) glycerol. Store in the freezer until used.

RuBP: 2.5 mM
The assay was initiated in a total volume of 0.75 ml. In a cuvet, 0.375ml buffer, sample and enough H_2O followed by 75 µl NADH, and 50 µl coupling enzymes was added and mixed thoroughly. Finally 150µl RuBP mixed quickly and effectively, and measured by spectrophotometer (Shimadzu UV-1800).

Protein assay:
The total protein was determined by the method of Lowry *et al.*[21], using casein as a standard (0.16 corresponds to 0.1 mg of protein). The spectrophotometric protein assay was measured at 280 nm on UV-visible spectrophotometer (Shimadzu UV-1800).

Kinetic Parameters:

Effect of Enzyme concentration on Rubisco activity:
To study the effect of the enzyme concentration on the rate of reaction and disappearance of NADPH, the various concentration of this enzyme ranging from 10 µL to 100 µL were measured. By plotting enzyme concentration versus activity, rate of reaction was determined. Assays were initiated with activated enzyme in triplicate.

For above purpose, all solutions were freshly prepared in MilliQ-beaked water, and buffer was adjusted with 50 % (w/v) NaOH.

Effect of Substrate/ RuBP concentration on Rubisco activity:
The catalytic properties of Rubisco were studied using RuBP (2.5mM) as a substrate at varying concentration range (10µl- 100µl).

Determination of K_m and V_{max}:
The reaction was carried out by the assay as noted above containing various concentration of substrate RuBP. Vmax and Apparent *Km,* values were determined from double reciprocal plots (Lineweaver-Burk) and wolf-Hofstee plots.

pH optimum:
Solanum xanthocarpum leaves powder was extracted in Tris buffer system for the determination of optimum pH at various pH ranging from 5-10 (50 mM). The concentration of buffer carried over the assay was 1 mM.

Optimum Temperature:
The most conventional method was used to study protein degradation is, supernant obtained from purification yield incubated at various temperature. The stability of Rubisco/protein (100µL) was estimated by studying irreversible thermal inactivation

at several temperatures from 0ºC -100ºC using 0ºC as a control. Protein assay was carried out to check the stability of Rubisco.

Detection of Activation energy of Rubisco:

The Arrhenius Activation energy expressed in term from the Arrhenius equation is best regarded as an experimentally determined parameter which indicates the sensitivity of the reaction rate to temperature. Activation energy may also be defined as the minimum energy required starts a chemical reaction. The activation energy of a reaction is usually denoted by E_a, and given in units of kilojoules per mole. The Arrhenius equation proposed by Svante Arrhenius (1829), relates the activation energy Ea to the rate constant K of a process.

$K = A\, e^{-E_a / RT}$

e – log to the base 10 (2.3)

Ea – activation energy of the reaction (J mol)

R – molar gas constant (8.3143 $JK^{-1}mol^{-1}$)

T – Absolute temperature (K)

The values Ea and A for the reaction was calculated from the equation:

$$Ea = \frac{2.3\ R\ X\ T_1\ X\ T_2}{T_1 - T2}\ \log K_1/K_2$$

Effect of Ca-1P on Rubisco activity:

The released Ca-1P/inhibitor concentration in the extracts was determined by measuring its inhibition of purified Rubisco. Various Ca-1P concentrations (10µl-100µl) incubated with Rubisco, the activity was recorded and quantified by comparing with assays containing identical amounts of Rubisco but in absence of Ca-1P and compared with standard Rubisco. It was assumed that each inactive Rubisco site was bound with one molecule of Ca-1P and that all Ca-1P was bound to Rubisco sites.

Storage Stability of Enzyme:

Purified Rubisco was stored at 4°C and used for the activity. Storage stability for the activity carried out periodically.

Result and Discussion:

This study presents an analysis of the biochemical characteristics of Rubisco from *Solanum xanthocarpum* leaves. The data include the most extensive measurements of the Michaelis–Menten constant for C_3 species (*Solanum xanthocarpum)* highlights that the activity of Rubisco.

Maximum activity of *Solanum xanthocarpum* Rubisco was observed at 20µl of enzyme. Its activity was gradually decreases but it was very negligible up to 80µl of enzyme. After 80µl of enzyme activity of it drop down. 20µl of enzyme was selected for further study (Figure 1A).

Measurements of the initial rates of reaction were run in triplicate at various substrate concentrations and their arithmetic mean used for evaluation. The plot Substrate concentration against activity was plotted which showed the activity of Rubisco with respect to RuBP as a substrate increases as the concentration of substrate increases. At varying concentration of substrate the Km value for Rubisco obtained from double reciprocal plot was 0.03µl. The results (Figure 1B) indicated that a rapid increase in Rubisco activity was delayed until the level of RuBP dropped below the Rubisco catalytic site concentration, which occurred after 2 to 3 min in this experiment. The RuBP remaining after this time must be bound, because the measured Rubisco activity was more than sufficient to 01 consume any free RuBP within seconds.

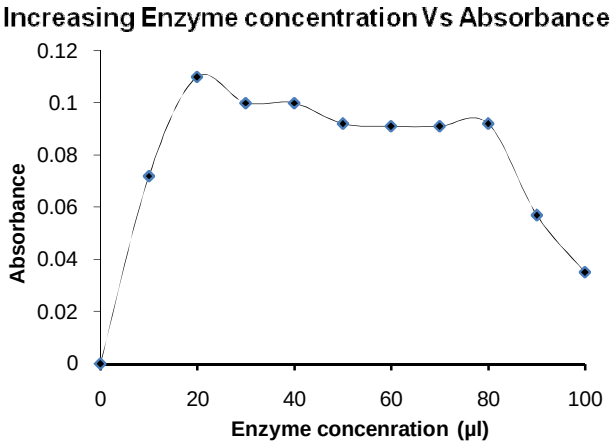

Figure 1A: Linearity curve of Rubisco

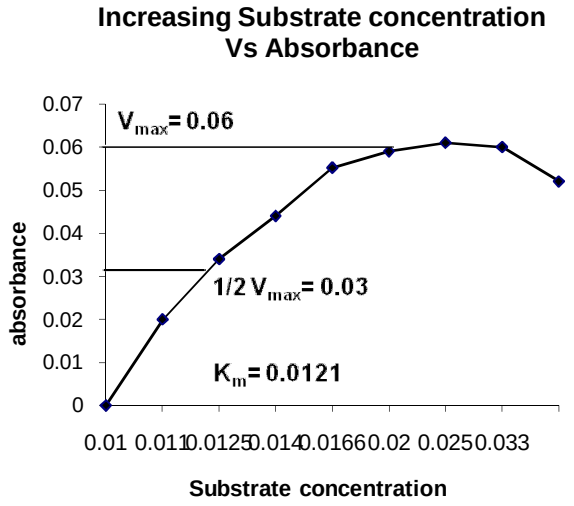

Figure 1B: Effect of Substrate concentration on Rubisco.

Temperature acclimation of Rubisco kinetic properties:

The Rubisco from *Solanum xanthocarpum* leaves is catalytically optimum active at 40°C. Test of thermostability of Rubisco activity showed that purified enzyme remained catalytically active over 20min at 60°C (Figure 2A). After 60°C, Rubisco activity was drastically decreased and gradually lost activity of Rubisco remain active up-to 100°C. Activity at 0°C and 100°C is equally attributed toward the pathway. Change in temperatures contributed to the increases in the photosynthetic rates. At moderately high temperatures, the photosynthetic rates were mainly determined by the Rubisco activation state, and that also differed depending on the growth temperature. These changes, depending on the growth temperature, were responsible, at least partly, for the differences in the temperature dependence of the photosynthetic rates because the photosynthetic rates are mostly limited by the RuBP carboxylation rates under a normal CO_2 condition. It is important to change the temperature dependences of the Rubisco kinetics and the Rubisco activation state for efficient photosynthesis at the growth temperature[22]. Rubisco kinetic properties acclimated to the growth temperature was examined by Arrhenius plot (Figure 2B).

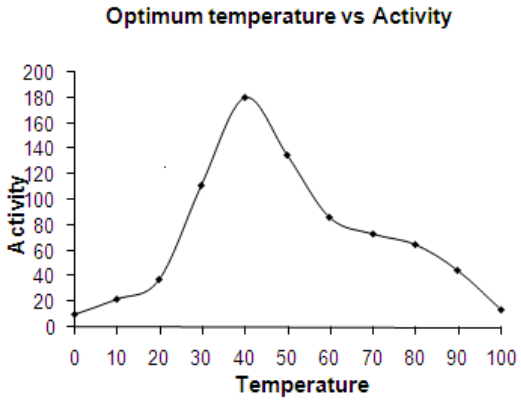

Figure 2A: Effect of temperature on Rubisco activity.

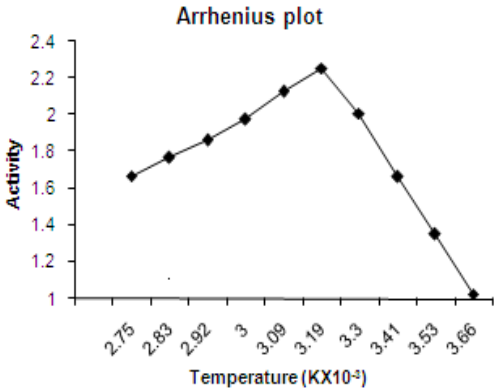

Figure 2B: Arrhenius plot shows the effect of pH on Slow inactivation of Rubisco.

Co-factor specificity:
Very low activities were detected with Co^{2-}, Ni^{2-} and Ca^{2-}, but Mn^{2-}, Fe^{2-}, Cu^{2-}, and Zn^{2-} were inactive. However, the substrate of the Rubisco reaction, RuBP, and its product, PGA, showed low but significant inhibitory activities. Mg^{2+} was needed in both assay and in activation processes. In absence of Mg^{2+} in reaction medium the activity of Rubisco was almost zero. It confirms Mg^{2+} is required for apparent inhibition of Rubisco assay but actives it upto 30mM (data on activation were not shown here). Catalysis of reaction proceeds maximally at 60mM of Mg^{2+}.

Effect of Inhibitor:
The activity of Rubisco from Phaseolus vulgaris and several other species[23] is influenced by mechanisms that control the in vivo concentration of an inhibitor which binds tightly to the active site of the enzyme rendering it catalytically inactive. This mechanism appears to function together with the reversible formation of the active (carbaminated) site in regulating the activity Rubisco in vivo. The rate of photosynthesis depends on the activity of Rubisco. In this study, the dissociation kinetics of Rubisco-bound RuBP was examined in the presence and absence of inhibitor. Effect of Ca-1p on Rubisco proves that presence of the Ca-1P which tightly binds to the active site of enzyme and decline the activity. The inhibition study performed with substrate and substrate-inhibitor concentration variation, from graph K_m and $1/V_{max}$ values was obtained for (Figure 3A). Regression factor R_1 and R_2 (for enzyme-substrate and enzyme-substrate-inhibitor respectively) indicates 0.974 and 0.976 values. Ca-1P has been concurrently identified in potato leaves as light dependent level which requires Rubisco. *Solanum xanthocarpum* leaves shows 3.2 K_i value for Rubisco. Surprisingly, K_i at 3.2 acts as very strong inhibitor. Its structural identification of the Ca-1P leads to control metabolism and mechanism of its synthesis and degradation. Literature review suggests that, inactivation of Rubisco is due to low light or darkness or due to decarbamylation of it. It resists due to the co-factors CO_2 and Mg^{2+}. It means C5 phosphate C3 phosphate group functions as a regulator, still the mechanism of degradation of Rubisco from *Solanum xanthocarpum* leaves in vivo are unknown.

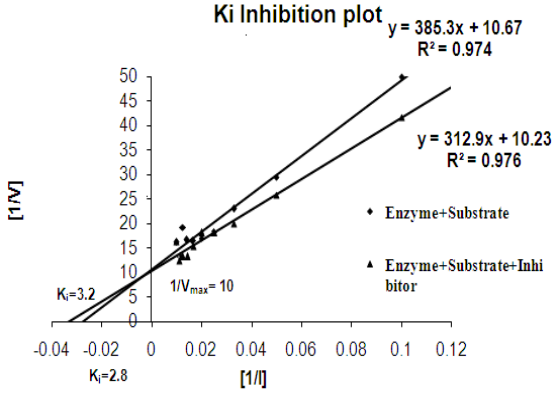

Figure 3A: Inhibition of Rubisco with substrate (.) and substrate inhibitor (▲).

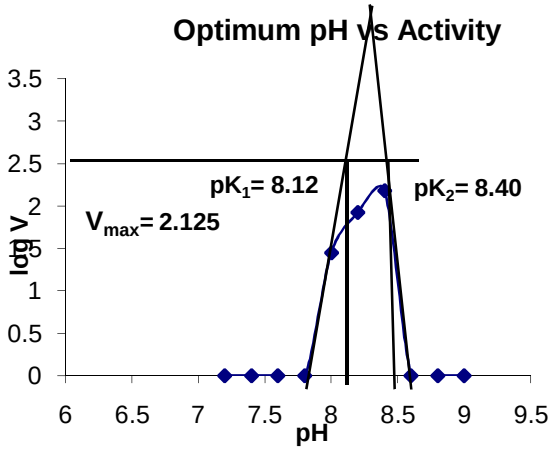

Figure 3B: Effect of pH on slow inactivation of Rubisco enzyme activity.

pH optima:

pH affect the enzyme activity were determined at pH range of 7.1 to 9.0. In *Solanum xanthocarpum* leaves Rubisco, activity is consistent and increased in the range 7.8-8.6 by procuring optimum pH is 8.2. The plot of optimum pH against Rubisco activity shows pK_1 value at 8.12 and pK_2 value at 8.40 (Figure 3B). pK_1 value at 8.12 and pK_2 value at 8.40 represents Lysine, Cysteine, Glycine, Tryptophan, Serine, Asparagine, Threonine, Histidine, Arginine amino acids sequence which serve as important nitrogen carriers in the vital process of plants and conserved amino acid residues are essential. Concentration variation in pH directly effects on Rubisco activity. According to various ionic strength of buffers system; Tris buffer (pH 7.1-9.0) is most suitable for Rubisco. Native SDS-PAGE and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure.

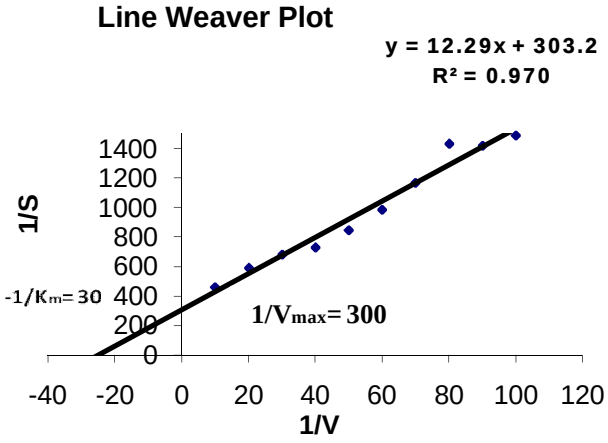

Figure 4A: Lineweaver-Burk plots of Rubisco activity.

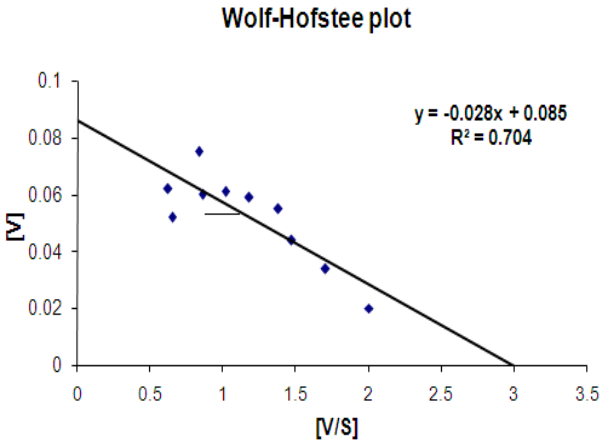

Figure 4B: Wolf-Hofstee plots of Rubisco activity.

Detection of Activation energy of Rubisco:

The Michaelis equation: $\nu = \frac{V}{1 + \frac{Ks}{s}}$

This equation can be plotted in several different ways for the determination of V_{max} and K_m from a set of measurements of velocity at different substrate concentrations.

A. If ν is plotted against s, as in fig 1A , the curve represents a section of a rectangular hyperbola. By substituing ν = V/2, the equation becomes s = K_m, and K_m thus be determined, being given by the 'Half-way point' of the experimental curve. Fig 1B shows curve for o-diphenol oxidase depending upon the values of V and K_m.
B. Second method of ploting is Lineweaver and Burk plot. According to this plot 1/ ν is plotted against 1/s as shown in fig 1C a straight line is obtained corresponding to the equation

$$\frac{1}{\nu} = \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V}$$

and this straight line cuts the base line at a point giving $-1/K_m$. This is easily shown by putting 1/ ν = 0 in the above equation which then gives $1/s = 1/-K_m$. The graph cuts the vertical axis at a point which gives 1/V and has a slope = K_m/V.

The maximum reaction rate V_{max} and Michaelis-Menton constant K_m were obtained from Lineweaver-Burk plot, which give rise to calculations at lower RuBP substrate concentrations. Rubisco has maximum velocity at 1 $X10^{-4}$respectively.

Storage Stability:

Rubisco samples frozen and stored in the presence of Mg^{2+} activators for 30 days for its stability. After 30 days, irreversible enzyme inactivation occurred and Rubisco losses its activity.

Conclusion:

This study consolidates that Rubisco kinetic properties acclimated the kinetic factors showed significant effect on enzyme activity. Understanding the response of photosynthesis to changing environmental conditions is of paramount weight for predicting the sound effects of global climate change on plant productivity. In the natural environment, the rate of photosynthesis is influenced primarily by temperature, the amount of light intercepted, the availability of water, nutrients and the amount of Rubisco available for photosynthesis (i.e. the activation state) changes in a predictable way to a host of environmental conditions, including temperature and CO_2 concentration. By adjusting the kinetics of Rubisco for changes in activation state, it is possible to accurately predict the response of photosynthesis at temperature and also to CO_2 and O_2[24]. Additional molecular studies are essential to quantify the effects of these factors on the kinetic properties of Rubisco.

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