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CHARACTERIZATION OF RUBISCO FROM VARIOUS PLANT SOURCES FOR EFFICIENT CATALYTIC ACTIVITY AGAINST CARBON SEQUESTRATION

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ABSTRACT

To combat the tremendous increase in atmospheric carbon dioxide level a wide number of technologies are being explored. The selection of natural and less destructive way would be by using plants as they are the most natural sinks present in Earth that have the ability to sequester carbon dioxide. This is mainly carried out by RuBisco, a protein found in all phototrophic forms as a major enzyme involved in photosynthesis. The present work focuses on isolating RuBisco protein from plant species taking into account one from each C3 and C4 plants. This was done in order to determine the effective plant with high carbon dioxide sequestering property. The experimental study was done both in normal and carbon dioxide stressed conditions. Rubisco was isolated from tomato and sugarcane plant and it was characterized by SDS PAGE, FT-IR and HPLC technique. To determine the carbon capture by the plant protein CHNSO analysis was carried out. The plant that absorbed higher concentration of carbon was can be identified.

Keywords: ***Solanum lycopersicum*; *Saccharum officinarum; RuBisco, Carbon dioxide; global warming; C4 plants***

INTRODUCTION

In recent years the climatic conditions have been drastically changing due to globalization and rapid industrialization. The concentration of greenhouse gases especially carbon dioxide are found to be increasing at an alarming rate. According to Intergovernmental Panel on Climate Change (IPCC) the level of global warming is increasing at a rate of 1.5°C per year. It was believed that about hundreds of millions of years ago the Earth's atmosphere includes carbon dioxide as the major component. Plants were able to utilize this carbondioxide to form sugar molecules, a process known as photosynthesis. The main by-product was oxygen and due to accumulation of oxygen in its course of time the atmosphere changed to oxygenic environment making it favorable for human life **[24]**. Therefore plants can be considered as the natural carbon sinks present on Earth.

Carbon dioxide fixation by plants was carried by Rubisco which is an abundant protein present in Earth. This is the major enzyme needed for photosynthesis and it catalyses the reaction between carbon dioxide and Ribulose 1,5, Bisphosphate to form two molecules of phosphoglyceric acid **[17]**. This protein has a unique property of having the ability to bind with both carbon dioxide and oxygen. Based on the substrate specificity it can either lead to photosynthesis or photorespiration **[16, 23]**. It is capable of fixing about more than 90% of inorganic carbon into biomass. Rubisco functions by catalyzing the carboxylation reaction followed by cleavage of the substrate Ribulose 1,5, Bisphosphate into two molecules of 3- phosphoglycerate. This enzyme is present as soluble protein in leaves which accounts for more than 30 to 50% **[7]**.

This protein is present in both C3 and C4 plants. C3 plants utilize carbon fixation pathway for conversion of carbon dioxide to organic compound. The C4 plants on the other hand avoid photorespiration by using PEP (Phosphoenolpyruvate) enzyme during carbon fixation. The majority of plants belong to the C3 group which involves the series of reactions in which the enzyme Rubisco fixes the carbon dioxide through Calvin –Benson cycle. However the major drawback of C3 photosynthesis is that it can also fix oxygen initiating the process called photorespiration which is a high energy process. Another disadvantage seen in C3 plants is sensitive to drought and temperature conditions. Studies were investigated on genetically modifying the RuBisco protein in order to increase the specificity towards carbon dioxide so as to overcome the disadvantage **[11]**. Examples of C3 plants include wheat, oats, rye, spinach, etc which are able to fix carbon dioxide into three carbon sugars. Plants can also evolve another form of photosynthesis that helps them to survive in hot and dry environment. They follow the C4 photosynthesis where- in a four carbon compound is produced. The main examples of C4 plants include maize, sugarcane, and sorghum. Carbon assimilation is significant step as it can store energy in huge volumes in living organisms thus producing most of the biomass in the biosphere.

The main limitation of Rubisco enzyme is that it is very slow and large amount are required to support photosynthetic rate. The property of Rubisco such as catalytic property, turnover rate, affinity and specificity of carbon dioxide are found to vary depending on the source and environmental conditions **[1, 20]**. The interaction of Rubisco with the molecular chaperone was studied by Tsai *et al*., 2015, **[21]** who observed that the leaf extract is dependent on Rubisco activase. During photosynthesis it can catalyze the carbon dioxide assimilation which indirectly influences the photosynthetic efficiency **[6, 9]**. Recently researches are carried out in NASA to improve Rubisco by protein engineering in order to increase the Rubisco ability to capture carbon dioxide. This may also lead to increase in biomass accumulation which can be met out the food demand. In the present study Rubisco protein was isolated from tomato (C3 plant) and sugarcane (C4 plant) and it was characterized by different methods. The absorption of carbon by these plants both in normal and carbon stress condition was investigated.

MATERIAL AND METHODS

Plant material and growth conditions

The tomato (*Solanum lycopersicum*) and sugarcane (*Saccharum officinarum*) seedlings were collected from Poovalur village of Laalgudi, Trichirappalli district, Tamil Nadu during the month of December. They were grown in pots at room temperature for a period of three months.

Protein extraction and determination

The leaves were thoroughly washed using sterile distilled water and dried at room temperature. The leaves were then placed at -4°C for 45minutes. The leaves were homogenised using motor and pestle, filtered and extracted using 20mL of ethanol consequently for three times and with 20mL of acetone two times. The supernatant was collected and the protein was partially purified using saturated 100% ammonium sulphate **[18]** precipitation. Estimation of total protein by Bradford method was done as per the standard protocol **[15]**.

SDS PAGE Protein Electrophoresis

The extracted protein was fractionated using 4% of stacking gel and 10% of separating gel. The sample was mixed in a ratio of 2: 1 ratio with sample buffer and heated up to 90°C for 3 minutes. Electrophoresis was conducted at 100V until the tracking dye reached three-fourth of the gel. Then the gel was stained in the Coomassie staining solution. The gel was incubated for 6 hours overnight in a staining solution. The gel was kept in a de-staining solution until the background was transparent **[14]**. The molecular weight protein ladder (24 to 200kD) for tomato sample and (10 to 250kD) for sugarcane sample was used to identify the sample's protein molecular weight.

Fourier Transform infra Red analysis (FT-IR)

FTIR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks ranging from 400 - 4000 cm-1 and their functional groups. The peak values of FTIR were recorded. Each and every analysis was repeated twice for the spectrum confirmation **[10]**.

High Performance Liquid Chromatography analysis

The liquid chromatographic system consists of a waters 600E automated gradient controller pump module, a waters wisp 717 automatic sampling device and a waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 image 466 computer. Millennium software was used to plot, acquire and analzye chromatographic data. Chromatographic process was performed with a Vydac C4 column (250mm). The mobile phase composed of water/trifluoroacetic acid (1000: 1 v/v) was used as eluent A, while acetonitrile/trifluoroacetic acid (1000:1 v/v) was used as eluent B. The flow rate was 0.6ml/minutes. Samples were filtered through 0.22mm filters and then injected **[12]**. The gradient applied was 0-10 minutes,100%A; 25-95minutes,21-81% B,96-110minutes,100-0% Band the HPLC system was equilibrated for 10 minutes with 100% A. On-line UV absorbance scans were performed between 215 and 325 nm at a rate of one spectrum per second with a resolution of 1.2nm. Chromatographic analyzes were completed with Millennium software.

CHNSO analysis

The tomato and sugarcane plants were kept in normal environment and one batch of plants were placed in chamber containing 2% carbon dioxide for about 24 hours. The leaves were taken and it was powdered using liquid nitrogen and further subjected to CHNSO analysis. The percentage of carbon absorbed by the leaves was determined using 98CHNS-O Analyzer as per standard protocol.

RESULT AND DISCUSSION

Protein extraction and determination

The protein present in the leaves of both tomato and sugarcane plant was extracted and the total protein concentration was estimated by Bradford method. The absorbance was measured at 595nm and the estimation of unknown concentration was calibrated using the standard as shown in **Figure 1a & Figure 1b**.

The graphs below indicates R^2 is 0.93 which means 97% of the values fit the regression analysis model. Generally, R squared of 95% or more is considered as a good fit. This implies that 97% of the dependent variables (absorbance) are directly proportional to the independent variables (concentration of proteins). The estimation of the amount of protein content was calculated using the equation $Y = mX + C$ which is obtained by plotting the concentration of standard protein against the absorbance. From the calibrated graph the concentration of the samples was estimated using the formula $X = \frac{Y-c}{m}$ as given in **Table 1**.

The total protein concentration present in leaves and root of tomato plant was studied by Vilhena *et al*., 2015 **[22]** to improve the proteomic analysis as only few reports are available regarding total protein estimation. He observed reported the higher levels of protein recorded in the treatment that was 4.24 mg mL^{-1} for leaves and 1.84 mg mL^{-1} for tomato roots. This result is similar to that reported in our study and decrease of protein observed may be due to different solvent used in our assay

SDS PAGE Protein Electrophoresis

SDS-PAGE was carried out to separate the proteins according to their molecular weight, net charges and electrophoretic mobility. The protein subunits were observed as bands after the destaining procedure and it compared with the protein marker to obtain the molecular weight **(Figure 2a, 2b)**. Three bands and seven bands were observed for tomato and Sugarcane sample respectively. On comparison with the molecular weight in tomato sample the first band corresponds to 75kD, the second band corresponds to -55kD and the third band corresponds to 36 kD. In sugarcane sample the major thick bands corresponding to 130kD, 110kD, 100kD, 55kD, 25kD and 15kD and faint bands were also observed at 250kD and 70kD respectively. From the result obtained it was evident that in both the plant species it contains Rubisco protein subunit at 56kD and in case of sugarcane two Rubisco subunits both at 56kD and 14kD were observed **[27]**. This is similar to literature studies reported by Yang *et al*., 2004 **[25]** in which the Rubisco subunit was found at 56kD isolated from fresh spinach leaves.

Alharby *et al*., 2016 **[2]** reported protein bands with molecular weights ranging from 14kDa to 134kDa. The SDS – PAGE method is found to be an important reliable tool for detection of Rubisco and it comparatively less cost effective as other methods include use of radio labelled compounds which need special equipment. The Rubisco extracted from plant leaves were denatured in the presence of sodium dodecylsulfate into small (15kDa) and large (55kDa) subunits. Further steps have to be followed for complete protein analysis.

Fourier Transform infra Red analysis (FT-IR)

FT-IR spectrum is carried out to determine the functional group present in the extracts tomato and sugarcane based on peak values obtained due to the infra-red radiation **(Figure 3a, 3b)**. The functional group present in tomato leaves include amine, ketone, methine, carbonyl group, thiocyanate and for sugarcane it includes alcohol group, ketone, sulfinyl, alkyl halide.

IR spectra of the Sugarcane was showed major peaks at 3435.454cm^{-1} for N-H amide usually strong, C-H methane at 1638.58cm^{-1} as strong peak was observed peak at 679.61cm^{-1} indicates c=c carboxyl group. For the IR spectra of the Tomato was showed major peaks at 3435.53cm^{-1} for N-H amide usually strong and broad, C-H methane at 1642.79cm^{-1} as strong peak,10556.97cm^{-1} sulfinyl was observed peak at 633.95cm^{-1} indicate alkyl halide. The major peak seen and from 3700-2000cm^{-1} in combination with highly characteristic medium at 1639.48cm^{-1} to weak absorption peaks between 2000-2500. FT-IR are mainly used to determine the functional groups such as hydroxy and amino group which link with the major atoms carbon, hydrogen, oxygen and nitrogen. The major peak seen at 3435cm^{-1} represents the hydroxy groups which can either bond intramolecular or intermolecular. These impacts broadening of the band as the mean adsorption frequency lowers and provides strength to the hydrogen bonding **[5]**. The peak shown at 1638cm^{-1} indicates amide (carboxylic acids) and exhibits strong hydrogen bond **[4]**. This is highly characteristic peaks as indicated in previous reports that shows presence of high level of amines in tomato leaves **[13]**. Flórez-Pardo *et al.,* 2019, **[8]** also observed similar FT-IR spectrum pattern and the band between 3000-2500cm^{-1} in sugarcane leaves. The peak locations correspond to the stretching and bending vibrations of the amides. Similar peak patterns were observed in sugarcane leaves with presence of an additional peak at 679cm^{-1}. This absorption may be less significant but can form another form of bending vibration of the O-H group. The OH bending vibrations are broadened by hydrogen bonding as is the stretching absorption, but often to a lesser extent. In some aspects, the infrared spectra and the characteristic group frequencies of amines tend to be parallel to those of alcohols.

High Performance Liquid Chromatography analysis

HPLC was performed to determine predominant activity of the components present in the sample **[26]**. The components identified were violaxanthin, catechin and gallic acid in which violaxanthin belongs to carotenoid were as catechin and gallic acid are flavonoids and proteins were present in moderate amount **(Figure 4a & 4b)**.

The chromatogram showed presence of gallic acid and cathechin which an important phytochemical constituent which has antioxidant, anti inflammatory and antineoplastic properties. Violaxanthin are natural xanthophuyll pigment which can impart an orange colour often used as food colouring agent. Ambati *et al*., 2009 **[3]** also reported the present of xanthophylls (1.5%) such as violaxanthin in plant leaves. RuBisCO was also submitted to a specific method of detection and analysis by HPLC. The purity of Rubisco and the potential effects of the extraction process on protein quality Rubisco can be further determined by reversed-phase high performance liquid chromatography analysis.

CHNSO analysis

On CHNSO analysis it was observed that the leaves of both sugarcane and tomato plant the absorption of carbon dioxide increased compared to the normal condition. Both tomato and sugarcane plants showed increase in carbon absorption of 35.4% & 42% for tomato and 39.6% & 45% for sugarcane respectively. This was in line with other literatures where the C, H, N, and O composition was studied. Stella *et al*., 2016 **[19]** showed a highest C of 40.43% in biomass wastes **(Table 3)**.

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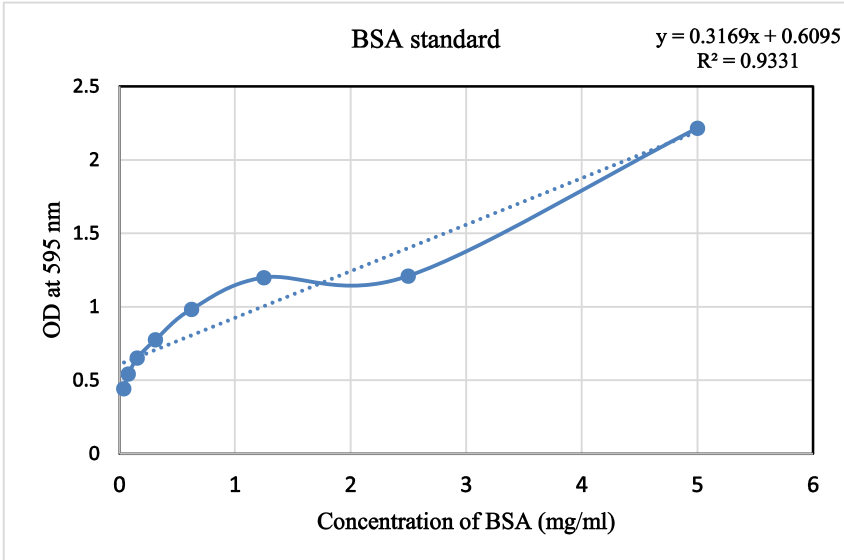


Figure 1-a: Standard calibration of tomato protein by linear least square analysis

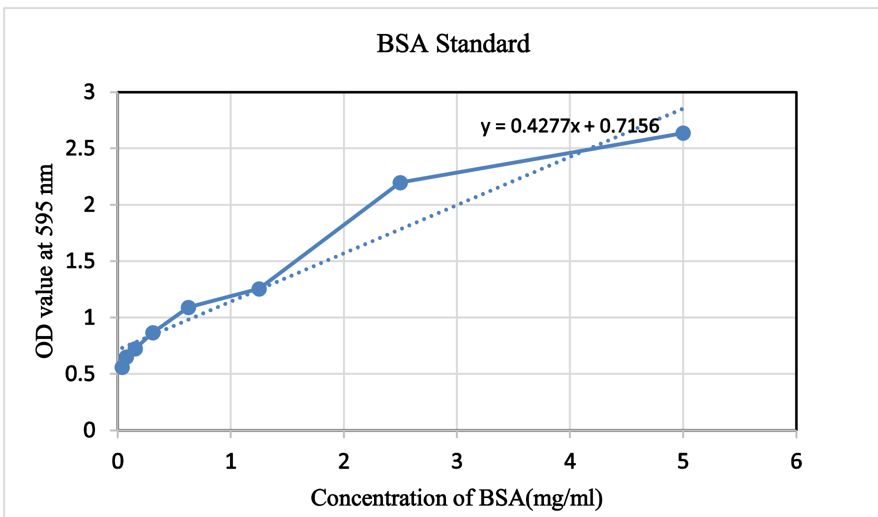
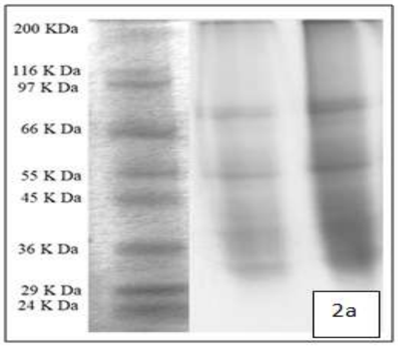


Figure 1-b: Standard calibration of sugarcane protein by linear least square analysis

Table 1: Estimation of protein concentration in Tomato and Sugar cane leaves

Name of the Sample	OD value at 595 nm	Total protein content	Mean value of total protein content (mg/mL)
Tomato	0.705	0.30	0.36
	0.708	0.31	
	0.762	0.48	
Sugarcane	0.805	0.20	0.26
	0.836	0.28	
	0.85	0.31	



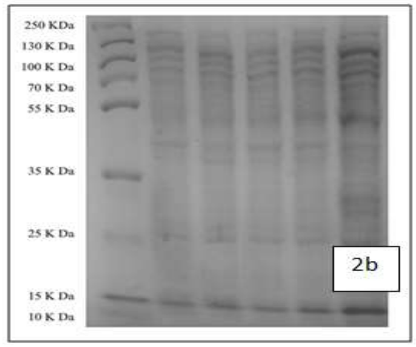
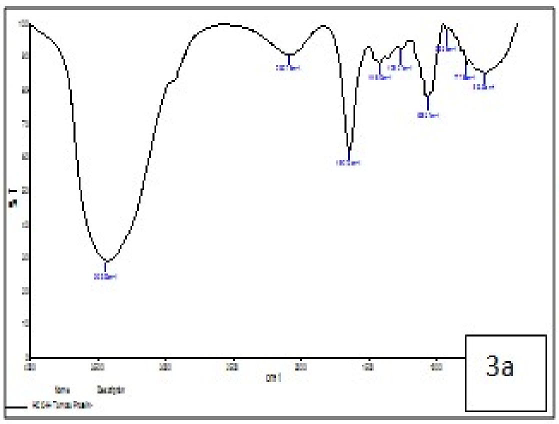


Figure 2a: SDS-PAGE analysis of Rubisco from tomato plant species

Figure 2b: SDS-PAGE analysis of Rubisco from sugarcane plant species



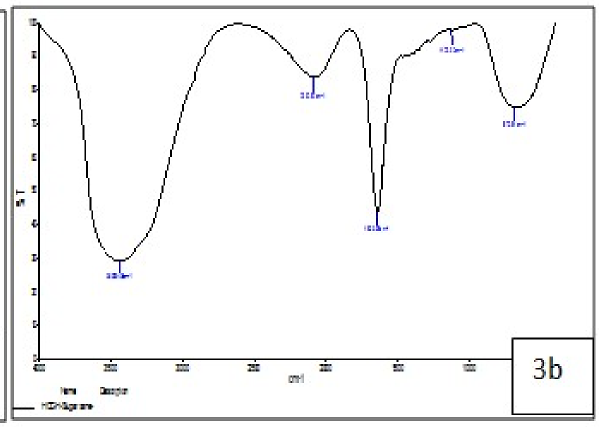


Table 2: IR spectra of tomato and sugarcane showing functional group

Frequency range cm-1	Bond	Compound type
	TOMATO	
3435.45	N-H stretching	Amine
1638.58	-O=CH(-NH_2)	Amide
679.61	C=C bending	Carbonyl group
	SUGARCANE	
3435.53	N-H Stretching	Amine
1642.79	-O=CH(-NH_2)	Amide
1056.97	-CH_2-NH	Aliphatic amine
633.95	C-Br stretching	Alkyl halide

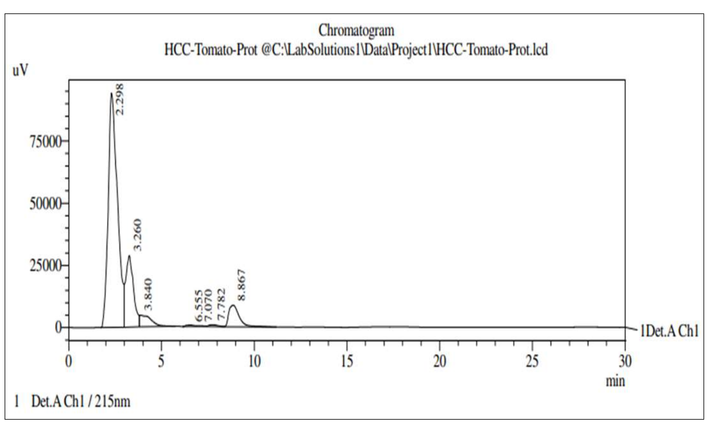


Figure 4a: The chromatogram of protein extract from tomato plant

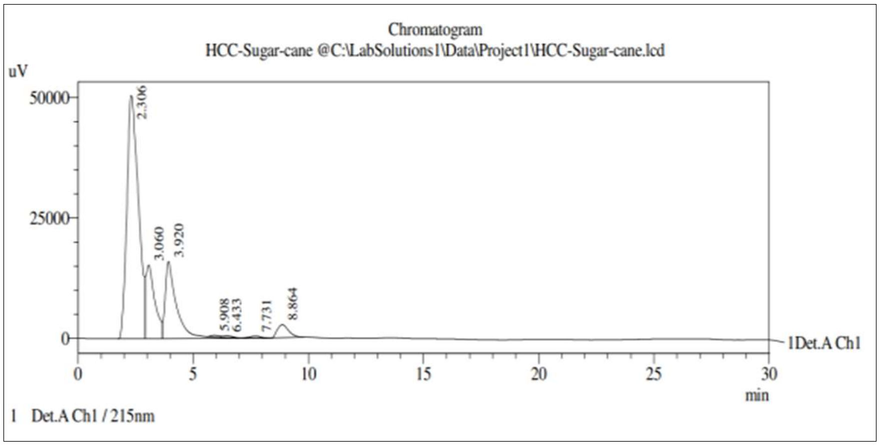


Figure 4b: The chromatogram of protein extract from sugarcane plant

Table 3

Sample	Carbon (wt%)	Hydrogen (wt%)	Nitrogen (wt%)	Sulphur (wt%)
SC-1	39.6	9.2	7.5	0.8
TM-1	35.4	9	5.6	0.4
SC-2	45	8.7	7.2	0.7
TM-2	42	8	7	0.9

SC-1 = Sugarcane in normal condition; TM-1= Tomato in normal condition
SC-2= Sugarcane in presence of carbon dioxide; TM-2= Tomato in presence of carbon dioxide

CONCLUSIONS

RuBisCo enzyme involved in Calvin cycle contributes mainly to carbon fixation, in which atmospheric carbon dioxide are converted to glucose, an energy-rich molecule. Therefore it plays an important part in terms of global net primary production. This enzyme is the most abundant protein present in Earth and is present in all photosynthetic plants. Both C3 and C4 plants have Rubisco but their substrate specificity and location in leaves changes. In the present study tomato plant was taken as an example for C3 plant whereas for C4 sugarcane plant was considered. Proteins were present in moderate amount and thus the activity of Rubisco was determined. The plants were subjected to increase in carbondioxide for 24 hours. The amount of carbondioxide absorbed by the leaves was determined by organic analyser to determine the effective plant that can sequester carbondioxide. On comparison the C4 plants are found to be effective in acting as effective natural carbon dioxide sink. This proves that the selected plant can be used as an effective natural carbon dioxide sink. Future genetically engineering studies in sugarcane plants can be carried out in order to combat global warming.

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