Bot. Bull. Academia Sinica 27: 77-84 (1986)

MOLECULAR AND KINETIC PROPERTIES OF RICE RIBULOSE-1, 5-BISPHOSPHATE CARBOXYLASE[1]

JEI-FU SHAW and SHU-YAW HSIEH

Institute of Botany, Academia Sinica
Nankang. Taipei, Taiwan 11529, Republic of China

(Received August 8, 1985; Accepted November 19, 1985)

Abstract

Rice RuBPCase was purified by ammonium sulfate fractionation, Sephadex G-50 and DEAE Sepharose CL-6B chromatography. The purified enzyme had small subunit and large subunit with molecular weight of 16 KD and 53 KD, respectively, as estimated by SDS-PAGE. The molecular weight of holoenzyme was estimated to be 580 KD by Disc-PAGE. When the enzyme was injected into HPLC column (TSK G 3,000 SW) and eluted with 50 mM Tris buffer (pH 7.0) containing 0.5 M NaCl, there are three peaks corresponding to molecular weight of 17 KD, 120 KD and >300 KD, respectively. The activation of enzyme was temperature dependent and it took 50 min at 30°C to reach maximal activation. The fully activated enzyme was denatured by heat and the first ordered thermal inactivation rate constants at 50°C, 55°C, 57°C and 60°C were 0.003, 0.020, 0.029 and 0.162 min^{-1}, respectively. The activation energy for thermal denaturation was 20.8 Kcal/mole. The optimal pH range for enzyme activity was between pH 7.2 and 8.7. The dramatic activity change between pH 6.3 and 7.2 could be involved in light-dark transition regulation of RuBPCase activty.

Key words: *Oryza. sativa*; Ribulose-1, 5-bisphosphate carboxylase.

Introduction

Ribulose-1, 5-bisphosphate carboxylase (RuBPCase) catalyzes photosynthetic CO_2 fixation: Ribulose-1, 5-bisphosphate + CO_2 = 2 3-phosphoglycerate, which is the key step in photosynthesis (Miziorko and Lorimer, 1983). O_2 can compete with CO_2 for the enzyme active site and lead to the formation of phosphoglycolate and 3-phosphoglycerate. Since the C_3 plant lacks the CO_2 concentrating mechanism the competition of O_2 for the reduced carbon leads to great loss of fixed carbon. In C_3 plants, the kinetic properties of RuBPCase might be the key determinant for their efficiencies of photosynthesis.

[1] Paper No. 303 of the Scientific Journal Series, Institute of Botany, Academia Sinica. The work was supported by National Science Council, R. O. C.

Rice is a C_3 plant and is one of the most important crop in the world. It has been shown that rice RuBPCase activity is highly correlated with the net rate of photosynthesis and the level of this enzyme could be a limiting factor in photosynthesis throughout the life span of the rice leaf (Makino *et al.*, 1983a). There are several reports on the relationship between RuBPCase activities and rice Physiology (Makino *et al.*, 1983a, 1983b; Kabaki *et al.*, 1979; Kabaki and Tajima, 1982; Nakamura and Saka, 1978; Saka, 1977; Shieh and Liao, 1985). Little informations concerning the molecular properties of purified rice RuBPCase have been reported. Although Makino *et al.* (1983b) purified the RuBPCase from a cultivar of rice (*Oryza sativa* L. cv. Sasanishiki), no serious attempt was made to study the enzyme properties in detail. In the present work, we have purified the rice leaf RuBPCase from a local cultivar (*Oryza sativa* L. cv. Hsingchu 56) and study its properties.

Materials and Methods

Materials

Leaves of rice cultivar (*Oryza sativa* L. cv. Hsingchu 56) were obtained from the experimental farm of Academia Sinica at four leaf stage. Ribulose-1,5-bisphosphate (RuBP), polyvinylpolypyrrolidone, sodium dodecylsulfate (SDS), acrylamide and N′-N-methylenebisacrylamide were obtained from Sigma Co. Sodium [^{14}C] bicarbonate was purchased from Amersham International. Ammonium sulfate (Enzyme grade), BICINE and HCl were obtained from E. Merk Co. Ferritin, catalase and thyroglobin were obtained from Pharmacia. Fine Chemicals. Other reagents were of reagent grade.

Enzyme Assay

RuBPCase activities were assayed according to Pierce *et al.* (1982). The reaction mixture was placed in a scintillation vial, which included 890 μl of 25.3 mM $MgCl_2 \cdot 6H_2O$ in 0.1 M BICINE buffer (pH 8.2), 50 μl of 0.5 M $NaH^{14}CO_3$, and 10 μl of the activated enzyme solution. The solution was incubated at 30°C for 50 min. The vials were stoppered with rubber serum caps. The reaction was initiated by the addition of 50 μl of 10 mM RuBP through the serum cap by Hamilton syringe. After 2 minutes, the reaction was stopped by the addition of 200 μl of 4 N HCl (or 300 μl of 6 N acetic acid). After removing the serum caps, the vials were slowly heated to dryness in an air-driven oven at 70°C (in a hood). After cooling, 1 ml of H_2O was added and followed by 10 ml of scintillation cocktail. The cocktail used was READYSOLV™ EEP (BECKMAN). Radioactivity was counted with BECKMAN LC 7800 counter.

Purification of The Enzyme

Seventy grams of rice leaf (taken at four-leaf stage) was homogenized in 100

mM BICINE buffer (pH 8.2) containing 1 mM DTT, 1 mM EDTA and 2% polyvinylpolypyrrolidone by polytron homogenizer. This and all subsequent steps were performed at 4°C. The ground slurry was filtered through 3–4 layers of cheesecloth and centrifuged for 30 min at 10,000 rpm. The supernatant from this centrifugation was filtered through glass wool to remove suspended material. The supernatant was subjected to ammonium sulfate fractionation and the fractions precipitated at 37% to 50% saturation were collected. After centrifugation, the precipitated enzyme was gently resuspended in a minimal volume (6 ml) of the BICINE buffer. The enzyme solution was desalted by Sephadex G-50 column (2.5×45 cm) and then loaded onto a DEAE-Sepharose CL-6B column (2.5×45 cm). After washing with 600 ml of BICINE buffer, the column was eluted with a two liter linear gradient (0.1 to 0.4 M $NaHCO_3$ in BICINE buffer). The fractions containing most enzyme activities were collected and precipitated by 50% saturated ammonium sulfate and stored at -20°C.

Assessment of Purity

Disc gel electrophoresis of polyacrylamide gel (7%) was carried out according to Gabiel (1971) to monitor the extent of enzyme purification. SDS polyacrylamide slab gel electrophoresis with 13% acrylamide was carried out according to Laemmli (1970) to estimate the molecular weight of subunits. The standard proteins used were phosphorylase b (94 KD), bovine serum albumin (67 KD), ovalbumin (43 KD), carbonic anhydrase (30 KD), soybean trypsin inhibitor (20.1 KD), α-lactalbumin (14.4 KD).

Determination of Holoenzyme Molecular Weight

The holoenzyme molecular weight was determined according to Hedrick and Smith (1968). Polyacrylamide gel electrophoresis with three different gel concentrations (5%, 6%, 7%) was carried out to determine the relative mobility of enzyme. The slope of log Rm vs gel concentration is a linear function of molecular weight. The standard proteins used were thyroglobin (669 KD), ferritin (440 KD), catalase (232 KD).

Results

Purification of RuBPCase from Rice Leaves

After 37%–50% ammonium sulfate fractionation step, we have tried four different ways to purify the enzyme. 1. Sepharose 4B (2.5×70 cm) and then DEAE-cellulose chromatography (2.5×40 cm, elution with 0.1–0.6 M $NaHCO_3$ in BICINE buffer, pH 8.2). The obtained enzyme was estimated to be 70% pure. 2. Removing the salt with Sephadex G-25 first and then chromatofocusing with Mono P. The obtained

enzyme was not pure and was inactivated. 3. Octyl-Sepharose CL-4B column chromatography and eluted with linear gradient of 35% $(NH_4)_2SO_4$ in buffer and 80% ethyleneglycol in buffer. The obtained enzyme was pure but was precipitated and showed little activity. 4. According to the procedure described in "Method", we obtained the purified enzyme with high activity.

Structure of Enzyme

Estimated by SDS polyacrylamide gel electrophoresis, the rice RuBPCase had small subunit and large subunit molecular weight of 16 KD and 53 KD respectively (Fig. 1). The molecular weight of the holoenzyme was estimated to be 580 KD by gel electrophoresis (Fig. 2). This suggested that the rice RuBPCase had a L_8S_8 structure similar to the enzyme from other plant source (Mizioroko and Lorimer, 1983).

The purified RuBPCase was injected into high performance size exclusion liquid chromatography column (TSK-gel G 3000 SW, 75 mm ID×60 cm, M. W. range 1,000–300,000) and eluted with 50 mM Tris buffer (pH 7.0) containing 0.5 M NaCl. As shown in Fig. 3, there are three peaks appeared at elution volume 9.77 ml (void volume), 10.975 ml and 21.968 ml. Comparing with standard proteins (Fig. 4), these

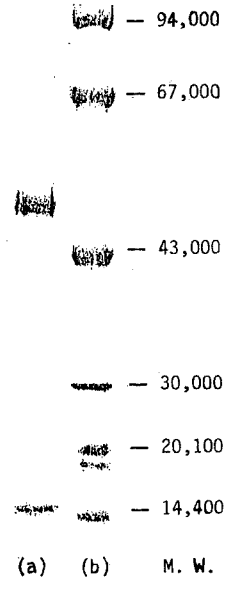

Fig. 1. SDS polyacrylamide gel electrophoresis pattern of purified rice RuBPCase (a) and standard protein(b) with 13% acrylamide according to Laemmli (1970).

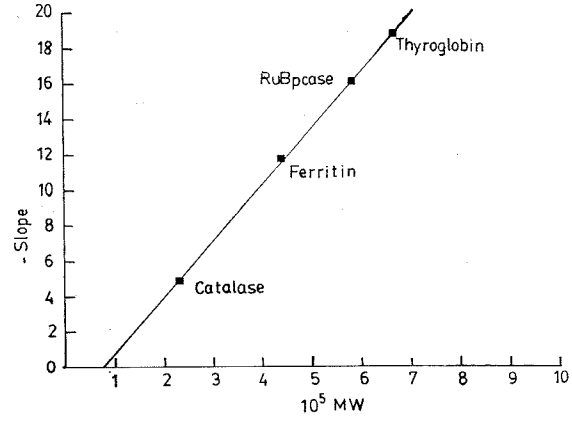

Fig. 2. Determination of holoenzyme molecular weight of rice RuBPCase according to Hedrick and Smith (1968). The slopes obtained from the plot of log mobility versus gel concentration (5%, 6% and 7%) were plotted against the protein molecular weight.

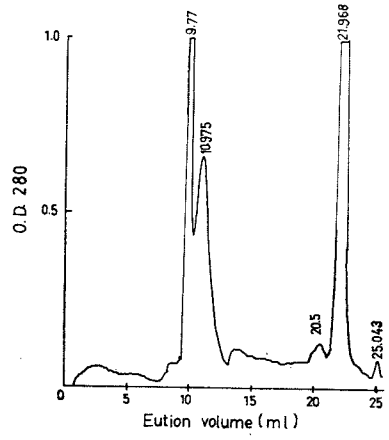

Fig. 3. High performance size exclusion liquid chromatogram of RuBPCase in TSK-gel (G-3,000 SW, 75 mm ID×60 cm). The elution buffer used was 50 mM Tris (pH 7.0) containing 0.5 M NaCl. The flow rate was 1 ml/min.

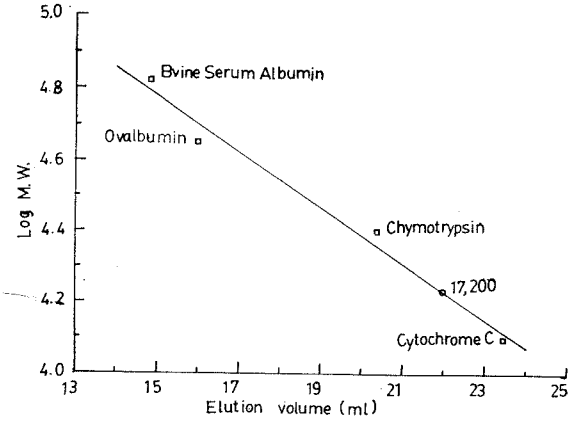

Fig. 4. Determination of molecular weight of RuBPCase by size exclusion HPLC. The last peak (Ve=21.968) in Fig. 3 was compared with standard proteins.

three peaks probably corresponded to holoenzyme (>300 KD), dimer of large subunit (M. W., 120 KD) and small subunit (M. W., 17 KD).

Effect of Temperature on the Activation of Enzyme

RuBPCase in activating medium at 4°C were incubated at different temperatures for various time intervals and then assayed for its activity. It is obvious that the kinetics of enzyme activation was temperature dependent (Fig. 5). At 22.5°C for 2 hours, the enzyme only reached 80% of maximal activity. It took 50 min at 30°C to reach maximal activation and the enzyme remained fully active without any denaturation for at least 2 hours.

Effect of pH on Enzyme Activity

RuBPCase was fully activated at 30°C, pH 8.2 and then placed in buffers of different pH to assay enzyme activity. As shown in Fig. 6, the optimal pH range was between pH 7.2 and 8.7. There were dramatic activity changes between pH 6.3 and 7.2; pH 8.7 and 9.4.

Thermostabilities

The fully activated enzyme was incubated at different temperatures for certain time and then assayed for the residual activity at 30°C. As shown in Fig. 7, the denaturation of RuBPCase followed first-order kinetics. The thermal inactivation rate constants at 50°C, 55°C, 57°C and 60°C were 0.003, 0.020, 0.029 and 0.162 min^{-1},

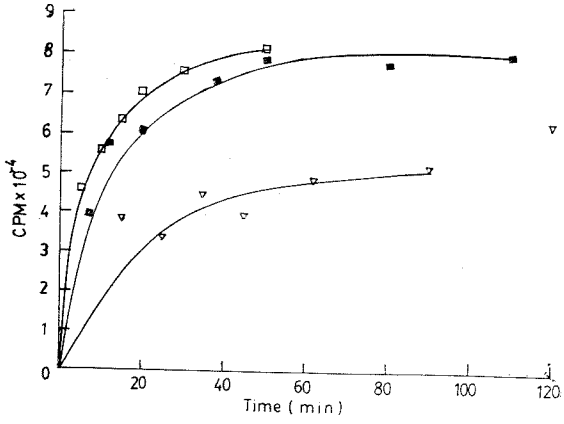

Fig. 5. The activation of rice RuBPCase at different temperatures. The enzymes were placed in activating medium at different temperatures for various time intervals and then assayed for its activity as described in "Methods". △, 22.5°C; ■, 30°C; □, 40°C.

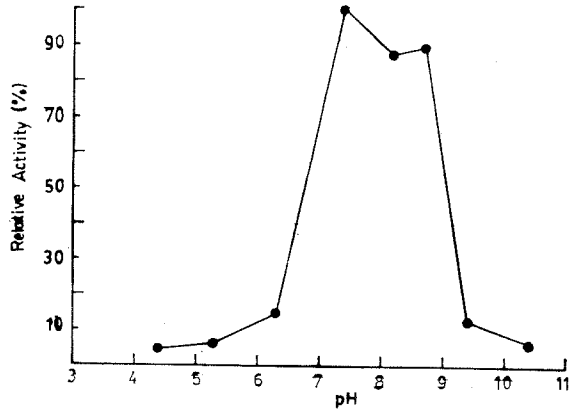

Fig. 6. PH-activity profile of RuBPCase. The enzyme was fully activated at 30°C and then placed in different pH buffers for enzyme assays. The buffers used were BICINE, CAPS, Sodium acetate and Bis-Tris Propane mixture (0.1 M each) containing 20 mM $MgCl_2$.

respectively. From the Arrehnius plot (Fig. 8), these data fitted a linear equation $\ln k = 121.8 - 41249 \times 1/T$ with linear correlation coefficient 0.99. The estimated activation energy for thermal denaturation reaction was 20.8 kcal/mole.

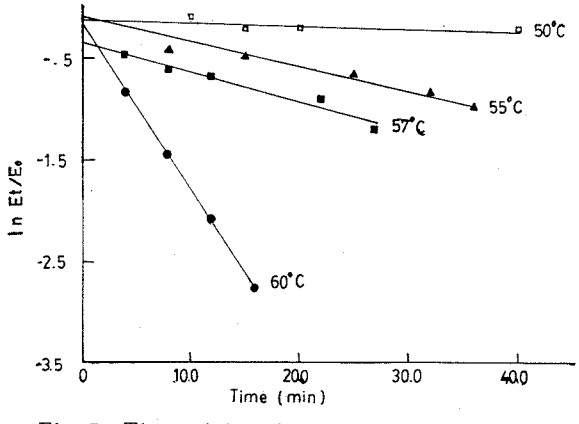

Fig. 7. Thermal inactivation of rice RuBPCase. The fully activated enzymes were placed at different temperatures for certain time intervals and then assayed at 30°C for residual activity.

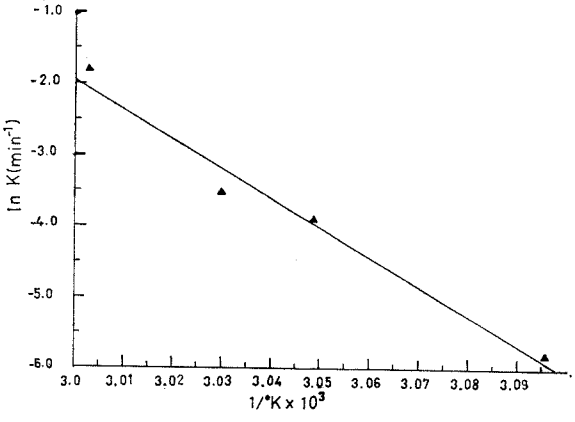

Fig. 8. Arrehnius plot of thermal inactivation rate constants at different temperatures. The data in Fig. 7 were used.

Discussion

The purified rice RuBPCase has a molecular weight of 580 KD as estimated by

disc gel electrophoresis (Fig. 2) and consists of two types of subunits with molecular weight 53 KD and 16 KD respectively as estimated by SDS-PAGE (Fig. 1). Therefore, this enzyme is similar to other plant RuBPCase with a L_8S_8 structure (Miziorko and Lorimer, 1983). The determination of molecular weight by different methods naturally subjected to some variation (Andrews and Abel, 1981). The holoenzyme was dissociated into three peaks in size exclusion HPLC column at pH 7.0 and 0.5 M NaCl. The last eluted peak (Fig. 3) is determined to be 17.2 KD which was presumably the small subunit. Therefore, the molcular weight determined by SDS-PAGE and HPLC column were somewhat different. The dissociation of RuBPCase under this condition is probably due to the high salt concentration (0.5 M NaCl) and suggests that the subunit interaction of RuBPCase could involve ionic force. Since some salt concentration is necessary to overcome the residual charge in TSK gel, it is difficult to study the effect of salts on the subunit association of this oligomeric enzyme. Other methods would be used to further study these phenomena.

It has been suggested that light-dark transition induced pH changes could be involved in the regulation of RuBPCase activity and CO_2 fixation. Werden *et al.* (1975) shown that illumination of spinach chloroplast increased the stroma pH from 7.2 to 8.0, which is just enough to switch CO_2 fixation by isolated chloroplast from zero to its maximal[2] rate. Since the rice RuBPCase showed sharp activity transition from pH 6.3 to 7.2, it is quite possible that the light-dark transition induced pH change in rice chloroplast is in this range and it might be the regulatory mechanism of CO_2 fixation through pH control of rice RuBPCase. Further studies on the pH changes and CO_2 fixation of rice chloroplast during light-dark transition would be necessary to answer this question.

Literature Cited

Andrews, T. J. and K. M. Abel. 1981. Kinetics and subunit interations of ribulose bisphosphate carboxylase from the cyanobacterium, *synechococcus* sp. J. Biol. Chem. **256**: 8445-8451.

Gabriel, O. 1971. Analytical disc gel electrophoresis. *In* W. B. Jakoby (ed.), Methods in Enzymol., Vol. 22. Academic Press, New York, pp. 565-568.

Hedrick, J. L. and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**: 155-164.

Kabaki, N., H. Saka, and S. Akita. 1979. Effect of nitrogen, phosphorus and potassium deficiencies on photosynthesis and RuBP carboxylase-oxygenase activities in rice plant. Japan. J. Crop. Sci. **48**: 378-384.

Kabaki N. and K. Tajima. 1982. Effect of phosphorus deficiency on RuBP carboxylase-oxygenase activity in rice plants. Japan. J. Crop Sci. **51**: 332-337.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Makion, A., T. Mae, and K. Ohira. 1983a. Photosynthesis and ribulose 1, 5-bisphosphate carboxylase in rice leaves. Plant Physiol. **73**: 1002-1007.

Makino, A., T. Mae, and K. Ohira. 1983b. Purification and storage of ribulose 1, 5-bisphosphate carboxylase from rice leaves. Plant Cell Physiol. **24**(6): 1169-1179.

Miziorko, H. M. and G. H. Lorimer. 1983. Ribulose 1, 5-bisphosphate carboxylase-oxygenase. Ann. Rev. Biochem. **52**: 507-535.

Nakamura, H. and H. Saka. 1978. Photochemical oxidants injury in rice plants III. Effect of ozone on physiological activities in rice plants. Japan. J. Crop Sci. **47**: 707-714.

Pierce, J. W., S. D. McCurry, R. M. Mulligan, and N. E. Tolbert. 1982. Activation and assay of ribulose 1, 5-bisphosphate carboxylase/oxygenase. *In* W. A. Wood (ed.), Method in Enzymology, Vol. 89. Academic Press, New York, pp. 47-55.

Saka, H. 1977. Changes in RuBP carboxylase activity of rice leaf blade at various growing stages. Japan. J. Crop Sci. **46**: 164-170.

Shieh, Y. J. and W. Y. Liao. 1985. Effect of nitrogen on growth and photosynthesis of rice of plants. Bot. Bull. Academia Sinica **26**: 127-146.

Werden, K., H. W. Heldt, and M. Milovancev. 1975. The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochim. Biophys. Acta **396**: 276-286.

水稻核酮醣二磷酸羧化酶之分子與動力學性質

蕭介夫 謝淑佑

中央研究院植物研究所

水稻核酮醣二磷酸羧化酶可經由硫銨分劃，Sephadex G-50 及 DEAE Sepharose CL-6B 層析純化。純化酵素含有大、小子單元而其分子量由 SDS-PAGE 估計各爲 5.3 及 1.6 萬。其全酵素分子量由 Disc-PAGE 估計爲58萬。在高效能液體層析管（TSK G 3000 SW）並以 50 mM Tris (pH 7.0) 含 0.5 M NaCl 緩衝液下層析，此酵素分成三個吸收帶相當於分子量爲 1.7，12 及 >30 萬。酵素之活化與溫度有關。在 30°C 下 50 分鐘酵素可以達到最大活性。充分活化之酵素可以加熱變性而其在 50°C, 55°C, 57°C 及 60°C 之一級變性反應速率常數各爲 0.003, 0.020, 0.029 及 0.162 min^{-1}，其變性反應活化能爲 20.8 Kcal/mole。酵素活性之最適 pH 值範圍爲 7.2 至 8.7。在 pH 6.3 至 7.2 間酵素之急遽活性變化可能與光-暗變化對其活性之調節有關。