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Short Communication

Ploidy Effects in Isogenic Populations of Alfalfa (*Medicago sativa* L.)[1]

IV. SIMILARITY IN PHYSICAL AND KINETIC PROPERTIES OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

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ABSTRACT

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) was isolated from isogenic diploid-tetraploid and tetraploid-octoploid sets of alfalfa (*Medicago sativa* L.) leaves. Molecular weights of RuBPCase subunits were similar across ploidy levels of both isogenic sets with subunits of 52,000 and 14,000. Apparent $K_m(CO_2)$ values and substrate specificity factors (V_cK_o/V_oK_c) of RuBPCase were similar across ploidy levels of both isogenic sets. These results indicate that ploidy had no effect on the kinetic properties of RuBPCase in alfalfa.

Increases in nuclear ploidy have been associated with changes in both the quantitative and qualitative expression of enzymes and isozymes (1, 4–6, 12, 22). It has been suggested that increased genetic expression of RuBPCase[3] (12) and a selective enhancement of carboxylase activity relative to oxygenase activity of RuBPCase (10, 11) may result in higher net photosynthetic rates and increased photosynthetic efficiency, respectively.

Joseph *et al.* (12) reported that the concentration of RuBPCase in leaf extracts and net photosynthesis increased significantly with ploidy in an allopolyploid series (4X, 6X, 8X, and 10X) of tall fescue (*Festuca arundinacea* Schreb.). However, concentrations of RuBPCase were not significantly different between ploidy levels of isogenic 2X–4X ryegrass (*Lolium perenne* L.) (21) and isogenic 2X–4X sets of alfalfa (*Medicago sativa* L.) (17). Cellular content of RuBPCase was found to increase proportionately with the amount of DNA per cell in these alfalfa polyploids (18).

Garrett (4) reported that purified RuBPCase from tetraploid ryegrass had a lower apparent $K_m(CO_2)$ value and a higher isoelectric point than RuBPCase from comparable isogenic diploids. Rathnam and Chollet (20) reported that tetraploid ryegrass protoplasts had a lower rate of photorespiratory glycolate formation relative to photosynthesis than did diploid protoplasts. However, subsequent studies by McNeil *et al.* (15) and Rejda *et al.* (21) demonstrated that there were no significant differences between isogenic diploid and tetraploid ryegrass in apparent $K_m(CO_2)$ values of RuBPCase. In addition, the diploid and tetraploid ryegrass cultivars were similar in mol wt and isoelectric points of RuBPCase subunits, RuBPCase activation and deactivation kinetics, and affinities for CO_2 of illuminated leaf protoplasts (15, 21).

The objectives of the present study were to compare several properties of RuBPCase isolated from each ploidy level of isogenic 2X–4X and 4X–8X alfalfa. Kinetic constants of RuBPCase were determined with a sensitive radiometric assay (10) for the simultaneous measurement of both RuBP oxygenase and carboxylase activities.

MATERIALS AND METHODS

Plant Material and Culture. The derivations of isogenic diploid-tetraploid (DDC 2X–4X) and tetraploid-octoploid (IC 4X–8X) alfalfa populations used in this study have been described (19). Plants were grown in an environmental chamber as in Meyers *et al.* (17).

Enzyme Preparation. RuBPCase was extracted and purified from leaves of each ploidy level of both isogenic sets of alfalfa according to Hall and Tolbert (7). Spinach (*Spinacia oleracea*) RuBPCase was purified as described (11). Further purification of the alfalfa RuBPCase was performed by eluting the RuBPCase-containing fractions through a Sephadex G-100 column (1.5 × 80 cm) equilibrated with 50 mM Tris (pH 7.6 at 4°C) and 10 mM 2-mercaptoethanol. The eluted protein was precipitated in 60% saturated $(NH_4)_2SO_4$ (pH 7.6) with 10 mM DTT, 2 mM EDTA and stored at −15°C. Prior to enzyme assays, the stored enzyme was precipitated by centrifugation at 10,000*g* for 25 min, dissolved in 20 mM Bicine (pH 8.0) with 0.1 mM EDTA, and dialyzed overnight at 4°C against 20 mM Bicine (pH 8.0) with 0.1 mM EDTA.

For electrophoresis, RuBPCase was rapidly isolated from leaf homogenates by immunoprecipitation. Alfalfa leaves (2 g) were homogenized in 10 ml of buffer (25 mM Tris, pH 8.6 at 5°C, 10 mM DTT) in a VirTis model 60K homogenizer (1 min at 30,000 rpm). Homogenates were filtered through 500 μm mesh nylon screen and centrifuged at 27,000*g* for 30 min at 4°C. Supernatants

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[3] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; PGA, 3-phosphoglycerate.

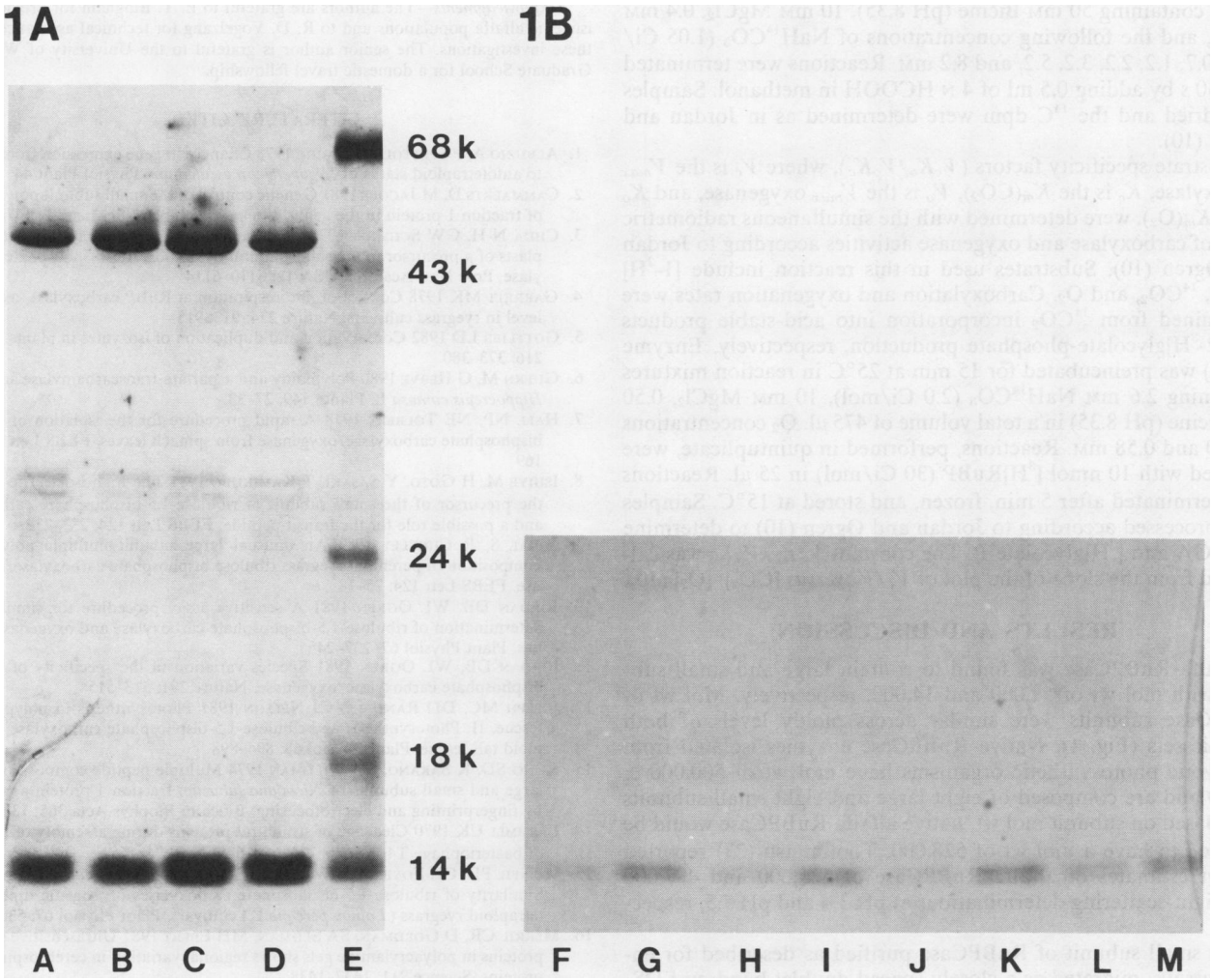

FIG. 1. A, SDS-electrophoresis in a 15% polyacrylamide slab gel (silver-stained) of RuBPCase (5 μg) isolated from: (A), DDC 2X; (B), DDC 4X; (C), IC 4X; and (D), IC 8X. Lane E contains the following mol wt standards: BSA, 68,000; ovalbumin, 43,000; trypsinogen, 24,500; β-lactoglobulin, 18,400; and lysozyme, 14,400. B, SDS-electrophoresis in a linear 10 to 15% polyacrylamide gradient (stained with Coomassie brilliant blue R-250) of RuBPCase small subunits from: (F, G), DDC 2X; (H, I), DDC 4X; (J, K), IC 4X; and (L, M), IC 8X. Lanes F, H, J, and L contain 5 g RuBPCase. Lanes G, I, K, and M contain 10 g RuBPCase.

(600 μl) were mixed with 200 μl of a solution containing 0.1 M NaCl, 15 mM NaN_3, and 400 μg rabbit anti-RuBPCase γ-immunoglobulins, prepared according to Meyers *et al.* (17), and stored at 4°C for 45 min. Immunoprecipitates were centrifuged at 3000*g* for 2 min and washed with homogenization buffer four times. Immunoprecipitates were dissolved into 100 μl of a solution containing 0.1 M DTT, 1 mM Na_2EDTA, 20% (w/v) sucrose, 2% (w/v) SDS, and 25 mM Tris (pH 8.6) for electrophoresis.

Electrophoresis. Subunits of RuBPCase were separated on 15% polyacrylamide gels and on a linear polyacrylamide gradient (10–15%) with a 4% polyacrylamide stacking gel in 0.1% (w/v) SDS, using the discontinuous buffer system described by Laemmli (14). Electrophoresis was performed at 20 mamp/gel for 4 h. Gels were stained for 12 h in 0.05% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid, and destained in 20% (v/v) methanol, 10% (v/v) acetic acid. The 15% gel was then silver stained according to Merril *et al.* (16) to show the small subunit better. Mol wts of RuBPCase subunits were estimated from R_F values using the following reference proteins as mol wt standards: phosphorylase B, 94,000; BSA, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsinogen, 24,500; soybean trypsin inhibitor, 21,000; β-lactoglobulin, 18,400; and lysozyme, 14,400.

Assay of RuBPCase. Apparent $K_m(CO_2)$ values were measured under a N_2 atmosphere using activated enzyme. Activation and assay solutions, in 3- and 9-ml serum stoppered vials, respectively, were flushed with N_2 for 7 min prior to the addition of CO_2-free buffer and $NaH^{14}CO_3$. RuBPCase (1.5 mg/ml was activated for 15 min at 25°C in 10 mM $NaH^{14}CO_3$ (1.05 Ci/mol), 50 mM Bicine (pH 8.35), and 10 mM $MgCl_2$.

Apparent $K_m(CO_2)$ values were determined from reactions at 25°C initiated with 20 μl of activated enzyme in a total volume of

Table I. *Kinetic Constants of Purified RuBPCase from Each Ploidy Level of Both Isogenic Alfalfa Sets and Spinach*

Species	$K_m(Co_2)$	V_cK_0/V_0K_c[a]
	μM	*ratio*
Alfalfa		
DDC 2X	14.9 ± 0.9[b]	85.5 ± 2.8[b]
DDC 4X	15.2 ± 0.6	79.0 ± 4.1
IC 4X	15.7 ± 0.2	81.6 ± 3.0
IC 8X	17.0 ± 1.2	77.1 ± 2.0
Spinach	14.6 ± 1.2	78.5 ± 3.2

[a] Substrate specificity factor, where V_c is the V_{max} carboxylase, K_c is the $K_m(CO_2)$, V_0 is the V_{max} oxygenase, and K_0 is the $K_m(O_2)$.

[b] Mean ± SE.

1.0 ml containing 50 mM Bicine (pH 8.35), 10 mM $MgCl_2$, 0.4 mM RuBP, and the following concentrations of $NaH^{14}CO_3$ (1.05 Ci/mol): 0.7, 1.2, 2.2, 3.2, 5.2, and 8.2 mM. Reactions were terminated after 30 s by adding 0.5 ml of 4 N HCOOH in methanol. Samples were dried and the ^{14}C dpm were determined as in Jordan and Ogren (10).

Substrate specificity factors (V_cK_o/V_oK_c), where V_c is the V_{max} carboxylase, K_c is the $K_m(CO_2)$, V_o is the V_{max} oxygenase, and K_o is the $K_m(O_2)$, were determined with the simultaneous radiometric assay of carboxylase and oxygenase activities according to Jordan and Ogren (10). Substrates used in this reaction include [1-^{3}H] RuBP, $^{14}CO_2$, and O_2. Carboxylation and oxygenation rates were determined from $^{14}CO_2$ incorporation into acid-stable products and [2-^{3}H]glycolate-phosphate production, respectively. Enzyme (60 μg) was preincubated for 15 min at 25°C in reaction mixtures containing 2.6 mM $NaH^{14}CO_3$ (2.0 Ci/mol), 10 mM $MgCl_2$, 0.50 mM Bicine (pH 8.35) in a total volume of 475 μl. O_2 concentrations were 0 and 0.58 mM. Reactions, performed in quintuplicate, were initiated with 10 nmol [^{3}H]RuBP (30 Ci/mol) in 25 μl. Reactions were terminated after 5 min, frozen, and stored at 15°C. Samples were processed according to Jordan and Ogren (10) to determine [^{14}C]PGA and [^{3}H]glycolate-P. The constant V_cK_o/V_oK_c was calculated from the slope of the plot of V_c/V_o *versus* $[CO_2]/[O_2]$ (10).

RESULTS AND DISCUSSION

Alfalfa RuBPCase was found to contain large and small subunits with mol wt of 52,000 and 14,000, respectively. Mol wt of RuBPCase subunits were similar across ploidy levels of both isogenic sets (Fig. 1). Native RuBPCase enzymes isolated from eukaryotic photosynthetic organisms have mol wt of 500,000 ± 50,000 and are composed of eight large and eight small subunits (25). Based on subunit mol wt, native alfalfa RuBPCase would be expected to have a mol wt of 528,000. Tomimatsu (23) reported mol wt estimates of alfalfa RuBPCase of 526,000 and 497,000 from light-scattering determinations at pH 3.4 and pH 7.5, respectively.

The small subunit of RuBPCase purified as described for enzyme assays, migrated as a closely spaced doublet band on SDS-polyacrylamide gels. Storage of purified RuBPCase in 60% saturated $(NH_4)_2SO_4$ (pH 7.6), 2 mM EDTA at 4°C had no apparent effect on the relative staining intensity of each band of the doublet (data not shown). Rapid isolation of RuBPCase from leaf homogenates by immunoprecipitation also resulted in the appearance of the doublet band in polyacrylamide gels (Fig. 1). The existence of the small subunit doublet in alfalfa may be related to specific proteolytic processing (3), a nonspecific proteolytic event, or the existence of different alleles (2, 8, 9, 13, 24).

Apparent $K_m(CO_2)$ values of alfalfa RuBPCase were nearly equal across ploidy levels of both isogenic sets (Table I). Alfalfa RuBPCase had $K_m(CO_2)$ values similar to those of RuBPCase from spinach (Table I), ryegrass (15), and other C_3 plants (11).

The substrate specificity factor (V_cK_o/V_oK_c) determines relative carboxylase and oxygenase reaction rates as a function of the CO_2 and O_2 concentrations (10). Specificity factors of alfalfa RuBPCase were similar across ploidy levels of both isogenic sets as well as to RuBPCase from other C_3 plants (10, 11).

In conclusion, ploidy had no effect on kinetic properties and subunit mol wt of RuBPCase from isogenic alfalfa populations. These results are consistent with comparisons between RuBPCases from isogenic 2X–4X ryegrass reported by McNeil *et al.* (15) and Rejda *et al.* (21).

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