Ribulose-1,5-bisphosphate Carboxylase/Oxygenase and Polyphenol Oxidase in the Tobacco Mutant Su/su and Three Green Revertant Plants

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ABSTRACT

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) was crystallized from a heterozygous tobacco (Nicotiana tabacum L.) aurea mutant (Su/su), its wild-type sibling (su/su), and green revertant plants regenerated from green spots found on leaves of haploid Su plants. No differences were found in the specific activity or kinetic parameters of this enzyme, when comparing Su/su and su/su plants of the same age, which had been grown under identical conditions. The enzyme crystallized from revertant plants was also identical to the enzyme from wild-type plants with the exception of one clone, designated R2. R2 has a chromosome number approximately double that of the wild-type (87.0 ± 11.1 versus 48). The enzyme from R2 had a lower V_{max} for CO₂, although the Kₘ values were identical to those for the enzyme from the wild-type plant. The enzyme from all mutant plants had identical isoelectric points, identical molecular weight as demonstrated by migration on native and sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the same ratio of large to small subunits as the enzyme from the wild-type. The large subunit of the enzyme from tobacco leaves exhibited a different electrophoretic pattern than did the large subunit from spinach: there were two to three bands on SDS-polyacrylamide gels for the tobacco enzyme whereas the enzyme from spinach had only one species of large subunit.

Total polyphenol oxidase activity was the same in leaves from the heterozygous mutant (Su/su) and wild-type (su/su) plants when correlated with developmental age as represented by morphology rather than by the chronological age of the plants. There was a marked increase in the soluble activity of this enzyme with increasing age of both plant types and also as a result of varying environmental conditions. Ribulose-1,5-bisphosphate carboxylase/oxygenase activity correlated inversely with increases in the soluble activity of polyphenol oxidase in crude homogenates from which the carboxylase/oxygenase was crystallized over a generation of Su/su and su/su plants. Criteria are outlined for determining if differences in activity of ribulose-1,5-bisphosphate carboxylase/oxygenase are caused by an effect of polyphenol oxidase activity and/or by some other extrinsic parameter.

The heterozygous tobacco aurea mutant Su/su (2), which resulted from a nuclear mutation, has been reported to have higher rates of photorespiration than its wild-type sibling. John Williams Broadleaf, su/su (19, 26). Kung and Marsho (11) examined the activity of ribulose-P₅ carboxylase/oxygenase (EC 4.1.1.39) and by isoelectric focusing looked for structural changes in the small subunit of the enzyme. Their results indicated lower activity for both reactions in the crystalline enzyme from the mutant plant Su/su; ribulose-P₅ carboxylase had approximately 57% and ribulose-P₂ oxygenase 40% of the activity found in the wild-type plant su/su. By isoelectric focusing, no differences were seen in the polypeptides of the large or small subunit of the enzyme from both plant types. Okabe (18, 19) has reported that ribulose-P₂ carboxylase/oxygenase from the mutant Su/su has a higher Kₘ(CO₂) and a lower Kₘ(O₂) in a crude desalted extract than does the enzyme from su/su. Because these results would be significant in understanding the ratio of photorespiration to photosynthesis, we sought to reproduce them with the crystalline enzyme prepared from these plants. Because of large fluctuations in the activity of the ribulose-P₂ carboxylase/oxygenase it was necessary to compare its total and specific activity from Su/su and su/su plants of the same age. This was done weekly, from the seedling state of development through the beginnings of senescence, for a single group of plants in an attempt to correlate changes in enzyme activity with plant age, growth conditions, and polyphenol oxidase activity.

Several kinetic properties of the ribulose-P₂ carboxylase/oxygenase from the wild-type su/su, the heterozygous mutant Su/su, and three different revertants isolated from haploid Su plants were examined. While our work was in progress, Garrett (6) reported that in ryegrass (Lolium) changes occur in the isoelectric point of the holoenzyme and in the Kₘ(CO₂) for ribulose-P₂ carboxylase/ oxidase due to a change in ploidy level in the plant from diploid to tetraploid. One of our tobacco revertants, R2, also has a high ploidy level (chromosome number of 87.0 ± 11.1). We, therefore, examined several properties of the enzyme from this clone of plants.

Polypheol oxidase is a chloroplast enzyme which is associated primarily with the thylakoids (16, 23). In the presence of soluble polyphenol oxidase in homogenates from leaves, other enzymes are inactivated (14, 16). Ribulose-P₂ carboxylase/oxygenase has been shown to be structurally altered in extracts, prepared for enzyme crystallization, in which there is high polyphenol oxidase activity (7). Hence we have compared the development of soluble polyphenol oxidase in the heterozygous mutant Su/su and the

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3 Abbreviations: ribulose-P₂, ribulose 1,5-bisphosphate; PVPP, insoluble polyvinylpolypyrrolidone; Bicine: N,N'-bis(2-hydroxyethyl)glycine; l-DOPA: l-dihydroxyphenylalanine.
wild-type plant su/su in an attempt to correlate its activity with changes in ribulose-P₂ carboxylase/oxygenase activity.

MATERIALS AND METHODS

Plants used included the semidominant aurea mutant of Nicotiana tabacum L., Su/su, first described by Burk and Menser (2), and its wild-type sibling, John Williams Broadleaf, su/su. Seeds were obtained from selfing of Su/su plants. Yellow haploid Su plants were derived from another cultures of Su/su plants and were maintained by heterotropic growth in a Linsmaier and Skoog medium (13) supplemented with 20% sucrose and 1 mg/l IAA. Revertant plants were regenerated from leaf tissue of haploid Su plants (10). These revertants were phenotypically distinct from both Su/su and su/su plants. Seeds from the heterozygous mutant Su/su were germinated and the segregating heterozygous mutant and wild-type plants were grown in Bacto potting soil in a growth chamber with a light intensity of 600 μE m⁻² s⁻¹. Between 60 and 70 days postgermination, plants were transplanted to pots containing a 1:1 mixture of Bacto potting soil with Vermiculite and placed in a greenhouse. Regenerated revertant plants were grown under the same conditions. Leaves used for comparing enzyme activity with plant age were taken from similar nodes on plants of different genetic character, but of the same age, grown under identical conditions.

Ribulose-P₂ Carboxylase/Oxygenase. This enzyme was crystallized as previously described (3, 21) except that 2% PVPP was added to the grinding buffer to protect partially against polyphenol oxidase activity released during grinding of the tobacco leaves. This procedure involved homogenization, filtration through Sephadex G-25, and protein concentration by vacuum dialysis. The enzyme was crystallized by dialysis in collodion bags against a NaCl-free buffer. Crystallization was done only once, as it was found that recrystallization had no effect on enzyme specific activity or purity as shown by native and SDS-polyacrylamide gel electrophoresis. The crystals were dissolved in a buffer containing 100 mM Bicine (pH 8.3), 10 mM MgCl₂, 0.25 mM EDTA, and 200 mM NaCl, and a stock solution was adjusted to 2 mg protein/ml as measured by A at 280 nm (22). A heat treatment at 50 C had no influence on enzyme activity. For experiments on changes in enzyme activity with plant age, assays for total activity of ribulose-P₂ carboxylase/oxygenase were run on the leaf extracts prior to passing the crude homogenate over a Sephadex G-25 column.

Before assay, the enzyme was activated by incubation for at least 30 min with 10 mM NaHCO₃ and 1 mM DTT in the dissolving buffer. The ribulose-P₂ carboxylase assays were initiated by the addition of 10 μl 12.5 mM ribulose-P₂ to 240 μl assay mixture containing 100 mM Bicine (pH 8.3), 20 mM MgCl₂, 0.25 mM EDTA, 10 mM NaH₄CO₃, 1 mM DTT, and 20 μg ribulose-P₂ carboxylase/oxygenase and were run at 30 C for 0.5 or 1 min. For Kₘ determinations, the ribulose-P₂ carboxylase assays were initiated by the addition of 10-μl aliquots of activated enzyme to the same reaction mixture, except that the concentrations of NaH₄CO₃ and ribulose-P₂ were varied. Activity was determined as ¹⁴C acid-stable product as measured by scintillation counting.

The ribulose-P₂ oxygenase assay was based on ribulose-P₂ dependent O₂ uptake as measured in a Rank Brothers O₂ electrode. Assays were initiated by the addition of 20 μl 12.5 mM ribulose-P₂ to a reaction mixture consisting of 480 μl 100 mM Bicine (pH 8.3), 20 mM MgCl₂, 0.25 mM EDTA, 1 mM DTT, and 40 μg ribulose-P₂ carboxylase/oxygenase which had been activated as described above and the reaction mixture was at temperature equilibrium for approximately 1 min. The reactions were stopped at 30 C and monitored for 50–60 s with a chart recorder; rates were taken only from the initial linear portion of the curves. These rates were not greatly lower than rates measured in reactions initiated by the addition of CO₂ activated enzyme.

After the initial experiments were run, another protocol for the oxygenase assay was developed (15), and was used when determining Kₘ (O₂) values. Reactions were initiated by the addition of 20 μl of activated enzyme to 480 μl of reaction mixture containing 100 mM Bicine (pH 8.3), 20 mM MgCl₂, 0.25 mM EDTA, 1 mM DTT, 0.5 mM ribulose-P₂, and variable amounts of O₂. The buffer was made from boiled distilled-deionized H₂O and was further degassed under vacuum and stored under N₂. Precise O₂ concentrations were obtained by aerating the reaction solution in the O₂ electrode chamber with O₂ and N₂ gases through a rubber serum stopper prior to addition of the enzyme. Calibrations were made using 1.12 mm as 100% O₂ saturation and 0.235 mm as the concentration obtained from 21% O₂ (24). There was a small amount of HCO₃⁻ in this assay because of carry over with the activated enzyme and the presence of K₂CO₃ in the KOH used to adjust the pH of the reaction buffer. However, Lorimer et al. (15) have demonstrated that the amount carried over is not enough to compete significantly with the oxygenase activity.

For comparisons of polyphenol oxidase activity from the heterozygous mutant Su/su and wild-type su/su plants, leaves were taken from plants of the same age grown either in a growth chamber or greenhouse as indicated. Leaves were ground with a mortar and pestle in a 2:1 ratio of grinding buffer to leaf weight. The grinding buffer was 50 mM Tris-HCl (pH 7.4), 1 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, and 2% PVPP. This is the same buffer used to grind tobacco leaves for the crystallization of ribulose-P₂ carboxylase/oxygenase. The crude extracts were either centrifuged at 27,000g for 30 min and used immediately or aged for approximately 40 h at 4 C prior to centrifugation during which time the amount of polyphenol oxidase activity increased (23).

Polyphenol oxidase was assayed spectrophotometrically (23) and also as l-DOPA-dependent O₂ uptake in a Rank Brothers O₂ electrode at 30 C. Assays were initiated by the addition of 20 μl of extract to 980 μl 30 mM phosphate and 50 mM citric acid buffer (pH 7.0), and 6 mM l-DOPA. Reactions were monitored for 1–2 min.

Polyacrylamide gel electrophoresis (5) was run with 10–50 μg protein applied/gel tube (100 × 5 mm). Gels consisted of 5% acrylamide and were run at 3.5 mamp/tube until the tracking dye (bromphenol blue) reached the bottom of the tube. SDS-polyacrylamide gel electrophoresis (25) was run in tube gels (100 × 5 mm). Gels consisted of 10% acrylamide, and 10–50 μg protein were placed on each gel with an applied current of 8 mamp/tube until the tracking dye reached the bottom of the tube. Nondenaturing isoelectric focusing was done in polyacrylamide tube gels according to the procedure of O'Farrell (17), except that urea was excluded from all buffers. Gels consisted of 7% acrylamide and were run for a total of 600–700 h/tube. All gels were fixed and stained overnight in 0.1% Coomassie blue R, 10% (v/v) trichloroacetic acid, and 25% (v/v) isopropanol, and then destained in 7.5% (v/v) acetic acid and 20% (v/v) methyl alcohol containing Rexyn 1-300 mixed bed ion exchange resin (Fisher Scientific Co.). Gels were scanned at 600 nm with a Gilford gel scanner.

Protein concentrations in crude extracts were determined using a Protein Lowry procedure (1). Ribulose-P₂ was prepared enzymically (9). Sephadex G-25 was from Pharmacia Fine Chemicals, NaH₄CO₃ from Amersham/Searle, and acrylamide gel materials from Bio-Rad. Other reagents were from Sigma.

RESULTS

Our initial objective was to examine the ratio of ribulose-P₂ carboxylase to oxygenase activity in leaves from the wild-type and mutant plants. In preliminary experiments, widely varying specific activities were obtained. Therefore the effect of plant age, the effect of growing plants in the growth chamber versus the greenhouse, and the effect of polyphenol oxidase content were examined. For the ribulose-P₂ carboxylase/oxygenase experiments, leaves were taken from a group of plants of the same age which
had been germinated and grown under identical conditions (Figs. 1, 2, and 4). Each weekly repetition of this experiment with one generation of plants was done with the same plants. A top leaf was removed at each cited age. This leaf was approaching a stage of full expansion and was approximately the fourth visible leaf from the apex. However, for the two earliest ages (20 and 40 days from planting), it was necessary to harvest entire plants to obtain enough material for crystallization of the enzyme. Thus, ages cited are for the whole plant, but the leaf examined was near the top of the plant and was of the same age, about 1 week old. Crystalline enzyme was prepared at each plant age for comparison of its specific activity from leaves of the mutant Su/su and the wild-type su/su plants.

Effect of Age and Growth Condition On Total and Specific Activity of Ribulose-P5 Carboxylase/Oxygenase. When analyzed on a weekly basis, the specific activity of isolated crystalline ribulose-P5 carboxylase (Fig. 1A) and ribulose-P5 oxygenase (Fig. 2A) had highly significant differences (P of 0.01 for the carboxylase and 0.05 for the oxygenase). Specific carboxylase activity of the enzyme from these top leaves was greatest in young plants, 44 days old, which were grown in the growth chamber, and then declined until values one-third to one-half the peak specific activity were measured with enzyme crystallized from plants 66 days old. At this time, the plants were transferred to the greenhouse; subsequently the specific activity of the crystalline enzyme from the top leaves increased again, reaching maximum values at 80–85 days of plant age. After this peak, there was a slow decrease in specific activity of the crystalline enzyme from plants of increasing age. These changes in specific activity of the crystalline enzyme were essentially the same in Su/su and su/su plants. Similar changes with age and growth conditions were also observed in the activity of the enzyme in the crude leaf extracts, expressed as activity per mg of total soluble protein, for both the carboxylase (Fig. 1B) and the oxygenase (Fig. 2B). These changes in activity indicate that a comparison between Su/su and su/su plants must be done with leaves from plants of the same age. Likewise, any changes in the ratio of carboxylase to oxygenase activity must be evaluated in leaves developed under exactly the same growth conditions.

In spite of a significant change in specific activity on a weekly basis, there was no significant difference in the specific activity of ribulose-P5 carboxylase when comparing all the data from the heterozygous mutant Su/su with the wild-type su/su by an F test. The mean carboxylase specific activity was 371 ± 121 and 330 ± 101 nmol CO2 min−1 mg−1 protein for Su/su and su/su, respectively. The mean oxygenase activity from the two plant types averaged 53 ± 12 nmol O2 min−1 mg−1 protein for Su/su and 45 ± 13 for su/su, under the assay conditions (air) employed. Although the differences for the oxygenase could be judged significant by an F test, there was wide variability as shown by the overlap in SD of the means. The oxygenase assay was subject to more variability than the carboxylase assay, especially since the enzyme was rapidly losing activity in the CO2-free medium necessary for the oxygenase assays.

Carboxylase activity in crude homogenates of these tobacco leaves averaged 189 ± 90 nmol CO2 min−1 mg−1 protein from Su/su plants and 276 ± 156 for su/su. Total oxygenase activity in the crude extracts was 13.2 ± 8.5 nmol O2 min−1 mg−1 protein for the Su/su mutant and 15.2 ± 12.8 for the su/su wild-type plants. Although these values varied widely, there was no significant difference in total activity on a protein basis between Su/su and su/su plants when the data were analyzed with an F test.

Two anomalies were observed. At 80–90 days, total activity in the crude extract from the wild-type Su/su plants was as high on a protein basis as was the specific activity for the crystalline enzyme from the same extract (Fig. 1, A and B). This might be explained by postulating activators in the crude extract or partial inactivation of the enzyme during crystallization, but the reason for low specific activity in the crystalline enzyme remains unknown. Another difficulty encountered was the endogenous rate of O2 uptake in the oxygenase assay. Ribulose-P5 oxygenase activity in the crude extract from plants older than 107 days is not given because the endogenous rate of O2 uptake became so high that accurate measurements were impossible. In fact, when ribulose-P5 was added to leaf extracts from these older plants, although the leaves were newly developed, the rate of O2 uptake was often depressed over the high endogenous rate.

Effect of Age and Growth Conditions on Polyphenol Oxidase. Measurable polyphenol oxidase activity in the leaf extracts appears to be affected by the developmental stage of the plant. In the wild-type Su/su plant grown in the growth chamber, soluble polyphenol oxidase activity first appeared in freshly prepared homogenates from top leaves when the plants were about 66 days old (Fig. 3). The appearance of activity in Su/su lagged behind...
The first appearance in su/su plants by approximately 1 week. However, when based on the morphological and developmental state of the plants rather than their chronological age, the time of appearance of soluble polyphenol oxidase is about the same in both plants.

Soluble polyphenol oxidase activity appeared at an earlier age in plants grown in the growth chamber than in those grown in the greenhouse. Activity was first present in aged extracts from su/su plants raised in the growth chamber for 40 days; by 47 days the activity (=60 nmol O₂ uptake min⁻¹ ml⁻¹ extract) was present in top leaves from both su/su and Su/su plants. Su/su and su/su plants of identical age, but grown in the greenhouse, had no soluble polyphenol oxidase activity at these ages.

It has been shown that polyphenol oxidase activity in leaf extracts is responsible for some enzyme inactivation (14), and that it modifies the polyepptide pattern of the large subunit of ribulose-P₂ carboxylase/oxygenase (7). In this study, correlation of changes in the activity of ribulose-P₂ carboxylase/oxygenase with induction and amount of soluble polyphenol oxidase activity in the crude extract was noted. The large decline in specific activity of the crystalline ribulose-P₂ carboxylase/oxygenase from plants grown in the growth chamber from 44 to 66 days occurred at the same time as the rise in soluble polyphenol oxidase activity (Figs. 1 and 3). When these plants were then transferred to the greenhouse, there was a concomitant drop in soluble polyphenol oxidase activity (data not shown) and a rise in the total and specific activity of the ribulose-P₂ carboxylase/oxygenase (Figs. 1 and 2). Thereafter, the gradual decline in specific activity of the ribulose-P₂ carboxylase/oxygenase from the top leaves of older plants correlated with the gradual increase in activity of soluble polyphenol oxidase. These fluctuations in the ribulose-P₂ carboxylase/oxygenase activity occurred in spite of the fact that the homogenizing medium contained 2% PVPP to remove the naturally occurring phenolic substrates of polyphenol oxidase, whose quinone oxidation products in part account for enzyme inactivation. Such extracts did not turn dark brown, but PVPP treatment did not remove the polyphenol oxidase which has been shown to modify other proteins (14).

Effects of Age and Growth Conditions on the Ratio of Ribulose-P₂ Carboxylase to Ribulose-P₂ Oxygenase from Su/su and su/su Plants. The data in Figure 4 that were used in calculating these ratios were taken from Figures 1 and 2. The assays had been run in identical buffers, at the same pH and temperature, under air, and as near in time as was possible (within 4 h of each other). The mean carboxylase/oxygenase ratio for the crystalline enzyme from the mutant Su/su was 7.1 ± 2.0 and 7.5 ± 2.0 for the wild-type su/su. This difference was judged not significant based on an F test. The variations in the ratio from week to week were highly significant (F = 0.01) ranging from 4.4 to 12.2 (Fig. 4A).

The reliability of the ratio determination using activity in crude extracts was not great due to high endogenous rates of O₂ uptake in extracts from increasingly older plants. The difference between the average ratios of 23.1 ± 9.3 for su/su and 17.0 ± 6.7 for Su/su (Fig. 4B) is significant according to an F test; however this may simply be a reflection of the earlier induction of polyphenol oxidase activity in su/su than in Su/su which would produce higher endogenous rates of O₂ uptake at an earlier age (previous section). Differences in the ratio of activity in crude extract from week to week are highly significant (Fig. 4B).

A much higher ratio of ribulose-P₂ carboxylase to ribulose-P₂ oxygenase activity was measured in crude extracts compared with crystalline enzyme preparations as we previously reported (ref. 8, Fig. 4). The reason for this change is not known. However, our data indicate that it occurs because ribulose-P₂ carboxylase in crude extracts is 50-85% as active as the crystalline enzyme, whereas ribulose-P₂ oxygenase is only 25-35% as active in crude extracts as in the crystalline enzyme, when each is measured on a per mg protein basis.

Kinetic Parameters of Ribulose-P₂ Carboxylase/Oxygenase from Su/su, su/su and Three Revertant Plants Recovered from Haploid Su. Vₘₐₓ and Kₐₑₜ for the crystalline enzyme from leaves of the mutant Su/su, its wild-type sibling, su/su, and three revertant plants isolated from haploid Su (10) are presented in Table I. There were no differences found in any of the kinetic parameters when comparing the carboxylase/oxygenase from su/su plants with the mutant Su/su or two of the revertants. The only exception was with the revertant R2. This plant has a chromosome number of 87.0 ± 11.1, is homozygous for Su, and phenotypically variant, although it is still green (10). The enzyme from the revertant R2 had the same apparent Kₐₑₜ(CO₂) and Kₐₑₜ(ribulose-P₂) as did the wild-type su/su, but its Vₘₐₓ for CO₂ was 50% lower. This lower Vₘₐₓ was observed in numerous preparations of the carboxylase from leaves of R2, but because of enormous changes which we have seen in the specific activity of the carboxylase/oxygenase from leaves of different developmental stages, and under different growth conditions, the reason for the apparently

![Figure 3](image-url)  
**Fig. 3.** Induction of polyphenol oxidase activity in tobacco plants grown in the growth chamber. Leaves were washed, de veneined and homogenized in a Waring blender for 1 min. The homogenate was then filtered through four layers of Miracloth and centrifuged at 27,000g for 30 min. Reactions were initiated with 20 µl of extract and were followed spectrophotometrically (30). (●●●); Wild-type, su/su; (■■■); mutant, Su/su.

![Figure 4](image-url)  
**Fig. 4.** The ratio of ribulose-P₂ carboxylase to ribulose-P₂ oxygenase activity as a function of plant age and growth conditions. (▲▲▲): Wild-type, su/su; (●●●); mutant, Su/su. These values were calculated from the data in Figures 1 and 2.
Table I. Properties of Crystalline Ribulose-1,5-Bisphosphate Carboxylase/ 
Oxygenase from Various Tobacco Mutants

Values obtained are means ± SD of at least three determinations, each on at least three enzyme preparations.

<table>
<thead>
<tr>
<th>Plant</th>
<th>(K_m (\text{HCO}_3^-))</th>
<th>(K_m (\text{ribulose-P}_2))</th>
<th>(K_m (\text{O}_2))</th>
<th>(V_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{su/su})</td>
<td>4.0 ± 2.6</td>
<td>47 ± 1</td>
<td>706 ± 186</td>
<td>330 ± 101</td>
</tr>
<tr>
<td>(\text{Su/su})</td>
<td>5.1 ± 1.4</td>
<td>44 ± 12</td>
<td>693 ± 283</td>
<td>371 ± 121</td>
</tr>
<tr>
<td>R1</td>
<td>4.2 ± 2.8</td>
<td></td>
<td>319 ± 136</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>4.1 ± 1.8</td>
<td>52 ± 17</td>
<td>148 ± 99</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>4.5 ± 2.8</td>
<td>55 ± 17</td>
<td>324 ± 187</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Protein Ratio of Large to Small Subunits of Ribulose-P2 
Carboxylase/Oxygenase

Large and small subunits were dissociated with SDS-polyacrylamide gel electrophoresis on 10% gels. Gel scans were made at 600 nm and ratios determined from relative areas under the peaks obtained.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Experiment</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>3</td>
<td>3.01 ± 0.25</td>
</tr>
<tr>
<td>(\text{su/su})</td>
<td>1</td>
<td>3.13 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.97 ± 0.16</td>
</tr>
<tr>
<td>R2</td>
<td>1</td>
<td>2.43 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.90 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.17 ± 1.17</td>
</tr>
<tr>
<td>R3</td>
<td>2</td>
<td>2.81 ± 0.15</td>
</tr>
</tbody>
</table>

* Ratios are means ± SD of at least two determinations.

variant \(V_{\text{max}}\) is uncertain. At present, the increased ploidy level of R2, which is a major difference between this plant and \(\text{Su/su}\), may be postulated to be related to the altered \(V_{\text{max}}\) of the carboxylase.

Physical Parameters of Ribulose-P2 Carboxylase/Oxygenase 
from \(\text{Su/su}, \text{su/su},\) and Three Revertants of Haploid \(\text{Su}\). Since our R2 tobacco plants had a high ploidy level and variant ribulose-P2 carboxylase activity, and since Garrett (6) had demonstrated altered kinetic and physical parameters for the enzyme in rye-grasses differing in ploidy level, several physical properties of ribulose-P2 carboxylase/oxygenase from \(\text{Su/su}\) and \(\text{su/su}\) were compared with those of enzyme from the revertant R2.

One hypothesis to explain Garrett’s results is that the ratio of large to small subunits may be different in the carboxylase/oxygenase from the tetraploid plants as compared to the enzyme from diploid plants. To examine this, the mol wt of the holoenzymes from several plants were compared on native gels (5% polyacrylamide). Enzyme from all the tobacco plants migrated identically, indicating that the mol wt of the holoenzymes were the same (data not shown). Carboxylase/oxygenase from tobacco leaves was dissociated with SDS and the large and small subunits were separated by SDS-polyacrylamide gel electrophoresis. The ratio of amount of large subunit to small subunit was estimated based on the area under the respective protein peaks obtained by scanning the gels at 600 nm after staining with Coomassie blue. There were no differences in this ratio for any of the plants (Table II). Quantifying the amounts of protein with this dye is not totally reliable, but our results are similar to those reported by Kung et al. (12) using three different methods. Isoelectric focusing of the holoenzyme was also done under nondenaturing conditions. Isoelectric points were identical for enzyme from all the plants (data not shown).

The large subunit of the crystalline enzyme from tobacco leaves dissociated into two to three bands when run on SDS-polyacrylamide gels with or without carboxymethylation. This occurred regardless of the method used to purify the enzyme (crystallization [3, 21] or the chromatographic purification used by Ryan and Tolbert [20]). Older preparations of the enzyme from tobacco had a greater number of these anomalous bands. Ribulose-P2 carboxylase/oxygenase from spinach leaves exhibited only one large subunit band.

DISCUSSION

Crystalline ribulose-P2 carboxylase/oxygenase from the heterozygous tobacco mutant \(\text{Su/su}\) and its wild-type sibling \(\text{su/su}\) had identical kinetic and physical parameters. Specific activities of enzyme preparations were examined weekly from a population of plants, both over a generation, and found to be identical for both plants. Kung and Marsho (11) have shown that there are no differences in the isoelectric points of polypeptides of denatured enzyme from the two plants. Therefore, a reevaluation of previous literature about the yellow \(\text{Su/su}\) tobacco mutant is in order.

Zelitch and Day (26) reported that heterozygous mutant \(\text{Su/su}\) leaves had a higher rate of photorespiration than wild-type \(\text{su/su}\). However, Chollet (4) has questioned the validity of the light/dark \(^{14}\text{CO}_2\) assay of photorespiration which they used. Kung and Marsho (11) reported differences in specific activity for both ribulose-P2 carboxylase and oxygenase, but they did not detail growth conditions and plant age. As reported here, large differences in specific activity can be attributed to differences in plant age, growth conditions, and levels of soluble polyphenol oxidase activity in tobacco leaves. Thus a comparison of ribulose-P2 carboxylase/oxygenase preparations from \(\text{Su/su}\) with \(\text{su/su}\) plants at different developmental stages could produce widely different activities, whereas no change actually would exist if the plants were grown under identical conditions and assayed at developmentally equivalent ages. Reasons for changes in total and specific activity of the enzyme during development are unknown but can probably be accounted for by artifacts. Our data indicated that a decrease in activity of the isolated enzyme correlated with an increase in soluble polyphenol oxidase in the leaf extract. Addition of PVPP to remove phenolic substrates does not remove the polyphenol oxidase which may partially alter or inactivate the carboxylase/oxygenase protein. However, the physiological or kinetic change was apparent in the isolated enzyme. Thus, it seems unlikely that a change in ribulose-P2 carboxylase/oxygenase is responsible for the \(\text{Su}\) phenotype as Okabe has speculated (18, 19).

Properties of ribulose-P2 carboxylase/oxygenase were identical in crystalline preparations from 2 phenotypic revertants isolated from haploid \(\text{Su}\) plants, which were homozygous for the mutant \(\text{Su}\) gene (10). For the enzyme from another revertant, R2, that had a high chromosome number of 87.0 ± 11.1, the \(V_{\text{max}}\) for the carboxylase activity was about half that for the enzyme from \(\text{su/su}\). The other physical and kinetic properties of the enzyme from this R2 revertant were similar to the enzyme from \(\text{su/su}\). Because of wide variations in activity of the enzyme when isolated, the reasons for and significance of the lower \(V_{\text{max}}\) in this revertant will require further examination. Genetically it is difficult to explain a kinetic change in an enzyme from a change in ploidy level, but since the carboxylase is encoded in both the nucleus and chloroplast, it could conceivably be modified by the nuclear ploidy level. On the other hand, as demonstrated in this paper, enormous variations in specific activity of the isolated enzyme may be artifacts of isolation. Gray et al. (7) have shown that change inoselectric focusing patterns may be artificial as well. To demonstrate an altered ribulose-P2 carboxylase/oxygenase, the change in kinetic properties must be correlated with changes in associated physiological processes, namely rates of photosynthesis, photorespiration and plant growth, as attempted by Garrett (6) with
ryegrasses of different ploidy level.

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