Differential activation of ribulose-1,5-bisphosphate carboxylase/oxygenase in non-radiolabelled versus radiolabelled sodium bicarbonate

Based on kinetic and physical studies of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a carboxylase assay for the enzyme was recommended, wherein the enzyme was to be activated in the presence of sodium bicarbonate for sufficient time period before proceeding for the actual assay. Papers published in the last decade showed that the activation was carried out either in the presence of non-labelled NaHCO₃ (refs 7–11), or in the presence of labelled NaH¹⁴CO₃ (refs 12–16). This opened up a domain to argue as to which of the two, NaHCO₃ vs NaH¹⁴CO₃, should be used for activation or whether both of them could be used? A discussion is indeed warranted in the present scenario of analysis of transgenic plants and mutants, keeping in view the far-reaching consequences of interpretation of the data and also to avoid discrepancy of the results globally.

Leaves at 3rd node of tea (Camellia sinensis (L.) O. Kuntze; clone Local Kangra), flag leaf of barley (Hordeum vulgare; variety, Dolma) and wheat (Triticum aestivum; variety, HS-240) and leaves of pea (Pisum sativum; variety, Azad; two opposite leaves adjacent to the stem at 3rd node position) were harvested from either biodiversity garden (for barley, wheat and pea) or the well-maintained tea garden (for Local Kangra clone of tea) of the Institute of Himalayan Bioresource Technology, Palampur (32° 6’N latitude, 76° 18’E longitude, altitude 1290 m above mean sea level) during morning hours between 10 and 11 am and stored in liquid nitrogen till further use. Leaves were homogenized in cold (4°C) grinding medium containing 50.0 mM Tris-HCl (pH 7.5), 1.0 mM MgCl₂, 5.0 mM DTT, 1 mM PMSF, 2% (w/v) insoluble PVP, 10% glycerol and 0.1% (v/v) Triton X-100. The extract was centrifuged at 12,000 g for 10 min at 4°C and the supernatant was used for determination of the protein and the enzyme activity. Protein in the crude extract was precipitated with 20% TCA followed by solubilization of the protein in 1 N NaOH and estimation using Folin’s reagent.

Rubisco was assayed by two different ways: In experiment (A) the enzyme was activated as described by Pierce et al. using 20 mM unlabelled NaHCO₃ and 10 mM MgCl₂ in Tris-HCl buffer (pH 8.0) for different periods at 30°C. After activating for 0, 2, 6 and 10 min, the enzyme, equivalent to 0.5–1 mg tissue fresh weight, was pipetted out in the assay medium [50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5 mM DTT, 1.3 mM ribulose bisphosphate (RuBP) and 20 mM NaH¹⁴CO₃ (specific activity of 1 x 10⁶ DPM/mmol)]. NaH¹⁴CO₃, (catalogue # LCC 35, specific activity 51.9 mCi/mmole) was obtained from Board of Radiation and Isotope Technology, Mumbai, India. After 1 min at 30°C, the reaction was terminated by the addition of tri-chloroacetic acid (TCA; 8% final concentration) to remove the unused NaH¹⁴CO₃. A control experiment with the boiled enzyme extract was also conducted to account for background counts. Before counting the acid stable radioactivity, hot air was passed through all the samples to remove unbound ¹⁴CO₂. Samples were counted in scintillation cocktail (Sisco Research Laboratories, India) using a Beckman Scintillation Counter LS 6000TA.

In a second experiment (B) the assay mixture contained 50 mM Tris-HCl 10 mM MgCl₂, 5 mM DTT, 20 mM NaH¹⁴CO₃ (specific activity of 1 x 10⁶ DPM/mmol) and enzyme extract. The assay mixture was incubated at 30°C for 0, 2, 6 and 10 min and the reaction was started by the addition of RuBP (1.3 mM final concentration). After 1 min at 30°C, the reaction was stopped with TCA and counted as above.

When the radioactivity incorporation into acid stable products by the enzyme of Local Kangra clone of tea was studied, radiocarbon fixed was higher by 8.9, 42.8, 70.16 and 72.6% when Rubisco was first activated in the presence of NaHCO₃ for 0, 2, 6 and 10 min and added later to the reaction mixture containing NaH¹⁴CO₃ (A, Table 1) compared to NaH¹⁴CO₃ included during the activation period (B, Table 1). Higher values at 0, 2, 6 and 10 min for the reaction A compared to reaction B were 5.8, 7.7, 29.5 and 33.3% for barley; 1.2, 31.6, 333

<table>
<thead>
<tr>
<th>Activation period (min)</th>
<th>Rubisco activity (μmol min⁻¹ mg⁻¹ of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Tea A</td>
</tr>
<tr>
<td>0</td>
<td>0.085 ± 0.0092</td>
</tr>
<tr>
<td>2</td>
<td>0.150 ± 0.007 (76.4)</td>
</tr>
<tr>
<td>6</td>
<td>0.211 ± 0.01 (148.2)</td>
</tr>
<tr>
<td>10</td>
<td>0.221 ± 0.005 (160)</td>
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</tbody>
</table>

Table 1. Effect of different activation periods on ¹⁴C fixation by Rubisco when assayed by the two different procedures. Enzyme was assayed essentially as described in the text. A, enzyme was first activated in the presence of NaHCO₃ and added later in the reaction medium containing NaH¹⁴CO₃, B, Rubisco was activated for different time periods in the presence of NaH¹⁴CO₃. Data represent the mean ± SD of 5 replicates. Values in parentheses indicate the % increase over the 0 min activation values.
42.4, and 54.9% for wheat and; 10.6,
26.96, 27.3 and 34.2% for pea (Table 1).

Interestingly, incubation period affected
the total activation of the Rubisco as well. In Local Kangra clone of tea, if
NaH[14]C]O3 was included during activation
period, the observed activation in 2,
6 and 10 min was 34.6, 58.9 and 64.1% higher compared to the 0 min activation
or unactivated enzyme (B, Table 1).
However, activation was prominent if
Rubisco was activated first in NaHCO3,
and NaH[14]C]O3 was included later at
assay phase. The present experiment
yields 76.4, 148.2 and 160.0% higher
activation in 2, 6 and 10 min compared to the 0 min activation or unactivated
enzyme for Local Kangra clone of tea (A,
Table 1). Enzyme activation in the reac-
tion B at 2, 6 and 10 min compared to the
0 min activation or unactivated enzyme
was 15.9, 23.5 and 29.6% in barley; 39.2,
61.2 and 66.5% in wheat; and 34.8, 60.6
and 63.6% in pea. Such higher activation
values at 2, 6 and 10 min compared to
the 0 min activation or the unactivated
enzyme for the reaction A were 17.9,
51.0 and 63.2% for barley; 81.2, 126.7
and 154.9% for wheat and; 54.7, 84.9
and 98.6% for pea (Table 1). Available
literature also supported our above results
that whenever activation was carried out in
the presence of NaH[14]C]O3 (as in B,
Table 1), the increase between initial and
the final Rubisco activity was lesser (25–
30%; see refs 13, 18 and 19) compared to
when activation was carried out in the
was added at carboxylation phase (as in
A, Table 1); activation to a tune of 2–
to 7-fold increase in the activity has been
reported.7–10 We have not encountered
any report wherein NaH[14]C]O3 was
included during activation phase and
substantial increase in activity between initial and the fully activated enzyme was
observed.

The reason for observed lower activa-
tion in the reaction B, compared to A in
Table 1 could be the fewer available
moles of CO2 + 14CO2 (*CO2) for carboxy-
lation reaction50 due to two reasons.
Firstly, the presence of carbonic anhyd-
rase in crude enzyme preparations, which
would catalyse conversion of HCO3 into
CO2, might lead to escape of *CO2 into
the atmosphere. Secondly, the reason for
such losses could be the way CO2 is util-
ized by Rubisco for activation process.

It is known that Rubisco has distinct CO2
activator and CO2 substrate sites. Kinetic
data of Lorimer4 clearly showed that
activator and substrate CO2 are different
moieties. Activator CO2 binds at e-
mino group of lysine 202 of the large
subunit to form carbamate3 and does not
participate in condensation reaction with
RuBP. This would imply that if
NaH[14]C]O3 was included during activa-
tion period, activator site of Rubisco
would utilize substantial moles of *CO2.
Upon denaturation of the enzyme, *CO2
would be released, leading to the loss of
reactivity from the reaction medium.
The results of Lorimer2 did show that a
ternary complex enzyme-*CO2-Mg2+ may
retain 20–75% of 14C, depending upon
turnover/site and temperature at which
the gel filtration was performed. 14C
bound to Rubisco disappeared if the
enzyme was denatured by sodium dodecyl
sulphate (SDS; see table 1 of ref. 3).

To conclude, our results show that
Rubisco should be activated for a suffi-
cient time period in the presence of
NaHCO3 rather than in the presence of
NaH[14]C]O3, to obtain proper data on
activated Rubisco. This becomes impor-
tant even for comparing mutants, trans-
genics or different plant species because
of varying number of activation sites in
Rubisco and varying amounts of carbonic
anhydride, etc.

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