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[1–6]. Papers published in the last decade showed that the activation was carried out either in the presence of non-labelled $NaHCO_3$ (refs 7–11), or in the presence of labelled $NaH[^{14}C]O_3$ (refs 12–16). This opened up a domain to argue as to which of the two, $NaHCO_3$ vs $NaH[^{14}C]O_3$, 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 msl) 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 $MgCl_2$, 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[17].

Rubisco was assayed by two different ways: In experiment (A) the enzyme was activated as described by Pierce *et al.*[6] using 20 mM unlabelled $NaHCO_3$ and 10 mM $MgCl_2$ 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 $MgCl_2$, 5 mM DTT, 1.3 mM ribulose bisphosphate (RuBP) and 20 mM $NaH[^{14}C]O_3$ (specific activity of 1×10^6 DPM/μmol)]. $NaH[^{14}C]O_3$, (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 $NaH[^{14}C]O_3$. 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 $^{14}CO_2$. 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 $MgCl_2$, 5 mM DTT, 20 mM $NaH[^{14}C]O_3$ (specific activity of 1×10^6 DPM/μmol) 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 $NaHCO_3$ for 0, 2, 6 and 10 min and added later to the reaction medium containing $NaH[^{14}C]O_3$ (A, Table 1) compared to $NaH[^{14}C]O_3$ 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,

Table 1. Effect of different activation periods on ^{14}C 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 $NaHCO_3$ and added later in the reaction medium containing $NaH[^{14}C]O_3$; B, Rubisco was activated for different time periods in the presence of $NaH[^{14}C]O_3$. Data represent the mean ± SD of 4 replicates. Values in parentheses indicate the % increase over the 0 min activation values

Activation period (min)	Rubisco activity (μmol min^{-1} mg^{-1} of protein)							
	Tea		Barley		Wheat		Pea	
	A	B	A	B	A	B	A	B
0	0.085 ± 0.0092	0.078 ± 0.0092	0.378 ± 0.05	0.357 ± 0.014	0.415 + 0.09	0.41 + 0.08	0.073 ± 0.009	0.066 ± 0.012
2	0.150 ± 0.007 (76.4)	0.105 ± 0.052 (34.6)	0.446 ± 0.03 (17.9)	0.414 ± 0.02 (15.9)	0.752 ± 0.06 (81.2)	0.571 ± 0.11 (39.2)	0.113 ± 0.005 (54.7)	0.089 ± 0.017 (34.8)
6	0.211 ± 0.01 (148.2)	0.124 ± 0.009 (58.9)	0.571 ± 0.06 (51.0)	0.441 ± 0.01 (23.5)	0.941 ± 0.17 (126.7)	0.661 ± 0.08 (61.2)	0.135 ± 0.009 (84.9)	0.106 ± 0.007 (60.6)
10	0.221 ± 0.005 (160)	0.128 ± 0.047 (64.1)	0.617 ± 0.11 (63.2)	0.463 ± 0.13 (29.6)	1.058 ± 0.02 (154.9)	0.683 ± 0.12 (66.5)	0.145 ± 0.004 (98.6)	0.108 ± 0.014 (63.6)

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 $NaH[^{14}C]O_3$ 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 $NaHCO_3$, and $NaH[^{14}C]O_3$ was included later at assay phase. The present experiment yielded 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 reaction 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 $NaH[^{14}C]O_3$ (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 presence of $NaHCO_3$ and $NaH[^{14}C]O_3$ 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 $NaH[^{14}C]O_3$ was included during activation phase and substantial increase in activity between initial and the fully activated enzyme was observed.

The reason for observed lower activation in the reaction B, compared to A in Table 1 could be the fewer available moles of $CO_2 + {}^{14}CO_2$ ($*CO_2$) for carboxylation reaction[20] due to two reasons. Firstly, the presence of carbonic anhydrase in crude enzyme preparations, which would catalyse conversion of HCO_3^- into CO_2, might lead to escape of $*CO_2$ into the atmosphere. Secondly, the reason for such losses could be the way CO_2 is utilized by Rubisco for activation process. It is known that Rubisco has distinct CO_2 activator and CO_2 substrate sites[3]. Kinetic data of Lorimer[4] clearly showed that activator and substrate CO_2 are different moieties. Activator CO_2 binds at ε-amino group of lysine 202 of the large subunit to form carbamate[5] and does not participate in condensation reaction with RuBP. This would imply that if $NaH[^{14}C]O_3$ was included during activation period, activator site of Rubisco would utilize substantial moles of $*CO_2$. Upon denaturation of the enzyme, $*CO_2$ would be released, leading to the loss of radioactivity from the reaction medium. The results of Lorimer[4] did show that a ternary complex 'enzyme-^{14}C-Mg^{2+}, may retain 20–75% of ^{14}C, depending upon turnover/site and temperature at which the gel filtration was performed. ^{14}C 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 sufficient time period in the presence of $NaHCO_3$ rather than in the presence of $NaH[^{14}C]O_3$, to obtain proper data on activated Rubisco. This becomes important even for comparing mutants, transgenics or different plant species because of varying number of activation sites in Rubisco and varying amounts of carbonic anhydrase, etc.

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NARINDER KUMAR
SANJAY KUMAR*

Biotechnology Division,
Institute of Himalayan Bioresource Technology,
P.O. Box 6,
Palampur 176 061, India
**For correspondence*
e-mail: sanjay_plp@usa.net