Reversed-phase ion-pair chromatographic separation of ribulose 1,5-bisphosphate from 3-phosphoglycerate and its application as a new enzyme assay for RuBP carboxylase/oxygenase

Robert Jakob and Wolfram Saenger*

Institut für Kristallographie, Freie Universität Berlin, Takustr. 6, 1000 Berlin 33, Germany

Received 28 January 1985

A new method for the detection of ribulose 1,5-bisphosphate (RuBP), the substrate of the prominent ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the carbon dioxide-fixing step in photosynthesis has been worked out. Within 15 min run time as few as 80 pmol RuBP can still be detected.

HPLC *Ion-pair chromatography* *Ribulose 1,5-bisphosphate* *Ribulose 5-monophosphate* *Ribulose-1,5-bisphosphate carboxylase/oxygenase* *3-Phosphoglycerate*

1. INTRODUCTION

The enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) attaches CO_2 to ribulose 1,5-bisphosphate (RuBP) to produce 2 moles 3-phosphoglycerate (3-PGA) [4,5]. The usual assays for testing the activity for Rubisco employ either $^{14}CO_2$ [1] or a cascade of 4 different enzymes with NAD^+ as spectrophotometric probe [2]. A new assay was tested where the decrease of the substrate RuBP is followed directly by ion-pair HPLC. The assay procedure was worked out as simply as possible, relying on ion-pair chromatography and separation in a single isocratic run.

Since RuBP is the product of the kinase reaction [6], the assay can also be used to measure kinase activity. Furthermore, the detection and separation of sugar phosphates is of value in synthetic and biochemical applications.

* To whom correspondence should be addressed

2. MATERIAL AND METHODS

2.1. *Apparatus*

The HPLC system consisted of a type 64.00 pump (Knauer, Berlin), a 20 µl sample loop (Knauer), a Rheodyne syringe loading sample injector (Rheodyne, Cotati, CA), an ODS Hypersil reversed-phase column (Shandon, Astmoor), 25 cm × 4 mm i.d., with particles of 5 µm diameter, spectrophotometer no. 87.00 and a strip chart recorder 42.20 (Knauer). Injections were made with a 20 µl injection syringe (Knauer).

2.2. *Chemicals*

These were all AnalaR grade (Merck, Darmstadt), except adenylyl-3′-5′-guanosine (Boehringer, Mannheim). RuBP carboxylases to be assayed were freshly isolated in our laboratory.

2.3. *System conditions*

With a flow rate of 1.2 ml/min and a sample volume of 20 µl, separations were performed at room temperature (approx. 22°C) in single isocratic runs using 0.05 M tetrabutylammonium hydrogensulfate (TBAHS) adjusted to pH 7.3 with

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

NaOH in 90% H_2O/10% CH_3OH as eluent. Detection was performed at 220 nm, where 3-PGA and especially RuBP show considerable absorption. Wavelengths below 220 nm were avoided because absorption by the eluent increases significantly in this range.

At a convenient sensitivity (0.04 units for full-scale deflection) RuBP in amounts as little as 80 pmol can still be detected.

2.4. *Calibration of the system*

For calibration purposes standard curves were made using various concentrations of RuBP and 3-PGA, ranging from 0.5 to 12.5 and 3 to 20 mM, respectively. As inner standard, adenylyl-3′,5′-guanosine (ApG) was used, which in HPLC runs is well separated from RuBP and 3-PGA.

For the 3-PGA standard curve a solution containing 2 μl of a 1% ApG solution and 25 μl of a 1% 3-PGA solution (12.5, 5, 2, 1 μl, respectively) were diluted to 50 μl with H_2O and boiled in a water bath for 3 min.

For the RuBP standard curve 2 μl of the 1% ApG solution and 25, 12.5, 5, 2 and 1 μl of a 0.025 M RuBP solution were diluted to 50 μl with H_2O and boiled in a water bath for 3 min. The injection volume was 20 μl for both standard curves. HPLC runs were performed under the above described conditions.

For quantification, the peak heights of the 3-PGA and RuBP peaks have to be divided by the peak height of the inner standard and a standard curve of RuBP and 3-PGA concentration against this quotient is drawn.

2.5. *Enzyme assay*

To determine Rubisco activity an assay mixture was prepared which contained the substrate RuBP, Mg^{2+} [7,8] and HCO_3^- [9] to maintain carboxylase activity, ApG as internal standard and the enzyme to be assayed. The stock solution consisted of: 150 μl 0.025 M RuBP, 12 μl 0.1% ApG, 12 μl 0.5 M $MgCl_2$, 24 μl 0.5 M $KHCO_3$, 24 μl 0.05 M NaH_2PO_4 adjusted to pH 7.5 with NaOH.

The reaction was started by addition of 78 μl Rubisco and was stopped by removing 50 μl of the enzyme assay at certain time intervals and boiling it in an Eppendorf reagent tube for exactly 3 min in a water bath. The denatured protein was removed by centrifugation and the supernatant was

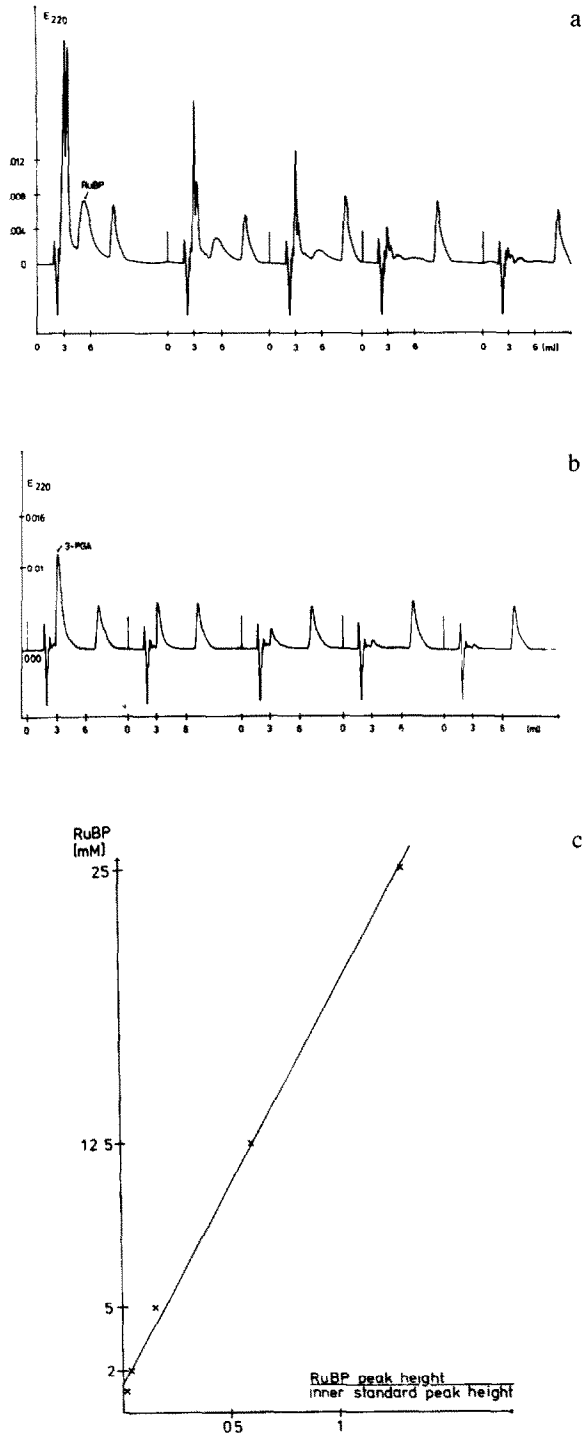

Fig. 1. (a) Chromatogram of a series of RuBP injections (12.5, 6.25, 2.5, 1, 0.5 mM). (b) Chromatogram of a series of 3-PGA injections (20, 10, 4, 2, 1 mM). (c) Standard curve derived from (a).

injected into the HPLC system. The RuBP peak heights at each of the time points were determined and divided by the heights of the inner standards. By comparison with the standard curve the quotients obtained yielded the amount of RuBP still present in the assay solution after a defined period of time. From several time points kinetic data could be obtained.

3. RESULTS AND DISCUSSION

Fig.1 shows a standard curve for 3-PGA and RuBP obtained by a series of injections of 3-PGA and RuBP of different concentrations as described in section 2.

Performance of the enzyme assay with Rubisco of spinach yielded a chromatogram as shown in fig.2 from which kinetic data of the enzyme were obtained.

As the reaction proceeded the RuBP peak height decreased since the substrate is converted to 3-PGA. An enzyme activity determination from the 6 time points in fig.2 yielded a value with about 15% deviation from the activity determined by the conventional method of Racker [2]. Fig.3 illustrates the course of the reaction by plotting the amount of reacted RuBP against time.

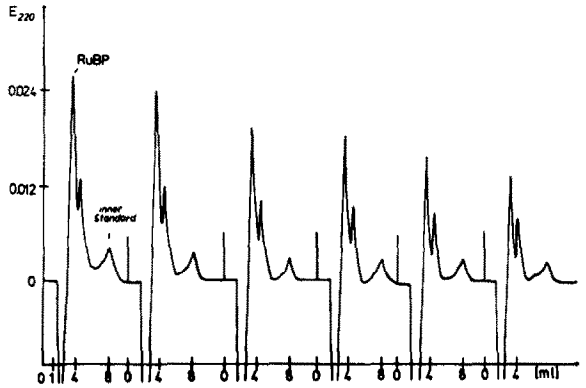

Fig. 2. Chromatogram of a spinach Rubisco assay mixture. The amount of enzyme added was 5.2 μl of a 2.2% (w/w) solution per injection. RuBP was eluted within 3.5 ml and adenylyl-3',5'-guanosine as the inner standard within 7.9 ml. A contamination of unknown origin was eluted within 4.5 ml. The column pore volume was 2 ml. Injections were made after incubation times of 3.5, 5, 9, 17, 24 and 41.5 min (from left to right). The concentration of the inner standard was one tenth of that used for the standard curves (fig.1).

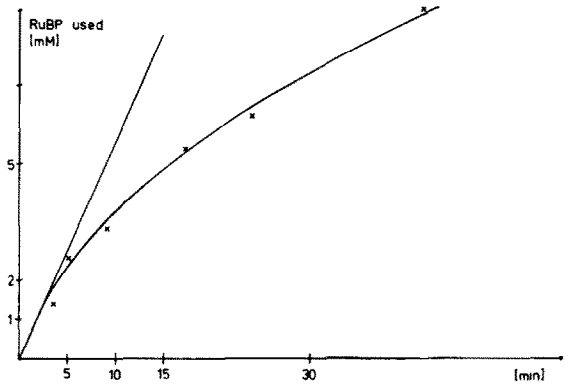

Fig. 3. By drawing the tangent through the origin of a plot showing the amount of RuBP converted against time the V_{max} of the reaction is calculated to be 100 nmol RuDP/min per mg enzyme.

By changing the assay composition [substituting 0.05 M NaH_2PO_4 (pH 7.5) by 1 M Tris-HCl (pH 7.8)] it was even possible to resolve 3-PGA but quantitative evaluation became difficult because most of this peak is obscured by another more prominent peak of unknown origin, as can be seen in fig.4.

A quantitative evaluation of this peak was hindered by peak interference with the contaminant. Should it be possible to resolve the 3-PGA peak from the contamination peak by suitable

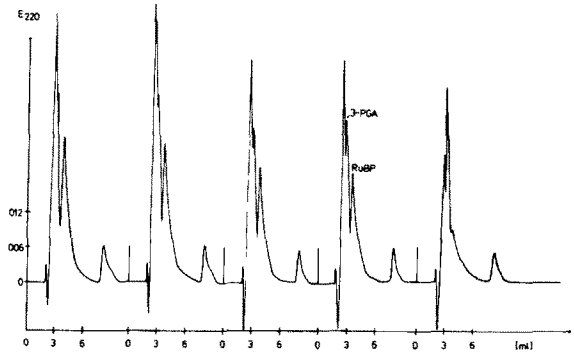

Fig. 4. Chromatogram of a spinach carboxylase assay mixture where NaH_2PO_4 is substituted by 1 M Tris-HCl (pH 7.8). RuBP is eluted within 3.6 ml and the inner standard within 7.9 ml. At an elution volume of 3 ml a second peak emerged while the amount of RuBP present in the assay decreased. This peak corresponds to 3-PGA (see fig.1c).

choice of column material and eluent, the amount of 3-PGA formed could be directly correlated to the amount of RuBP disappearing during the reaction.

Further studies with tetraalkylammonium salts of varying chain length showed that with smaller chains like methyl and ethyl groups, separation of RuBP and 3-PGA could not be optimized. Variation of the mobile phase polarity towards higher and lower methanol contents leads to smaller retention volumes and decreased resolution.

The above system can also be used to separate ribulose 5-phosphate (RuMP) from RuBP. It is known that RuBP is unstable in aqueous solutions [10] and is hydrolyzed to RuMP. Fig. 5a shows a chromatogram of a 3-week-old RuBP solution. At an elution volume of 2.5 ml a second peak emerged, corresponding to RuMP, as indicated in fig. 5b.

4. CONCLUSION

The separation of RuBP and 3-PGA in a single isocratic HPLC run could be demonstrated as well as their concentration decrease and increase respectively, in enzyme assays. Further improvements can make this semiquantitative enzyme test a quantitative one. Its applicability also for the determination of RuMP kinase activity is based on the fact that RuBP is the product of the RuMP kinase-catalyzed reaction: RuMP + ATP → RuBP + ADP. Thus, a single assay procedure can be employed to test the activity of two different enzymes. Furthermore, the quality of RuBP solutions can be examined by HPLC chromatograms.

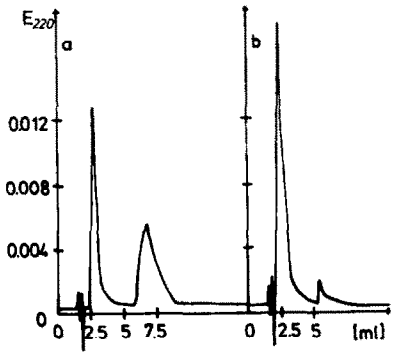

Fig. 5. (a) Chromatogram of 20 μl of a 3-week-old 0.025 M RuBP solution. The elution volume of RuBP is 7 ml. (b) Chromatogram of 20 μl of a freshly prepared 0.025 M RuMP solution, showing that the peak at 2.5 ml in (a) was RuMP. The small peak at about 7 ml elution volume is RuBP contamination.

ACKNOWLEDGEMENTS

This work was supported by Sonderforschungsbereich 9 and by Fonds der Chemischen Industrie.

REFERENCES

[1] Lorimer, G.M., Badger, M.R. and Andrews, T.J. (1977) Anal. Biochem. 78, 66–75.
[2] Racker, E. (1962) Methods Enzymol. 5, 266–282.
[3] Akazawa, T. (1979) in: Encyclopedia of Plant Physiology, New Series (Gibbs, M. and Latzko, eds) vol. 6, pp. 208–229, Springer, Berlin.
[4] Andrews, T.J. and Lorimer, G.M. (1978) FEBS Lett. 90, 1–9.
[5] Chollet, R. (1977) Trends Biochem. Sci. 2, 155–159.
[6] Siebert, K., Schobert, P. and Bowien, B. (1981) Biochim. Biophys. Acta 658, 35–44.
[7] Kawashima, N. and Wildman, S.G. (1970) Annu. Rev. Plant Physiol. 21, 325–358.
[8] Weissbach, A., Horecker, B.L. and Hurwitz, J. (1956) J. Biol. Chem. 218, 795–810.
[9] Dailey, F. and Criddle, R.S. (1980) Arch. Biochem. Biophys. 201, 594–602.
[10] Peach, C.I., Pierce, S.D., McCurry, S.D. and Tolbert, N.E. (1978) Biochem. Biophys. Res. Commun. 83, 1084–1092.