

Ribulose-1,5-bisphosphate Carboxylase-Oxygenase: New Aspects Respective the pH-Dependence of the Carboxylation Reaction

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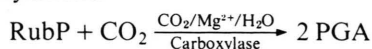
Ribulose-1,5-bisphosphate Carboxylase-Oxygenase, Crude Enzyme Extracts, Reaction Conditions,
pH Activity Profile

The investigation was directed towards the effects of reaction conditions, substrates and pH on the carboxylation reaction of ribulose-1,5-bisphosphate carboxylase-oxygenase in the crude enzyme extracts from several plants. Optimal substrate concentrations (HCO_3^- and RubP) were determined. The highest carboxylase activity was attained with Tris/HCl buffer. The pH activity profile was quite sharp with an optimum at pH 7.8. Purified and crystallized carboxylase yielded a broad optimum curve under the same reaction conditions.

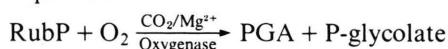
Introduction

Ribulose-1,5-bisphosphate carboxylase-oxygenase (EC.4.1.1.39) – by its action all photosynthetic organisms assimilate CO_2 to form carbohydrates – has been the object of intense research for many years. Wildman and Bonner [1] revealed the presence of a major protein component in plant leaves having a large molecular size. It was named fraction-1-protein which often comprises more than 50% of the soluble leaf protein. Further research showed its ubiquitous distribution in higher plants and green algae, and investigations into the enzymic machinery of the reductive pentose phosphate cycle explained its main function as a CO_2 fixing enzyme [2] catalyzing the reactions:

Photosynthesis:



Photorespiration:



Abbreviations: DTE, dithioerythritole (2,3-dihydroxy-1,4-dithiolbutane); EDTA, ethylenedinitrilotetraacetic acid disodium salt; HEPES, N-2-hydroxyethylpiperazine-N-ethane sulfonic acid; MES, 2-(N-morpholino)-ethanesulfonic acid; NADP, nicotinadeninucleotidphosphate; PGA, phosphoglycerate acid; RubP, ribulose-1,5-bisphosphate; RubPC-ase, ribulose-1,5-bisphosphate Carboxylase-Oxygenase; TRICIN, N-tris-(hydroxymethyl)-methylglycine; TRIS, tris-hydroxy-methylaminomethane.

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From now on it was called RubP Carboxylase-Oxygenase. Many investigations have been undertaken in the following years to determine molecular and catalytic properties. While former research focussed on the carboxylase reaction, attention is now paid to the oxygenase reaction of the enzyme.

In a study of the influence of growth regulators on wheat plants we looked for a carboxylase enzyme assay to be carried out without loss of time.

Therefore, we checked crude leaf homogenates in respect of RubPC-ase activities. To compare the carboxylase reaction in the crude homogenate with that of purified preparations, we tested homogenisation conditions and some catalytic properties. This study reports of the observations concerning the pH-optima during the carboxylase reaction carried through with the crude enzyme extract and with the purified enzyme.

Materials and Methods

Test material

Crude enzyme extracts were prepared with leaves of the following plants: *Triticum aestivum* (var. Kolibri), *Spinacia oleracea*, *Sinapis alba* and *Nicotiana rustica*. All plants were grown as hydroponic cultures with Hoagland solution (culture conditions: temperature 22 °C, light intensity 17 W · m⁻² (400–700 nm) with a photoperiod of 16 h and relative humidity of 60%). For Spinach preparation field grown plants were used. RubPC-ase was purified and crystallized from tobacco. Spinach-carboxylase was purchased from Sigma Chemical Company (R-8000).



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Preparation of crude enzyme extracts

1.0 to 2.5 g leave material was prepared either by grinding in a mortar with 3 g sand and 15 ml buffer solution for 2 min or by cutting in a special device constructed according to the instructions of Kannan-gara [3] with 30 ml buffer solution for 1 min. (Buffer solution: 50 mmol · l⁻¹ Tris/HCl pH 8.0, 10 mmol · l⁻¹ MgCl₂, 10 mmol · l⁻¹ KCl, 5 mmol · l⁻¹ DTE, 0.2 mmol · l⁻¹ EDTA.) The homogenate was filtered through a nylon net (10–20 µm, Scrynel) and centrifugated at 30000 × *g* for 10 min. The residue was discarded and the supernatant used for assay of carboxylase activity.

Content of soluble proteins of the homogenate was estimated with Bio-Rad Protein Assay[®] founding on a protein determination method by Bradford [4].

Isolation of the RubPC-ase from tobacco

The procedure was described by Wildner and Fedtke [5].

The leaves were homogenated in an omni-mixer with 1 mol · l⁻¹ Tris/HCl pH 8.5, 1 mmol · l⁻¹ EDTA, 2 mmol · l⁻¹ MgCl₂, 40 mmol · l⁻¹ 2-mercaptoethanol and 2% Polyclar AT, filtered through a nylon gaze and centrifugated at 45000 × *g* for 30 min.

The purification of the supernatant included following steps: ammonium sulfate precipitation up to 55% saturation; centrifugation of the precipitate at 20000 × *g* for 20 min; dissolution of the residues in 25 mmol · l⁻¹ Tris/HCl pH 8.5, 0.2 mol · l⁻¹ NaCl and 0.5 mmol · l⁻¹ EDTA (Gel buffer) followed by an ultracentrifugation at 100000 × *g* for 60 min; Sephadex G-50 filtration of the supernatant (equilibration with gel buffer, see above); dialysis of the first protein fraction (1 mmol · l⁻¹ with MgCl₂) against 25 mmol · l⁻¹ Tris/HCl pH 7.4, 0.5 mmol · l⁻¹ EDTA. RubPC-ase crystallized within 48 h.

RubP-carboxylase assay

Before performing an assay the crystallized enzyme was dissolved in 50 mmol · l⁻¹ Tris/HCl pH 8.5, 200 mmol · l⁻¹ NaCl, 10 mmol · l⁻¹ MgCl₂, 10 mmol · l⁻¹ NaHCO₃ and activated for 30 min at 40 °C. An assay of carboxylase activity of the crude enzyme extract, or rather the activated purified enzyme, followed after a 5 min preincubation at

25 °C, pH 8.2 with 20 mmol · l⁻¹ MgCl₂, 10 mmol · l⁻¹ KCl, 18 mmol · l⁻¹ NaHCO₃ (0.05 µCi per 0.8 ml assay solution) and the subsequent addition of 50 mmol · l⁻¹ Tris/HCl, 5 mmol · l⁻¹ DTE, 0.25 mmol · l⁻¹ EDTA. The reaction was started with 0.6 mmol · l⁻¹ RubP and stopped after 30 s with acetic acid.

After the evaporation of the non-fixed ¹⁴CO₂, the residues were dissolved in 0.8 ml H₂O. The scintillator solution (10 ml) was added and the present ¹⁴CO₂ was measured using a liquid scintillation counter.

Results and Discussion

At first we tested the influence of various buffers (Mes, Hepes, Tricin, Tris) on carboxylase activity during homogenisation of the leaf material.

In Tris-buffer, enzyme activity was about 30% higher than in the other buffers (Fig. 1).

With regard to the enzyme activity the homogenisation method (grinding or cutting) did not make any difference. Likewise the enzyme concentration had no significant influence on the activity (the protein concentration ranged from 1 to 3 mg protein per ml extract). After an 80-minutes-experiment a slight decrease in carboxylase activity

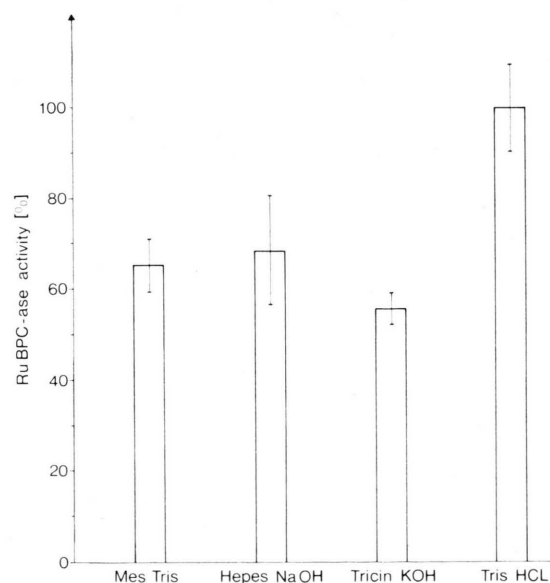


Fig. 1. Influence of different buffers during homogenisation on RubPC-ase activity. All buffers were used with 50 mmol · l⁻¹; the pH was adjusted to 8.1 before homogenisation and did not alter severely during preparation. (100% adequate to 400 µmol fixed CO₂ × h⁻¹ × mg Chl⁻¹).

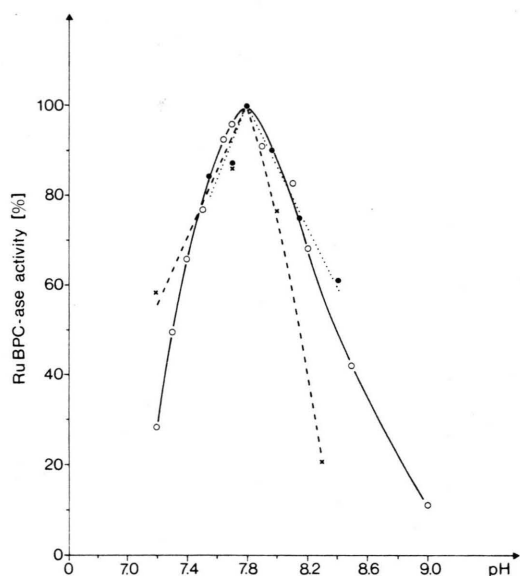


Fig. 2. pH Activity profile of RubPC-ase in the crude homogenate of wheat (○—○), spinach (×---×) and mustard plants (●---●). Assays were carried through at different pH values (checked in the reaction vial after all components were mixed) with optimal HCO_3^- concentrations (100% see Fig. 1).

Subsequently we tested crude extracts from spinach, tobacco and mustard plants under the same test conditions. We always attained similar pH profiles (Figs. 2, 4, 5). These results were confirmed with purified and crystallized RubPC-ase from spinach and tobacco which was dissolved and activated as described above. Under the same conditions the pH curves became broad (Figs. 4, 5). This

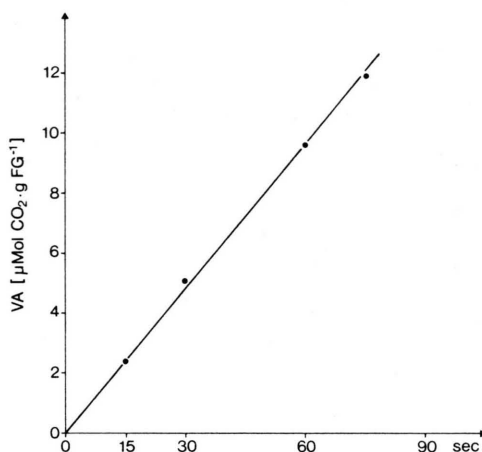


Fig. 3. Time course of CO₂-fixation; a slow decline of RubPC-ase activity became evident at 90 s.

was discovered, which was likely due to the presence of proteolytic enzymes in the extract.

First pH investigations have been made with crude homogenate of wheat leaves. Fig. 2 (outlined graph) shows the pH activity profile. Carboxylase activity is optimal at a pH of 7.8 (20 mmol·l⁻¹ MgCl₂) and drops sharply on the acid and the alkaline side. Depending on the Mg²⁺ concentration the optimum shifted as described earlier [6].

We did additional research on the pH-optimum for the carboxylase reaction based on publications of other authors like Lorimer [7] and Andrews [8] who described broad pH-optima. Lorimer [7] presumed that the failing activation of the enzyme is responsible for the sharp drop on the acid side of the pH optimum. We could prove that the enzyme is sufficiently activated because the reaction began without any discernible lag (Fig. 3). Estimates on the basis of the Henderson-Hasselbach equation show sufficient CO₂ concentrations for the alkaline side. Varying the bicarbonate concentration to higher values at alkaline pH [7] did not alter our results. Likewise a loss of CO₂ at low pH (pH 7.2) is not responsible for the sharp drop.

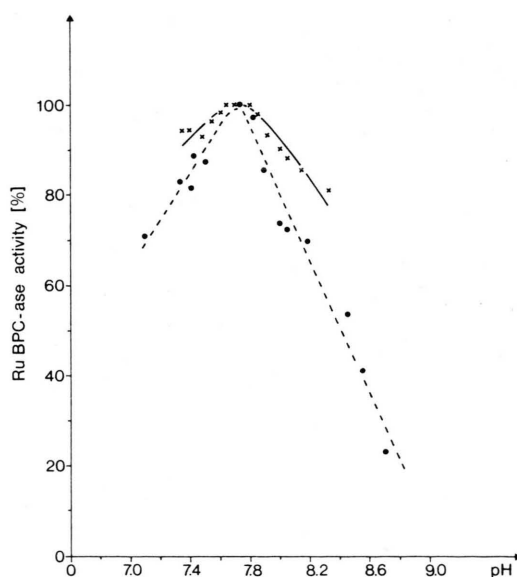


Fig. 4. pH Activity profile of RubPC-ase in the crude extract (●---●) and of a purified and crystallized preparation from tobacco (×---×). (100% adequate to 30 μmol CO₂ × h⁻¹ × mg sol. prot⁻¹).

proved the assay to be adequate for these measurements. The pH profiles of crystallized spinach and tobacco RuBPC-ase correspond to that of Andrews [8] which they got with the spinach enzyme (Fig. 6). Many positions of the pH optimum have been published in scientific literature, so far. This is due to the dependence on the Mg^{2+} concentration [6] and the CO_2 concentration [9] because of the pH sensitivity of the $K_m(CO_2)$.

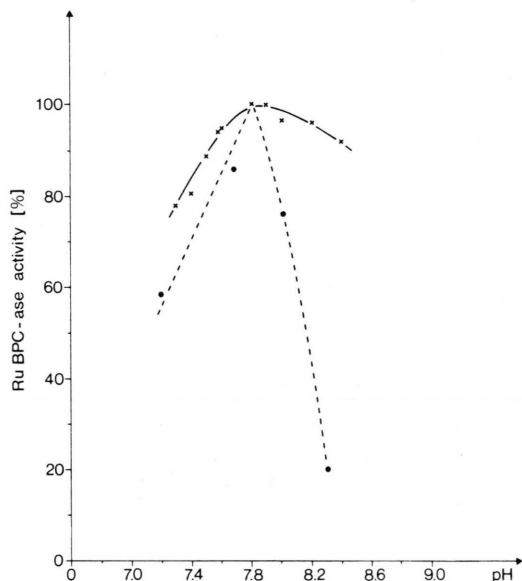


Fig. 5. pH Dependence of RuBPC-ase activity in the crude homogenate (•---•) and with crystallized carboxylase from spinach (x—x).

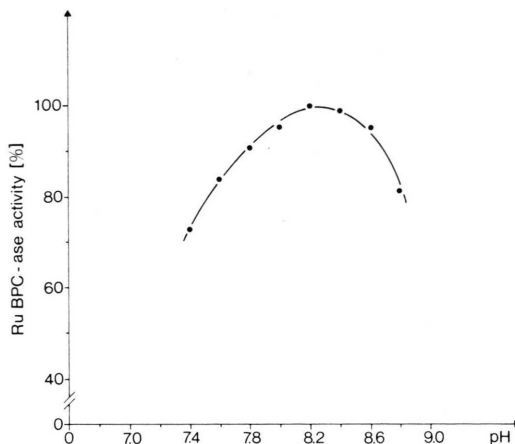


Fig. 6. pH Activity profile of purified RuBPC-ase from spinach (graph drawn from a publication of Andrews [8]).

Judging from recent scientific knowledge the question has not been answered yet if there exists a broad or sharp pH optimum for RubPC-ase. A change of its properties caused by purification and crystallization processes cannot be excluded. In this connection Ashton's hypothesis [10] might be of special interest: He discusses an extensive fixation of Calvin cycle metabolites, *e.g.* fructose-1,6-biphosphate, seduheptulose-1,7-biphosphate and NADPH by RubPC-ase.

These conditions, being not valid for RubPC-ase *in vitro*, might help to influence the properties of the carboxylase *in vivo*.

Present publication is part of the first authors thesis.

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