Phytochemistry, 1979, Vol. 18, pp. 561–564. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0401-0561 $02.00/0

ISOLATION AND CHARACTERIZATION OF WHEAT RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

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(*Received* 24 *August* 1978)

Key Word Index—*Triticum aestivum*; Gramineae; wheat; ribulose-1,5-diphosphate carboxylase; purification; biochemical properties.

Abstract—Wheat ribulose-1,5-diphosphate carboxylase purified to homogeneity had a MW of 540000, sedimentation coefficient ($S_{20,w}$) of 18.5 S, apparent diffusion constant (D_{app}) of 3.07 × 10^{-7} cm^2/sec, Stoke's radius 5.44 nm, and fractional ratio of 1.17. Electron microscopy revealed particles of 10–12 nm diameter. The enzyme was dissociated by sodium dodecyl sulphate into two subunits of MW 53000 ($S_{20,w}$ = 3.0 S) and 13500 ($S_{20,w}$ = 1.7 S). The total amino acid residues in the large and small subunits were 481 and 117, respectively. Tryptic peptide maps of the two subunits confirmed the estimated numbers of Arg and Lys residues. Although the amino acid pattern of the large subunit closely resembled that from barley, rather than that for spinach, beet or tobacco, the pattern of the small subunit was markedly different from those of all the other species.

INTRODUCTION

Ribulose-1,5-diphosphate (RuDP) carboxylase (EC 4.1.1.39) catalyses the carboxylation of RuDP to form two molecules of 3-phosphoglycerate during photosynthesis and also catalyses the oxygenation of the same substrate to form one molecule each of 3-phosphoglycerate and phosphoglucolate [1, 2]. *In situ* immunofluorescent labelling experiments show that this enzyme is located mainly in the PCR (usually 'Kranz') cell chloroplasts of C_4 plant species, and in the chloroplasts of all leaf chlorenchymatous cells of C_3 and CAM plant species [3]. The enzyme, as isolated from higher plants, has a MW of *ca* 550000 and can be dissociated into two serologically non-identical subunits of 52000–58000 and 12000–18000 [4]. It has been established that the large subunit is encoded by the chloroplast genome whereas the small subunit is encoded by the nuclear genome, and that the assembly of the enzyme takes place in the chloroplast [5]. A recent report indicates that the small subunit may be synthesised as a higher MW precursor which is cleaved to its final size when it enters the chloroplast [6]. The large molecule varies in its detailed structure and composition according to plant species [7, 8], and it seems worthwhile investigating more thoroughly the extent to which, and at what levels (division, order, family) such variation is taxonomically predictable. Although wheat RuDP carboxylase has been studied previously [9, 10] there has been no thorough investigation of its physiocochemical properties. In this paper, we report the isolation of the enzyme from wheat leaves and discuss its molecular properties and enzyme kinetics, comparing these with information available for the enzyme as isolated from other higher plants.

RESULTS AND DISCUSSION

Isolation and purity

Table 1 shows the sp. act. of our enzyme at each stage of purification. Since only 55% of the total enzyme activity was recovered by centrifugation of the 35–60% satd. $(NH_4)_2SO_4$ fraction in 35% (w/v) sucrose–60% satd $(NH_4)_2SO_4$, not all the RuDP carboxylase was sedimented and/or passed through the sucrose dense region. DEAE-cellulose chromatography separated the nucleic acids (A_{280}/A_{260} < 0.5, fractions 16–22) from the protein (A_{280}/A_{260} > 1.6, fractions 40–60) with the

Table 1. Purification of RuDP carboxylase from wheat leaf

Fraction	Total protein* (mg)	Total activity*	Specific activity‡	Purification (fold)	Recovery %
Crude extract	777	93.2	0.12	1.0	100
35–60% satd $(NH_4)_2SO_4$	355	92.3	0.26	2.2	99
35% sucrose–60% satd $(NH_4)_2SO_4$	160	51.2	0.32	2.7	55
DEAE-cellulose chromatography	30	10.2	0.34	2.8	11
20–35% (w/v) sucrose gradient	24	8.6	0.36	3.0	9

*Starting material, 50 g fr. wt leaf.
†Total activity expressed as μmol CO_2 fixed/min.
‡Sp. act. expressed as μmol CO_2 fixed/min/mg protein.

enzyme activity. Selective pooling of fractions with high enzyme activity resulted in recovery of only 30 mg protein. Sucrose density gradient ultracentrifugation of the DEAE-cellulose chromatography fraction indicated a homogenous protein band with RuDP carboxylase activity.

However, there was some UV absorbing material at the top of the gradient which was not attributable to protein, this being too little to cause a significant A reading. The purified enzyme has a sp. act. of 0.36 μmol CO_2 fixed for spinach enzyme and 0.15 μmol CO_2 fixed for barley enzyme [11, 12]. It is homogenous by criteria of polyacrylamide gel electrophoresis in different gel concentrations and by sedimentation velocity ultracentrifugation.

Kinetics

HCO_3^- and Mg^{2+} concentrations in excess of 25 and 10 mM respectively are inhibitory to enzyme activity. With RuDP concentration up to 0.4 mM, 'first-order' kinetics approaching 'zero-order' kinetics were observed. The apparent K_ms for CO_2, RuDP and Mg^{2+} are 27 μM, 107 μM and 2.3 mM, respectively; those for CO_2 and RuDP thus being similar to values reported for other plants, while that for Mg^{2+} is higher than that for spinach [4, 13, 14].

Molecular properties

The MW as determined by electrophoresis is 540000 and the $S_{20,w}$ value by sedimentation velocity is 18.5 S. The dimer of the enzyme ($S_{20,w}$ = 27.3 S) was also detected by the above methods, after leaving it to stand in the absence of dithiothreitol. The D_{app} of the enzyme is 3.07 × 10^{-7} cm^2/sec and the partial specific volume is 0.731 g/ml. Thus, the MW based on MW = $RTS/D(1 - v\rho)$ is *ca* 550000, in close agreement with the value obtained by electrophoresis. The Stoke's radius is 5.44 nm and the fractional ratio is 1.17. Assuming the water of hydration to be 0.33 g/g for globular proteins, the enzyme molecule seems to be either a prolate or oblate ellipsoid [15]. These physical features of the wheat enzyme are similar to those reported for spinach and oat [4, 6, 13, 16, 17].

Electron microscopy

Electron microscopic examination of the phosphotungstic acid stained enzyme reveals particles with diameter range 10–12 nm. This correlates well with the diameter of the Stoke's sphere (10.9 nm). The particles show a central dark region, perhaps due to penetration of the stain in a pit or depression in the protein surface [8, 9].

Subunit composition

The wheat enzyme consists of two subunits of MW 53 000 and 13 500, as determined by SDS-polyacrylamide gel electrophoresis. When the subunits are separated on Sephadex G-100 in the presence of SDS, the elution profile shows 3 peaks (Fig. 1). The elution volume of peak A corresponds with that of Blue Dextran, SDS-treated albumin and SDS-treated ovalbumin, while peak B coincides with the elution volumes of SDS-treated haemoglobin and SDS-treated cytochrome *c*, thus indicating the relative molecular sizes of the two peaks. SDS-polyacrylamide gel electrophoresis confirms peak A to be the large subunit and peak B to be the small subunit,

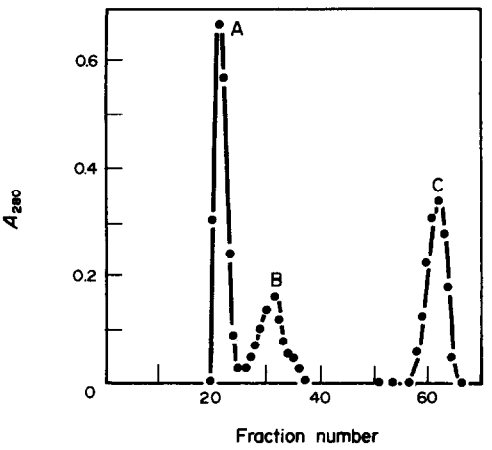

Fig. 1. Separation of subunits of RuDP carboxylase by gel filtration on Sephadex G-100 in the presence of SDS. The large and small subunits are represented by A and B, respectively, C being excess mercaptoethanol.

peak C being mercaptoethanol, present in the sample during the dissociation of the enzyme. The ratio of the total absorbances at 280 nm of peak A to peak B is 2.1:1, compared to 1.9:1 obtained for spinach enzyme [18].

Using urea to dissociate the enzyme for study by sedimentation velocity ultracentrifugation, one protein peak ($S_{20,w}$ = 3.0 S) was observed, notwithstanding that the enzyme consists of two subunits with different MWs [19] with the large and small subunits dissolved in urea and subjected separately to sedimentation velocity ultracentrifugation, only the large subunit showed a protein peak. However, when the subunits were prepared in the absence of urea, the large subunit exhibited an $S_{20,w}$ = 3.0 S, and the small subunit, a protein peak of $S_{20,w}$ = 1.7 S, these being comparable with 3.0 and 1.8 S, as previously reported for spinach [18]. The small molecular size and the high viscosity of the urea medium seemingly does not allow the small subunit to sediment under the conditions employed.

Attempts to determine the N-terminal amino groups of the subunits have been unsuccessful, suggesting that they may be blocked or have substituted groups [20, 21]. The N-terminal amino group for the small subunit of the enzyme for other plant species is Met; as yet there is no report of the N-terminal amino group for the large subunit [22, 23].

Amino acid composition of native enzyme and subunits

The amino acid composition and the probable numbers of each amino acid in the native wheat enzyme and its subunits are presented (Table 2). Amino acid composition is expressed as molar ratio relative to Phe for comparison with previously published analyses [7, 8] and the estimations of amino acid residues for the native enzyme assume a stoichiometry of 8 large and 8 small subunits [24]. We find the total amino acid residues in the large and small subunits to be 481 and 117, respectively. In the large subunit we estimate the Arg and Lys content per molecule to be 31 and 25 residues, respectively. Tryptic peptide maps of the large subunit indicate the presence of 27 Arg and 21 Lys containing peptides. With the small subunit, tryptic peptide maps reveal the presence of 5 Arg and 9 Lys containing peptides, against the estimated 6 Arg

Table 2. Amino acid composition of wheat RuDP carboxylase and its subunits

Amino acid	Molar ratio to Phe (residues per molecule)		
	Native enzyme	Large subunit	Small subunit
Lys	1.18 (264)	1.07 (25)	1.13 (8)
His	0.54 (128)	0.59 (14)	0.25 (2)
Arg	1.28 (296)	1.33 (31)	0.85 (6)
Asx	1.96 (440)	1.96 (46)	1.28 (9)
Thr	1.13 (264)	1.17 (28)	0.72 (5)
Ser	1.01 (224)	0.91 (21)	0.92 (7)
Glx	2.03 (472)	1.93 (45)	1.89 (14)
Pro	1.30 (272)	1.12 (26)	1.14 (8)
Gly	2.09 (480)	2.15 (51)	1.31 (9)
Ala	1.86 (416)	1.93 (45)	0.94 (7)
Cys	0.33 (72)	0.30 (7)	0.30 (2)
Val	1.20 (304)	1.26 (30)	1.11 (8)
Met	0.50 (88)	0.34 (8)	0.44 (3)
Ile	0.88 (204)	0.93 (22)	0.58 (4)
Leu	1.67 (352)	1.48 (35)	1.24 (9)
Tyr	0.74 (168)	0.62 (15)	0.79 (6)
Phe	1.90 (248)	1.00 (24)	1.00 (7)
Trp	0.35 (88)	0.34 (8)	0.45 (3)
Total	(4784)	(481)	(117)

and 8 Lys residues per molecule. Thus, there is satisfactory correlation between the predicted number of Arg and Lys residues in the subunits and the tryptic peptides observed. In turn, this supports the reliability of the estimated number of amino acid residues in the two subunits.

The overall amino acid pattern of the native enzyme is rather similar to that reported for barley, oat, spinach, beet and tobacco, except that Trp, Try, Val and Lys are lower in the wheat enzyme. However, a comparison of our wheat enzyme data with those available for other higher plants shows much closer resemblance between the grasses wheat, barley and oat, than between them and the dicotyledons spinach, beet and tobacco. The enzyme from wheat, barley and oat has lower Thr, Val, Leu and Tyr contents, but higher Pro content as compared to that from spinach, beet and tobacco [8].

The amino acid pattern of the large subunit of the wheat enzyme is distinctly different from that of the small subunit, His, Arg, Asx, Thr, Gly, Ala and Ile being low in the latter. On the other hand, the large subunit is quite similar to that from barley, spinach, beet and tobacco, expect that Glx, Val and Leu are slightly lower in wheat [8]. Again, however, we observe that the pattern of the large subunit from wheat enzyme shows greater similarity to that of barley (lower levels of Tyr, Lys, and Leu but higher Ser) than to that of spinach, beet or tobacco. A comparison of the amino acid pattern of the small subunit of wheat enzyme with that of barley, spinach, beet and tobacco however shows great variations. Nevertheless, while the small subunit of the wheat enzyme has significantly lower Asx, Thr, Glx, Gly, Ala, Ile, Leu, Lys and Trp but higher Pro, as compared to that of the barley enzyme, the small subunits from the two grasses show lower Pro, Leu and Tyr, as compared to those from the spinach, beet and tobacco enzyme [8].

Comparison with RuDP carboxylases from other higher plants

Comparison of our observations on the wheat enzyme with data from that of other higher plants [7, 8, 11–14, 16–19] shows that, as expected, all the RuDP carboxylases studied share many similarities at gross molecular level and in enzyme kinetics. However, there is variation in detailed amino acid patterns of the native enzyme and its subunits from different species, and this shows some correlation with the taxonomic distinction between grasses and dicotyledons.

EXPERIMENTAL

Enzyme preparation. This method was modified from that of ref. [9], with all stages carried out at 0–4. Freshly harvested leaves (50 g) from 14-day-old wheat seedlings were ground in liquid N_2 and extracted in 100 ml of 0.2 M Tris–SO_4, 10 mM EDTA, and 5 mM dithiothreitol (DTT) pH 8. The homogenate was filtered, then centrifuged at 30000 *g* for 15 min. The pH of the supernatant was adjusted to 7.8 by addition of N NaOH. $(NH_4)_2SO_4$ fractionation followed. The ppt. from the 35–60% satd. fraction was dissolved in 38 ml of 50 mM *N*-2-hydroxyethyl piperazine *N'*2-ethanesulphonic acid (HEPES), 1 mM EDTA, 1 mM DTT pH 7.8 (50 μm HEPES buffer), of which 9 ml was then pipetted into a 76 × 15 mm cellulose nitrate centrifuge tube containing 2 ml of 35% (w/v) sucrose in 50 mM HEPES buffer satd to 60% $(NH_4)_2SO_4$, overlayered with 1 ml of 35% (w/v) sucrose in 50 mM HEPES buffer. Centrifugation was carried out at 190000 *g* for 17 hr, using the Beckman SW rotor. The pellet, recovered in 6 ml of 50 mM HEPES buffer, was dialysed against 2 l. of 1 mM HEPES buffer for 6 hr. Dialysis was then continued for 18 hr in 2 l. of 10 mM HEPES buffer containing 2.5 mM $(NH_4)_2SO_4$. After elution through a DEAE-cellulose column, (2.5 × 22 cm) which had been equilibrated in 10 mM HEPES buffer containing 2.5 mM $(NH_4)_2SO_4$, with a linear gradient of 2.5 mM to 0.3 M $(NH_4)_2SO_4$ in 10 mM HEPES buffer, 5 ml fractions were collected and assayed for enzyme activity and protein content. Active fractions were combined and precipitated with $(NH_4)_2SO_4$ at 60% satn. The ppt. was recovered in 4 ml 50 mM HEPES buffer, of which 1 ml was layered on top of 36 ml linear sucrose density gradient, 20–35% (w/v) sucrose in 0.2 M Tris–SO_4, 10 mM EDTA and 5 mM DTT pH 8. Centrifugation was carried out at 100000 *g* for 24 hr using a Beckman SW 27 rotor. Fractions of 2 ml were collected and assayed for enzyme activity and protein content.

The enzyme was assayed by following the oxidation of NADH at A_{340} nm [25]. The reaction mixture at pH 7.8 (vol. = 0.9 ml) consisting of 50 mM HEPES, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 2 mM ATP, 62.5 μM NADH, 25 mM $NaHCO_3$ and coupling enzymes was contained in a 1 ml curvette. The coupling enzyme mixture (50 μl) comprised 12.5 units 3-phosphoglyceric phosphokinase, 12.5 units 3-phosphoglyceraldehyde dehydrogenase, 25 units triose phosphate isomerase and 12.5 units α-glycerol phosphate dehydrogenase and 50 μg BSA in 50 mM HEPES. An aliquot (50 μl) of a suitably diluted enzyme prepn was added to the reaction mixture and pre-incubated for 10 min, while the A_{340} was constant. The reaction was then initiated by the addition of 3.6 mM RuDP (50 μl). The change in A_{340} was followed on a spectrophotometer with a linear recorder. It was then converted to μmol CO_2 fixed [25].

To study its kinetics, the enzyme was activated by incubation in 50 mM HEPES, 10 mM $MgCl_2$ 0.2 mM EDTA, 0.5 mM DTT and 12.5 mM $NaHCO_3$ pH 7.8. The reaction mixture containing RuDP (vol. 0.95 ml) was equilibrated to 25° before the reaction was started by addition of 50 μl of the activated enzyme [26, 27].

Protein was estimated using modifications of Lowry's method and Coomassie-Blue staining [28, 29]. The latter procedure is free from interference by DTT and $(NH_4)_2SO_4$, and is useful when the enzyme sample is limiting and its protein content low. In both methods, BSA served as a reference protein.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out following the methods of refs [30–32]. SDS-polyacrylamide gel electrophoresis was carried out following the method of ref. [33] except that the acrylamide:bis (acrylamide) ratio was 74.3 and no stacking gel was used.

Ultracentrifugation. The purified enzyme (1 mg/ml) was dialysed in 0.2 M Tris–SO_4, 0.1 mM EDTA, and 0.1 mM DTT pH 8 for 24 hr with 1 change of medium. For dissociation, the enzyme was precipitated with cold Me_2CO at 80% and the ppt. was redissolved in 8 M urea, 50 mM borate–NaOH buffer pH 9. Subunits of the enzyme separated on Sephadex G-100 in the presence of SDS, were precipitated with cold Me_2CO, and the ppts. were taken up in the appropriate medium. The native enzyme was centrifuged at 46000 rpm at 20°, while the subunits were centrifuged at 58000 rpm with the aid of synthetic boundary cells. The $S_{20,w}$, D_{app}, Stoke's radius and frictional ratio were calculated by standard procedures [34]. The partial sp. vol. was calculated from the amino acid composition of the enzyme.

Electron microscopy. A drop of the purified enzyme (0.3 mg/ml) was pipetted onto a Cu grid and left to stand for 1 min before excess sample was removed. A drop of 1% (w/v) aq. phosphotungstic acid was placed on the grid for 1 min, excess stain being removed before examining the sample.

Separation of subunits. The enzyme was dissociated in 2 ml of 1% SDS, 1% mercaptoethanol in 0.2 M Tris–SO_4 pH 8 at 100° for 3 min. The subunits were separated on a Sephadex G-100 column (45 × 2.5 cm) equilibrated with 0.1% SDS, 0.1% in mercaptoethanol in 40 mM Tris–SO_4, 2 mM EDTA pH 8 at room temp. Fractions corresponding with the subunits were combined for further analyses.

Amino acid analysis. The purified enzyme and subunits were precipitated with cold Me_2CO, and washed with cold 80% Me_2CO. Hydrolysis was carried out in 3 N mercaptoethane sulphonic acid as well as in 6 N HCl [35]. Cys was determined as Cys(O_3H) after performic acid oxidation [36]. Amino acid analysis was carried out on a Beckman-Spinco amino acid analyser Model 120B.

Tryptic digestion and mapping. The subunits were carboxymethylated according to the procedure of ref. [37]. The carboxymethylated protein (0.3 mg) was suspended in 10 µl 0.1 M *N*-ethylmorpholine acetate pH 8, TPCK-Trypsin in 5 µl NH_4 formate pH 8 was added in the ratio of 1 Trypsin:100 protein (w/w), and incubated at 37° for 2 hr. A similar quantity of the enzyme was added and further incubated for 2 hr. The digest was then lyophilised and redissolved in 5 µl of 30% HOAc. The recovered sample (5 µl) was spotted on a 10 × 10 cm pre-coated cellulose TLC plate and developed in *n*-BuOH–Py–HOAc–H_2O (15:10:3:12) for *ca* 2 hr. The plate was dried, then electrophoresed in the second dimension at 46 V/cm^{-1} for 25 min with 8.7% HOAc in 2.5% aq. HCO_2H pH 2 as electrolyte buffer. The dried chromatogram was stained in phenanthrene quinone reagent to detect Arg containing peptides, and then in Cd-ninhydrin reagent for total peptides [8, 38, 39]. *N*-terminal amino group analysis was carried out as given in ref. [40].

Acknowledgements—We thank Mr. M. Perri and Mr. R. G. Whitty (Central Services, RSBS), for carrying out the sedimentation velocity ultracentrifugation and electron microscopy respectively, Mr. L. B. James (Biochemistry, JCSMR) for the amino acid analyses, and Prof. C. B. Osmond for helpful comments.

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