RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE FROM OENOTHERA. PURIFICATION AND A PEPTIDE MAPPING PROCEDURE FOR THE SUBUNITS

by

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Key words: Fraction I protein, plant enzyme isolation peptide mapping, Oenothera

Ribulose 1,5-diphosphate carboxylase has been purified from fully expanded leaves of *Oenothera*. The isolation procedure overcame the problems which resulted from a high content of mucilage and phenolic compounds in the leaves. Protein extraction into dilute buffer containing a phenol adsorbant and reducing agents followed by a combination of gel filtration and ion exchange chromatography allowed the enzyme to be essentially purified to homogeneity. The purified RuDP carboxylase had a specific activity of 1.5 μmoles CO_2 fixed. min^{-1}. mg^{-1} at pH 7.8 and 25°. The two subunits could be dissociated in detergent solutions and were found to have molecular weights and amino acid compositions similar to the respective subunits from other sources. A two-dimensional mapping procedure employing ion exchange and paper chromatography was used to partially characterize the tryptic peptides of the RuDP carboxylase subunits; this technique may be useful in interspecific comparisons of the primary structure of the two subunits from *Oenothera* species.

1. INTRODUCTION

Ribulose 1,5-diphosphate [1]) carboxylase (Fraction I protein, E. C. 4.1.1.39) catalyzes the photosynthetic fixation of carbon dioxide in higher plants. The enzyme from a number of species has been shown to have a molecular weight of about 550,000 and can be dissociated into two types of subunit (8,18); the larger of about 55,000 MW contains the catalytic site (28) and the smaller of about 13,000 MW which may have a regulatory function (29). Isoelectric focusing (23,36) and peptide mapping (3,21) studies of the subunits from some *Nicotiana* species have shown that the large subunit is maternally inherited, possibly coded by chloroplast DNA and that the small subunit is inherited in a Mendelian manner characteristic of a nuclear DNA product. The large subunit has been shown to be synthesized from chloroplast mRNA (12,35) by chloroplast ribosomes (2), whilst the small subunit is probably synthesized on cytoplasmic ribosomes (11,33).

The prominent position of RuDP carboxylase in the photosynthetic process and in the interaction between nuclear and extranuclear DNA provides a unique tool to investigate aspects of the cooperation between chloroplast and cytoplasm in a classical system: species of the subgenus *Oenothera*, the evening primrose. In the subgenus *Oenothera* the chloroplast organelle genetic information, the plastome has been clearly shown to be a discrete and independently transmitted genetic system (32). Among the studied species 5 heritable plastome types have been recognised which are adapted to particular genome classes (41). When the organelle genetic system is transferred, by interspecific crossing into a cell with a different genome class then various degrees of plastome-genome incompatibility may be observed as embryonic breakdown or aberrant pigment and plastid properties (37). Prevention of viable interspecific hybridization by this extranuclear system in *Oenothera* has played a role in the evolution of the subgenus (42). This paper reports the isolation of RuDP carboxylase from some *Oenothera* species and a peptide mapping technique to compare the primary structure of the subunit polypeptides from the different species.

2. MATERIALS AND METHODS

2.1 Plant Material

The investigations were carried out on four species: *Oenothera hookeri, O. parviflora ammophila, O. argillicola* and *O. grandiflora Tuscaloosa*. Seeds were swollen at 4° and germinated on moist filter paper in petri dishes or capped glass vials. When the seedling cotyledons were free of the seed coat the plants were transfered into soil in the greenhouse. Leaves which were fully expanded or approaching full expansion were used for protein isolation.

2.2 Chemicals

Ribulose diphosphate (tetrasodium salt) was purchased from Sigma Chemical Co., USA. [^{14}C]-sodium bicarbonate was from the Radiochemical Centre, Amersham, UK. Soluble polyvinyl pyrolidone (PVP) was either PVP-10 from Sigma Chemical Co. or K25 (pharmaceutical grade) from Fluka AG, Switzerland. 4-vinylpyridine was bought from Riedel-de Haën, Germany; »Aristar« guanidinium hydrochloride was from B. D. H. Chemicals, UK and L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketonetrypsin (TRCK-trypsin) was from Worthington, USA. Sepharose^{R} 6B and Sephadex^{R} G25 and G100 were products of Pharmacia Fine Chemicals, Sweden; DE52 DEAE-cellulose was from Whatman Biochemicals Ltd., UK and Dowex AG50W-X4 (200-400 mesh) resin was from Biorad Laboratories, USA. All other chemicals were of analytical grade.

All buffers were made up with deionized water. Pyridine was redistilled from ninhydrin before use and iodoacetic acid was rendered iodine-free by drying from water *in vacuo*.

2.3 Enzyme Assay

RuDP carboxylase activity was assayed by measuring fixation of $^{14}CO_2$ into acid-stable products at 25° in a solution containing 0.1M Tris-Cl, pH 7.8: 15mM $MgCl_2$; 25 mM $NaH^{14}CO_3$ (ca. 0.4 μCi/μmole) and 0.4 mM RuDP. The total volume was 0.5 ml and the reaction was performed in 3 ml capped serum vials (Pierce Reactivials). The enzyme was preincubated in the reaction mixture for 6 minutes prior to the addition of RuDP which was used to initiate the reaction. Aliquots (50 μl) were taken at various time intervals up to 5 minutes and placed directly into 0.1 ml acetic acid in a scintillation vial. After drying the samples were redissolved in 0.1 ml water and fixed ^{14}C was determined by scintillation counting. The specific activity of the sodium bicarbonate was determined in each assay by pipet-

1. Abbreviations: BAWP, butanol-acetic acid-water-pyridine; PVP, polyvinylpyrolidone; RuDP, ribulose 1,5-diphosphate; SDS, sodium dodecyl sulfate; SSA, 5-sulfosalicylic acid; TCA, trichloroacetic acid; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone.

ting a 10 µl aliquot of the reaction mixture into 0.1 ml saturated barium hydroxide solution and measuring the radioactivity. Enzyme activity was measured from the initial linear rate of ^{14}C fixation.

All assay solutions were made up with a solution containing 0.1M Tris, 15 mM $MgCl_2$ which had been adjusted to pH 3 with HCl, degassed under vacuum and extensively flushed with nitrogen to remove endogenous dissolved carbon dioxide and oxygen and subsequently adjusted to pH 7.8 with 2M NaOH (1).

2.4 Protein estimations

In the initial stages of purification protein was determined by the turbidity produced with 2% 5-sulfosalicylic acid (SSA) (25) as suggested by LOOMIS (26). With the purified enzyme this method gave results in reasonable agreement with other methods assuming that the concentration of protein by absorbance at 280 nm equals 1.3 x concentration by SSA turbidity. The presence of soluble PVP and phenolic compounds interfered with other methods. 1% PVP was precipitated with 5% trichloroacetic acid (TCA) but not with 2% SSA and interfered with the Biuret procedure. The Biuret reagent was reduced to cuprous oxide by crude extracts.

Purified RuDP carboxylase was estimated by amino acid analysis or by using the formula: conc. (mg/ml) = 0.59 x $A^{1\,cm}_{280}$ based on an extinction coefficient determined as $E^{1\%}_{280} = 16.9$

2.5 Extraction of soluble protein from leaves

100 gm mature leaves were chopped into small pieces and homogenized with 500 ml buffer using a Sorvall Omnimixer or a Polytron (Kinematica GmbH) at full speed for 5 periods of 30 seconds such that the temperature did not rise above 2°. The fuffer contained 0.1M Tris-HCl, 0.2M NaCl, 10 mM sodium metabisulfite, 1 mM KCN, 5 mM EDTA, 40 mM mercaptoethanol and 1% soluble PVP such that the final pH was about 7.2. The homogenate was squeezed through a nylon mesh (30 µm pore size) and then centrifuged at 32,000 g for 5 minutes in a Sorvall SS34 head.

2.6 Enzyme Purification

The homogenate supernatant after centrifugation was applied directly to a Sephadex G25 (medium) column equilibrated with 16.7 mM sodium phosphate, pH 7.2; 10 mM sodium metabisulfite and 20 mM mercaptoethanol. Sample volumes of up to 25% of the column volume were applied. The transmission at 280 nm and the conductivity of the eluate were recorded. Protein content of alternate fractions was determined with the SSA turbidity method. Fractions eluted after the void and containing protein were pooled. Between each use the column was washed with deionised water and subsequently reequilibrated with buffer.

Whatman DE52 DEAE-cellulose was equilibrated with 16.7 mM sodium phosphate, pH 7.2; 10 mM sodium metabisulfite and 20 mM mercaptoethanol (buffer 1) and packed into a column such that about 200 ml of packed resin was used for the extract from 100 gm leaves. The pooled fractions from the G25 column were run onto the column which was then washed with buffer 1 until the transmission of the eluate at 280 nm was almost that of buffer 1. The enzyme was eluted with a linear gradient of buffer 1 to 167 mM sodium phosphate, pH 7.1; 10 mM sodium metabisulfite and 20 mM mercaptoethanol. Those fractions containing the majority of the enzyme activity were pooled and concentrated to 15 ml in an Amicon 402 ultrafiltration device fitted with a PM30 filter.

The concentrated sample was applied to a Sepharose 6B column (51 cm x 5 cm) equilibrated with 133 mM Tris-Cl, pH 7.5; 10 mM KCl and 0.1 mM EDTA and eluted with this buffer. Fractions which contained RuDP carboxylase activity and with an absorbance at 280/260 ratio of greater than 1.6 were pooled and used for subsequent studies on the subunits.

All steps in the purification were performed at 2° and all buffers were degassed under vacuum and flushed with nitrogen before use.

2.7 Subunit Separation

The two subunits of the reduced or reduced and modified protein were separated at 22° by gel filtration on a Sephadex G100 column (85 cm x 5 cm) equilibrated with 50 mM ammonium bicarbonate, 0.5% SDS adjusted to pH 8.0 with ammonia or equilibrated with 50 mM Tris-Cl, pH 8.5; 0.5% sodium dodecyl sulfate (SDS)

(34). Fractions containing the two subunits were pooled and freeze dried. SDS was removed by dissolving the freeze dried powder in 1 volume of 10 mM HCl and precipitating the protein with 4 volumes of acetone at -18°. The precipitate was collected by centrifugation, dialyzed against 50 mM ammonium bicarbonate and freeze dried.

2.8 SDS-polyacrylamide gel electrophoresis

The homogeneity of the enzyme during purification and of the subunits was analysed with the system of CHUA and BENNOUN (4) using gels with a 10-15% gradient of acrylamide. Electrophoresis was performed at a constant current of 17.5 mA for 6 hours at room temperature and the gels were stained with Coomassie G250 brilliant blue.

2.9 Reduction and Chemical Modification

Samples of subunit were reduced and carboxymethylated as described by HIRS (14) or reduced and S-pyridylethylated as described by FRIEDMAN *et al.* (9). After the reaction the samples were dialyzed extensively against deionised water or 50 mM ammonium bicarbonate, using acetylated dialysis tubing (44) for samples containing small subunit.

2.10 Amino acid analyses

Samples were hydrolysed *in vacuo* at 110° with 6M HCl containing 0.3% phenol for 16 and 48 hours. Amino acid analyses were obtained with a Durrum D-500 automatic amino acid analyser. 4-pyridylethyl-cysteine was eluted immediately after lysine with the buffer system used and was quantified using the colour value calculated according to FRIEDMAN *et al.* (9).

2.11 Trypsin digestion

Digestion of the polypeptide chains was performed at 25° in 50 mM ammonium bicarbonate with 2% (w/w) TPCK-trypsin. The trypsin was added as a solution (1 mg/ml) in 1 mM HCl. After 6 hours the digestion was stopped by adjusting to pH 3.5 with acetic acid and subsequently freeze dried.

2.12 Peptide mapping

A two-dimensional technique was used to characterize the tryptic peptides.

The peptides were first separated by ion exchange chromatography on Dowex AG50W-X4 using a pyridine acetate gradient. The resin was equilibrated with 0.05 M pyridine acetate, pH 2.5 and packed into a column (19 cm x 0.0 cm) thermostated at 40°. The peptides were dissolved in 1 ml of this buffer and, after any insoluble material had been removed by centrifugation, applied to the column. Elution was commenced with about 20 ml 0.05M pyridine acetate followed by an exponential gradient (290 ml) from 0.05M pyridine acetate, pH 2.5 to 2M pyridine acetate, pH 4.9 generated by a LKB Ultrograd gradient maker. When the gradient was finished elution was continued with about 15 ml 2M pyridine acetate and a final 4M pyridine wash to remove any tightly bound peptides. The flow rate was 13.5 ml h^{-1} and 2.2 ml fractions were collected. The transmission at 280 nm of the eluate was recorded with a LKB Uvicord.

For the second dimension the procedure described by WOOTTON *et al.* (45) was used. Aliquots of alternate tubes were taken, dried down and spotted onto duplicate sheets of Whatman 3 MM chromatography paper in a small volume of 50 mM ammonium bicarbonate. The papers were developed by descending chromatography using butanol: acetic acid: water: pyridine (15:3:10:12 v/v). After drying the papers were stained with 0.25% ninhydrin in acetone followed, after bleaching with 2% HCl in acetone by the phenanthrenequinone arginine stain (46) or with cadmium ninhydrin (5) followed by 1-nitroso-2-napthol and HNO_3 as a tyrosine stain (5). The composite map was produced from the two staining procedures.

3. RESULTS AND DISCUSSION

3.1 Isolation of native ribulose diphosphate carboxylase

Fully expanded *Oenothera* leaves were found to contain large quantities of mucilage which made extraction of soluble proteins difficult. Homogenization of the leaves in 2 volumes of

buffer produced a very viscous homogenate in which the majority of the cells were judged to be still intact by light microscopy. At least 5 to 10 volumes of homogenization buffer were required to rupture the cells and extract the soluble proteins.

It has been shown that *Oenothera* leaves contain phenolic compounds (47) and flavonoids (16) and tannin vacuoles have been described within the leaves (7). In the absence of adsorbants of phenolic compounds and tannins and nonreducing conditions during homogenization, the extracts turned brown very rapidly. Such extracts contained very little protein which could be precipitated with SSA or TCA and very little RuDP carboxylase activity. The use of the reducing agents 0.1M sodium ascorbate or 40 mM mercaptoethanol alone preserved about 7% of the activity in crude extracts compared to the activity preserved if 2% soluble PVP was also included to adsorb phenolic compounds. Insoluble PVP (up to 40 gm/100 ml) was less effective in preserving enzyme activity; only 14% of the activity of extracts containing soluble PVP was obtained, although the bulk of insoluble PVP may also have interfered with cell breakage during homogenization. Best results were obtained if the homogenization buffer contained 1-2% soluble PVP as a phenol adsorbant, as well as sodium metabisulfite and mercaptoethanol to maintain reducing conditions and to inhibit phenol oxidase. Because of the large extract volumes a concentration of the protein prior to fractionation was attempted by pressure dialysis or precipitation. The extract supernatant was concentrated in an Amicon 402 cell fitted with a PM30 filter (nominal retention over 30,000 MW). However the compounds responsible for the high viscosity of the extracts were retained by the membrane and filtration flow rates were very low. Attempts to concentrate the protein by ammonium sulphate precipitation were also unsuccessful. In crude homogenates the addition of ammonium sulphate at constant pH to 50% saturation caused the solution to brown almost instantaneously, although the addition of reducing agents such as 0.1M sodium ascorbate decreased this effect.

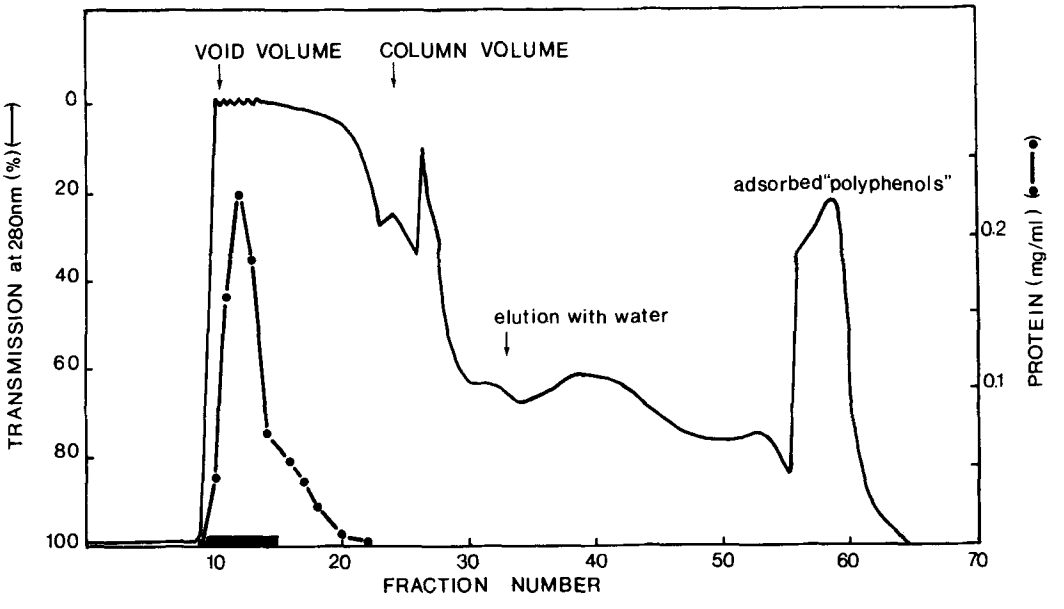

Figure 1. *Chromatography of a homogenate of* Oenothera hookeri *leaves on a Sephadex G25 column* (2.6 cm x 32 cm). The column was equilibrated with 16.7 mM sodium phosphate, pH 7.2; 10 mM sodium metabisulfite and 20 mM mercaptoethanol and eluted with this buffer at a flow rate of 59 ml.h^{-1}. Fractions of 7.3 ml were collected. The sample (10% of the column volume) was prepared by filtration and centrifugation of a crude homogenate, as described in the text. The transmission of the eluate at 280 nm was recorded with a LKB Uvicord (3 mm optical path length), 100% transmission was set with the elution buffer. At the point indicated the elution was continued with deionised water. Fractions were pooled as indicated by the bar.

The brown material was adsorbed onto Sephadex G25 like the polyphenolic material (Fig. 1) suggesting that high concentrations of ammonium sulphate catalyse the oxidation of phenolic materials causing polymerization. If soluble PVP was present in the homogenization buffer it was also precipitated with ammonium sulphate as a yellow precipitate which could be dissolved very slowly to yield a dark brown solution. Such preparations contained protein associated with red pigmented material which underwent further aggregation and precipitation.

It was essential to separate the proteins from the phenolic compounds as quickly as possible to prevent protein modification. Therefore the extract supernatant after centrifugation was applied to a Sephadex G25 column to separate the protein from low molecular weight compounds in the homogenate and also to equilibrate the protein with the starting buffer for the DEAE-cellulose step. Figure 1 shows the resultant column profile with an extract from *O. hookeri*. Some polyphenolic compounds were removed due to their adsorption to the Sephadex gel (6). Such compounds eluted slowly and incompletely from the G25 column unless elution was continued with deionized water which abolished the affinity of these compounds for the gel. It was essential to be able to apply a large sample volume because of the extraction problems. Adequate resolution was achieved with sample volumes of up to 25% of the column volume as judged by the protein content and conductivity of the eluant fractions.

The pooled fractions from the G25 column containing protein and soluble PVP were loaded onto the DEAE-cellulose column. All of the enzyme activity was bound to the resin together with some green material, retained as a band at the top of the resin. A considerable amount of brown pigmented material, possibly phenol oxidation products and denatured protein was irreversibly retained on the resin. The majority of the PVP was not bound to the column and is the

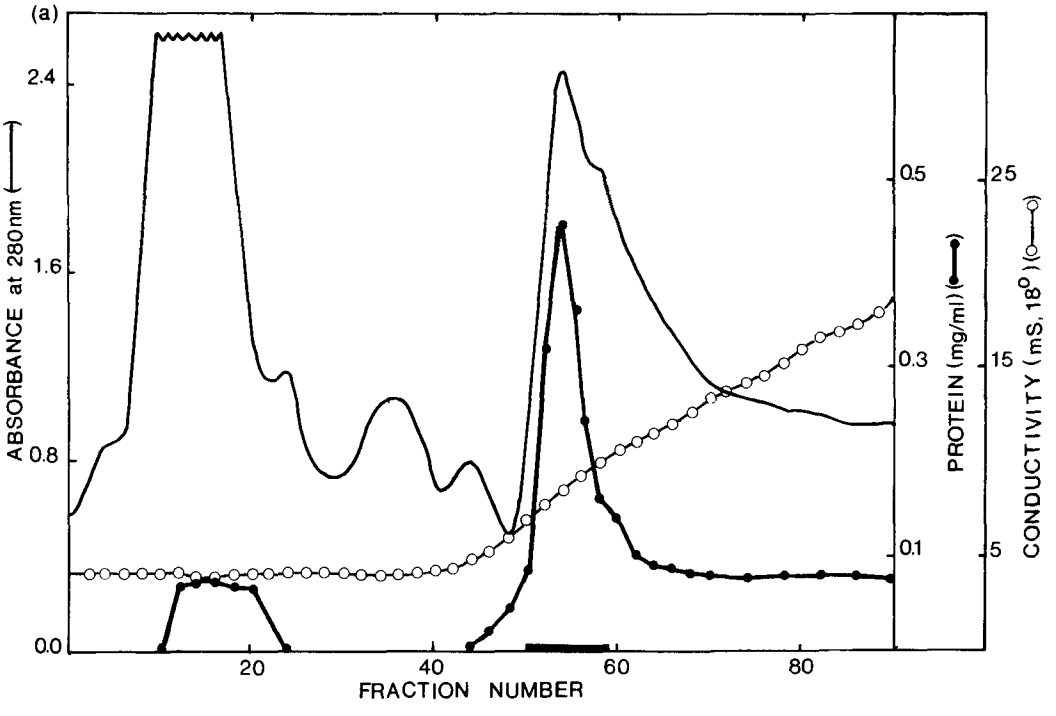

Figure 2a. *DEAE-Cellusose (Whatman DE52) chromatography of a homogenate from* O. hookeri *after its passage through a Sephadex G25 column.* The gel was equilibrated with 16.7 mM sodium phosphate, pH 7.2, 10 mM sodium metabisulfite, 20 mM mercaptothanol (buffer 1) and packed into a column (13 cm x 2.6 cm). Fraction size was 7.6 ml and the flow rate was 36 ml. h^{-1}. The sample (65 ml) was loaded onto the column and elution commenced with buffer 1. At fraction 30 a linear gradient of 250 ml buffer 1 to 250 ml 250 mM sodium phosphate, pH 7.1, 10 mM sodium metabisulfite, 20 mM mercaptoethanol was begun. Fraction were pooled as indicated by the bar.

major component of the first absorption peak in Figure 2(a) which shows the separation pattern obtained with an extract from *O. hookeri* leaves. The enzyme was eluted with a phosphate concentration gradient and the first protein peak eluted by the phosphate buffer was found to have RuDP carboxylase activity. Figure 2(b) shows the elution pattern of RuDP carboxylase from *O. parviflora*. The peak protein fractions also contained the highest carboxylase activity, the specific activity of these fractions being almost constant at 4.2 μmoles $^{14}CO_2$ fixed min^{-1}. mg $protein^{-1}$ in the assay system described and with the protein determined by the SSA procedure. Fractions containing the majority of the enzyme activity were pooled and their homogeneity was determined by SDS polyacrylamide gel electrophoresis. Although the two RuDP carboxylase subunits were the main components, significant contamination with other proteins was observed.

The pooled and concentrated fractions from the DEAE-cellulose column were applied to a Sepharose 6B column. A typical elution profile, obtained with *O. argillicola* RuDP carboxylase is shown in Figure 3. A small amount of green material, probably membrane and aggregated protein eluted at the void volume. A discrete peak of RuDP carboxylase (K_{av}=0.39) was eluted followed by a broad peak which contained some protein as well as some soluble PVP. The carboxylase protein was colourless in solution and white when precipitated. The ratio of absorbance at 280 nm to the absorbance at 260 nm of the peak fractions was 1.9 indicating no contamination of the protein with materials which absorb more strongly at 260 nm than at 280 nm such as nucleic acids and some phenolic compounds (26). The specific activity of the enzyme was 1.5 μmoles CO_2 fixed. min^{-1}. mg^{-1} which is in good agreement with values reported for the spinach (38) and bean (11) enzymes. The high specific activity of the enzyme immediately after DEAE-cellulose chromatography was par-

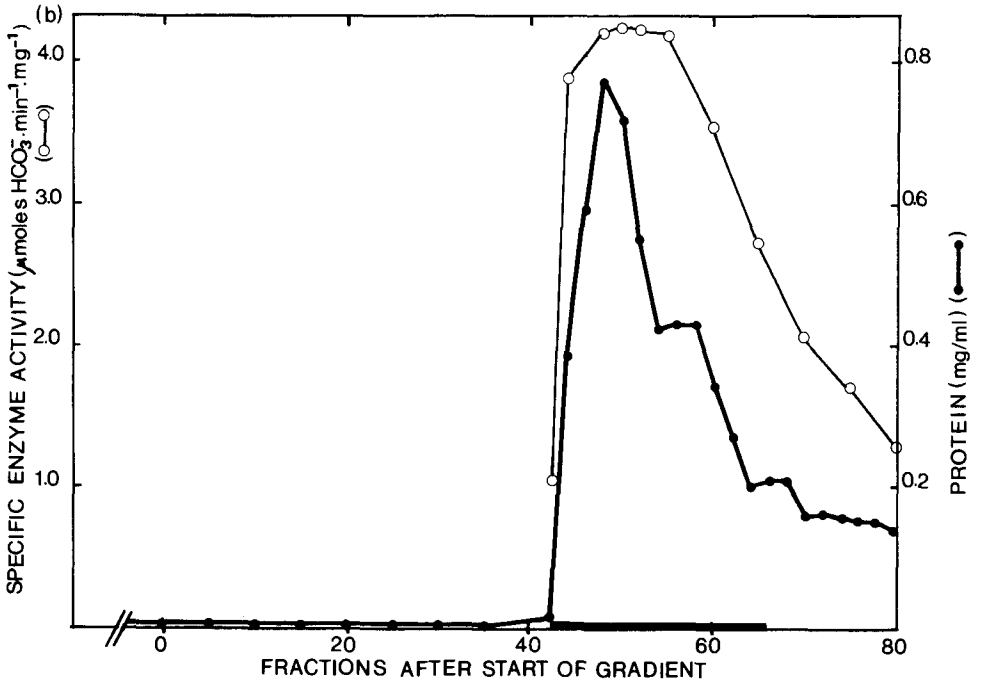

Figure 2b. *As in (a) except that the homogenate was obtained from 100 gm of leaves of* O. parviflora ammophila. The column (15 cm x 4.4 cm) was equilibrated with buffer 1. 1600 ml sample was applied to the column at a flow rate of 400 ml. h^{-1}. Elution commenced with 415 ml buffer 1 followered by a linear gradient of 500 ml buffer 1 to 500 ml 165 mM sodium phosphate, pH 7.1, 10 mM sodium metabisulfite, 20 mM mercaptoethanol. During the gradient the flow rate was 48 ml. h^{-1} and 9.2 ml fractions were collected. The specific carboxylase activity of selected fractions was determined in the assay system described. Fractions were pooled as indicated by the bar.

tially due to an underestimation of the protein concentration but the possibility of a decrease in the specific activity during purification was not excluded. Fractions with an absorbance 280nm/260nm ratio greater than 1.6 were pooled and examined by SDS polyacrylamide gel electrophoresis. By this criterion the protein was essentially pure.

The application of this purification procedure to obtain RuDP carboxylase from four species of *Oenothera (hookeri, parviflora ammophila, argillicola, grandiflora Tuscaloosa)* has been successful. The behaviour of the protein from different species was essentially similar and in general approximately 1 mg/gm fresh wt leaf was obtained.

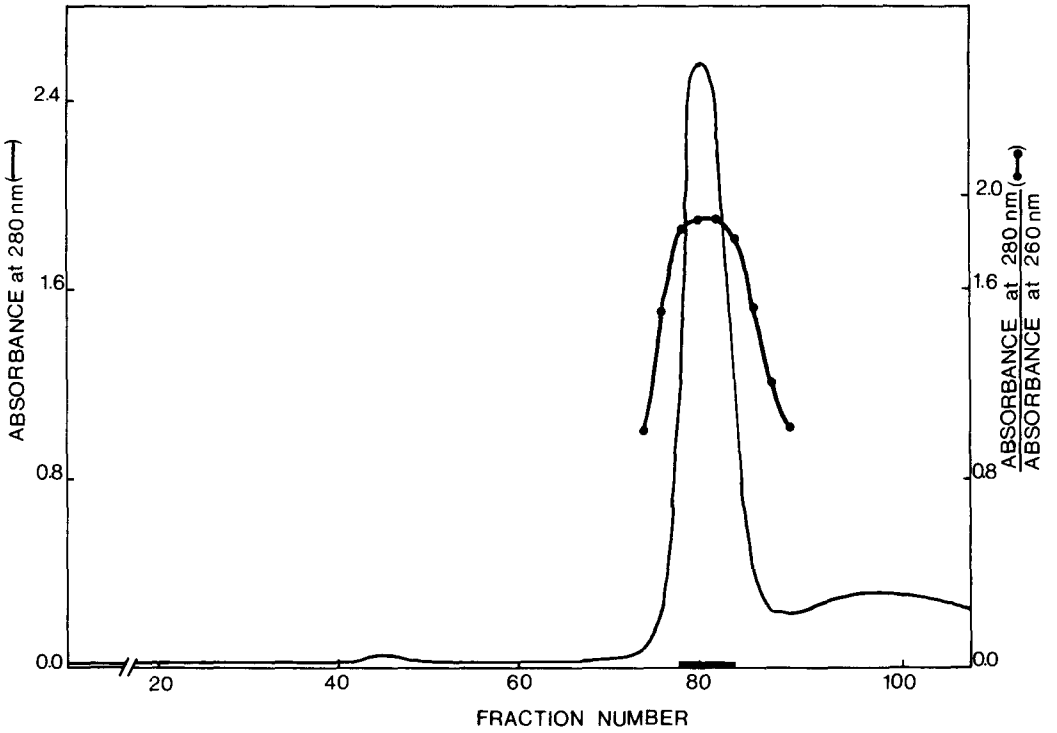

Figure 3. *Chromotography of RuDP from* O. argillicola *on Sepharose 6 B.* The column (51 cm x 5 cm) was equilibrated with 133 mM Tris-Cl, pH 7.5, 10 mM KCl, 0.1 mM EDTA. The flow rate was 24 ml. h^{-1} and the fraction size was 7.5 ml. The concentrated pooled fractions after DEAE-Cellulose chromatography (15 ml) were applied. The absorbance of alternate fractions was determined at 280 nm and 260 nm. Fractions containing RuDP carboxylase with an $A_{280/260}$ ratio greater than 1.6 were pooled and concentrated.

3.2 Studies on the Subunits of RuDP Carboxylase

The separation of the two subunits of *O. argillicola* RuDP carboxylase by chromatography in SDS-containing buffer after reduction and dissociation of the protein is shown in Figure 4. The homogeneity of pooled fractions containing the subunits was checked by SDS polyacrylamide gel electrophoresis. The large subunit preparation contained two faint bands of lower molecular weight and the small subunit preparation contained a faint large subunit band on heavily loaded gels. However it was judged that the levels of contamination were not significant to interfere with subsequent studies on the subunits.

The two subunits of *O. grandiflora* RuDP carboxylase were also run on a 7.5% to 10% acrylamide gel calibrated with bovine serum albumin (67,000 MW), catalase (57,500), ovalbumin (45,500) and cytochrome c (12,500). The large subunit had measured molecular weight of 54-57,000 and the small subunit had a mobility

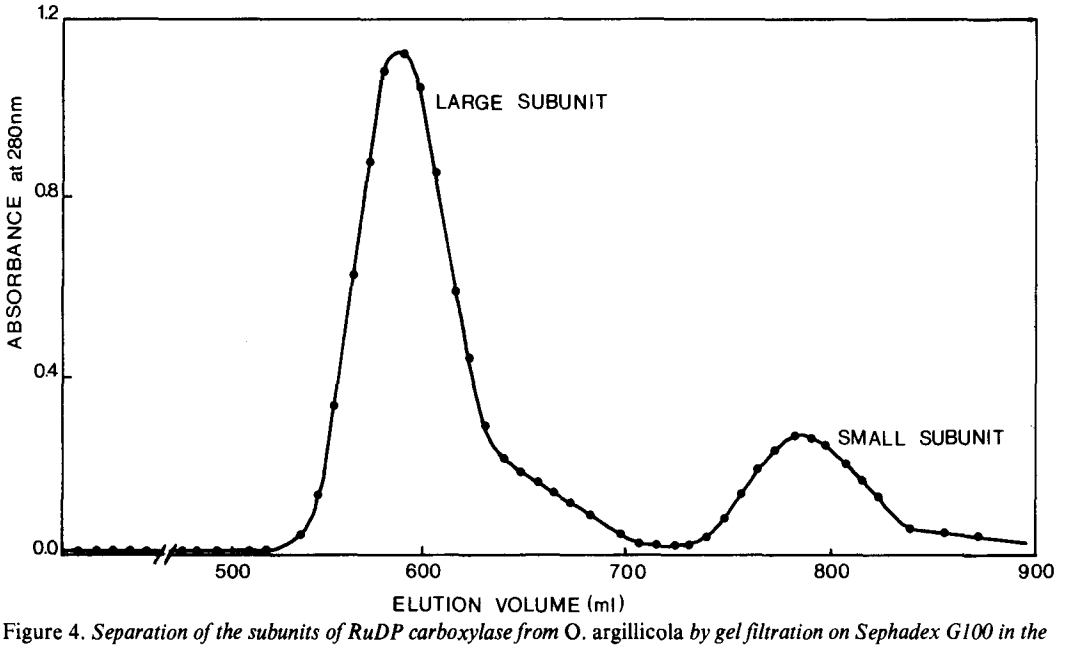

Figure 4. *Separation of the subunits of RuDP carboxylase from* O. argillicola *by gel filtration on Sephadex G100 in the presence of SDS.* The protein (in 30 ml, 2.3 mg.ml^{-1}) was incubated in 0.2M mercaptoethanol, 1% SDS at 55° for 1 hour and then applied to the column (84 cm x 5 cm) which was equilibrated with 50 mM Tris-Cl, pH 8.5, 0.5% SDS. Elution was with this buffer at a flow rate of 28 ml. h^{-1} and the fraction size was 8.4 ml. The absorbance at 280 nm of alternative fractions was recorded and fractions containing the two subunits were pooled by this criterion.

consistent with a molecular weight of 13,000, in good agreement with published values for other sources (8).

The amino acid compositions of the two subunits from *O. hookeri* together with the calculated residue numbers for the observed molecular weights are presented in Table I. In Table II the compositions of the two subunits from *O. hookeri* are displayed together with the amino acid compositions of the subunits from barley, spinach and *Chlamydomonas reinhardtii.* Previous comparisons have shown that the amino acid composition of the small subunit exhibits considerable inter-specific variation. Automatic determination of the N-terminal sequence of the small subunits from barley, pea, bean, and tobacco (10,13,31) have shown that the small subunits are homologous polypeptides but with a substantial number of amino acid substitutions between them. As shown in Table II the amino acid compositions of the large subunits are very similar between the species confirming that these are also homologous polypeptides but which are too large for small differences between them to be resolved by amino acid analysis.

The pyridylethylated small subunit of *O. hookeri* was digested as a suspension which gradually disappeared during the digestion. No insoluble peptides could be observed in the pyridine acetate buffer, pH 2.5 and the complete digest was applied to a Dowex AG50W column. The pyridylethylated large subunit of *O. hookeri* was soluble in 50 mM ammonium bicarbonate and no insoluble digestion products were observed in this buffer; however, at a lower pH, for example pH 2.5 in pyridine acetate, a considerable amount of material was insoluble and only the soluble peptides were applied to the AG50W Column.

Altogether 20 peptides from the small subunit and 33 peptides from the large subunit of RuDP carboxylase from *O. hookeri* were visualized by the staining regime used (Figure 5). Together with the tryptic peptides the fingerprints were also complicated by the presence of a few minor peptides not shown in Figure 5 and some free amino acids. Free arginine and free lysine were indicated in the region of fractions 100 and 85, respectively. In addition free neutral amino acids, probably contaminants, were possibly represented in fractions 30-40. The num-

Table I

The amino acid compositions of O. hookeri RuDP carboxylase subunits

Amino acid	Large subunit		Small subunit	
	molar ratio a)	no. residues/ polypeptide b)	molar ratio a)	no. residues/ polypeptide b)
Aspartic acid	2.11	46.3	1.42	9.6
Threonine c)	1.41	31.0	0.71	4.8
Serine c)	0.90	19.7	0.74	5.0
Glumaic acid	2.40	52.7	2.17	14.8
Proline	1.04	22.8	1.06	7.2
Glycine	2.38	52.3	1.60	10.9
Alanine	2.14	47.0	0.99	6.7
Valine d)	1.53	33.6	1.21	8.2
Methionine	0.39	8.5	0.34	2.3
Isoleucine	1.10	24.1	1.19	8.0
Leucine	2.08	45.7	1.53	10.4
Tyrosine	0.88	19.3	1.20	8.1
Phenylalanine	1.00	21.9	1.00	7.4
Histidine	0.67	14.7	0.33	2.3
Lysine	1.10	24.1	1.18	8.0
Arginine	1.39	30.5	0.77	5.3
Cysteine	0.40 e)	8.7	0.11 f)	0.7

a) molar ratios relative to phenylalanine = 1.00
b) calculated for a large subunit of 55,000 MW and a small subunit of 13,000 MW assuming an average residue wt of 110 daltons.
c) extrapolated to zero time.
d) value after 48h hydrolysis.
e) average value from S-pyridylethyl and S-carboxymethylated derivatives.
f) determined as S-pyridylethyl derivative.

ber of small subunit peptides was broadly consistent with the calculated arginine + lysine content (13 residues/polypeptide), whereas only about half the expected number of peptides was obtained from the large subunit based on a calculated arginine + lysine content of 54 residues/polypeptide.

Previous reports of the numbers of tryptic peptides obtained by fingerprint analysis from the two subunits of several plant species show considerable variation which to some extent seems dependant upon the technique used and the interpretation of the resultant map. For the small subunit 9 (19), 13-16 (19,20,21,27), 23-28 (13,22,39,40) and 32 (24) peptides have been reported, whereas for the large subunit 19-27 (3,19,20,22,27), 42-43 (3,40) and 55 (24) peptides have been reported. In all cases peptides were identified as material which was stained with ninhydrin. This illustrates the caution which must be exercised when a peptide mapping technique is used to compare mutant and wild type proteins or make phylogenetic relationships between homologous proteins.

The fingerprint technique is used to characterize peptides produced by multiple cleavage of the polypeptide usually with proteolytic enzymes. Certain limitations are inherent in this approach which should be considered in an evaluation of the results and choice of system. The specificity of the proteolytic enzyme and the rate of hydrolysis of individual peptide bonds may by partial hydrolysis produce multiple redundant peptides containing essentially the same sequence. Trypsin is one of the more specific enzymes but partial hydrolysis can result from adjacent basic amino acid sequences as for example in the N-terminal sequence

Table II

Amino acid composition of RuDP carboxylase subunits

Amino acid	*O. Hookeri*	Barley	Spinach	*Chlamydomonas*	
Aspartic acid	1.42	1.58	2.20	2.14	
Threonine	0.71	0.95	1.23	0.96	
Serine	0.74	1.06	0.64	1.08	
Glutamic acid	2.17	2.48	2.71	2.08	
Proline	1.06	1.01	1.56	1.16	
Glycine	1.60	1.50	1.07	1.09	
Alanine	0.99	1.37	0.79	1.96	
Valine	1.21	1.46	1.12	1.83	
Methionine	0.34	0.37	0.45	0.65	SMALL
Isoleucine	1.19	0.87	0.66	0.74	SUBUNIT
Leucine	1.53	1.49	1.78	1.19	
Tyrosine	1.20	0.93	1.74	0.91	
Phenylalanine	1.00	1.00	1.00	1.00	
Histidine	0.33	0.26	0.54	0.18	
Lysine	1.18	1.31	1.09	1.05	
Arginine	0.77	0.83	1.10	1.02	
Cysteine	0.11	0.35	0.64	0.72	
Tryptophan	-	0.78	0.96	-	
Aspartic	2.11	1.98	2.20	2.42	
Threonine	1.41	1.32	1.73	1.39	
Serine	0.90	1.07	0.70	0.87	
Glumatic acid	2.40	2.26	2.28	2.40	
Proline	1.04	1.15	1.12	1.27	
Glycine	2.38	2.20	2.33	2.62	
Alanine	2.14	2.07	2.18	2.61	
Valine	1.53	1.57	1.67	1.80	
Methionine	0.39	0.30	0.39	0.66	LARGE
Isoleucine	1.10	1.12	0.92	1.07	SUBUNIT
Leucine	2.08	1.90	2.16	2.02	
Tyrosine	0.88	0.70	0.94	0.96	
Phenylalanine	1.00	1.00	1.00	1.00	
Histidine	0.67	0.61	0.77	0.63	
Lysine	1.10	1.06	1.13	1.25	
Arginine	1.39	1.25	1.48	1.62	
Cysteine	0.40	0.28	0.44	0.93	
Tryptophan	-	0.70	0.48	-	
Reference:	present work	(40)	(43)	(17)	

Compositions are molar ratios relative to phenylalanine = 1.00

of some RuDP carboxylase small subunits (10,13,31). Partial hydrolysis is also known from basic-acidic amino acid sequences and basic amino acid-proline sequences. At long digestion times the results of pseudochymotryptic activity can be significant. Resultant tryptic peptides may not be soluble in the buffer system used and most mapping techniques are not suitable for large peptides so that parts of the sequence are not represented as peptides. Chemical changes can occur during the treatment of peptides, for example oxidation of labile residues, deamidation of amide residues, cyclization of N-terminal glutamine or peptide bond hydrolysis at sensitive sequences such as Asp-Pro (30). When comparing essentially homologous proteins it is desirable that differences detected between the two by peptide map-

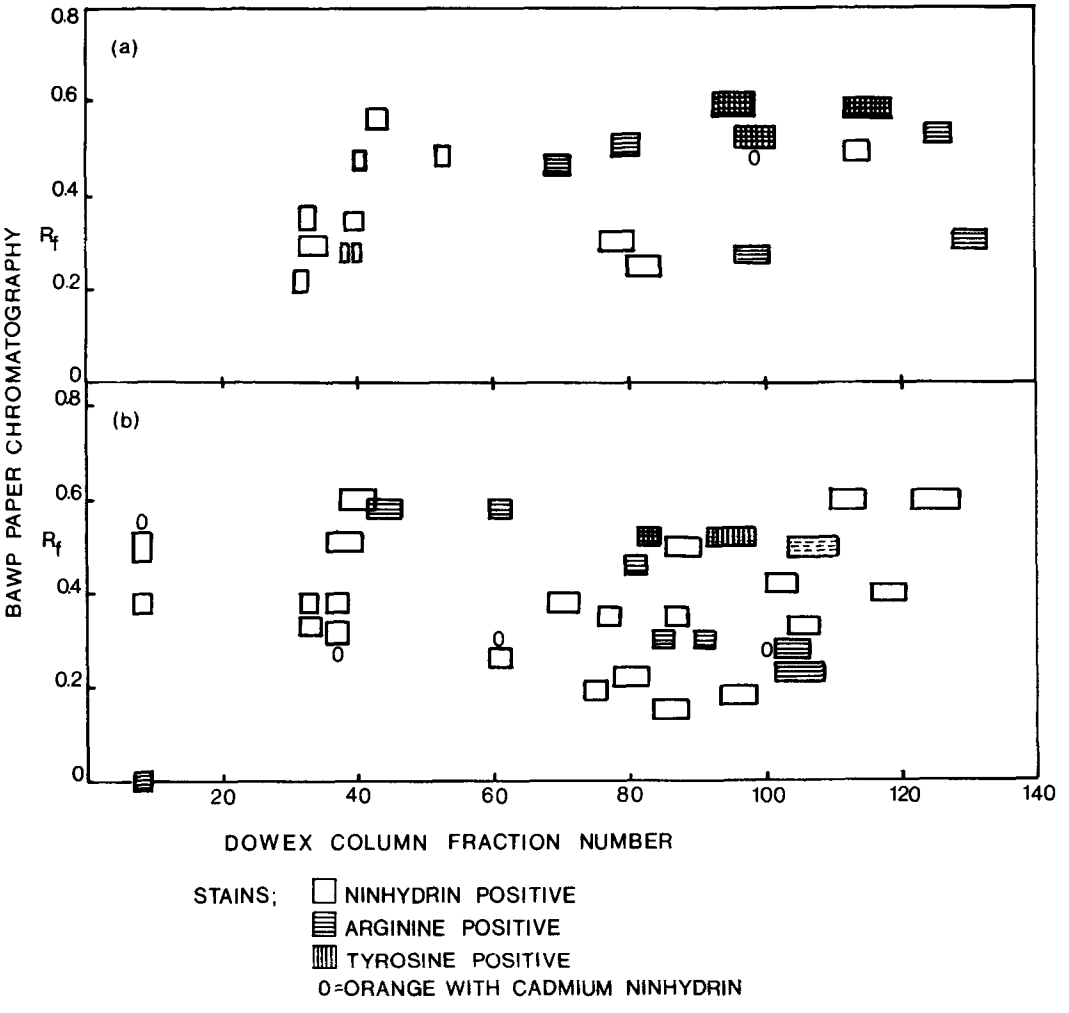

Figure 5 a. *Two-dimensional »map« of the tryptic peptides of S-pyridylethylated small subunit from* O. hookeri *RuDP carboxylase.* The digest was first fractionated on AG50W-X4 resin as described in the text and then samples from alternate fractions were chromatographed on paper using butanol-acetic acid-water-pyridine (BAWP). Peptides were located with ninhydrin and stained for specific amino acids as indicated.

Figure 5b. *Two-dimensional »map« of the tryptic peptides of S-pyridylethylated large subunit from* O. hookeri *RuDP carboxylase.* The procedure used was identical to that Figure 5 (a).

ping should be confirmed by isolation of the peptides containing amino acid differences and the determination of their sequences.

A two-dimensional procedure employing column ion exchange chromatography and BAWP paper chromatography can be used to resolve a large number of peptides (15). Peptides can be further characterized in the second dimension, for example by paper elctrophoresis and different specific amino acid stains or isolated for sequence determination. The resolution of the small subunit tryptic peptides by this method can be used to screen for species-specific amino acid sequence differences. Probably only half of the tryptic peptides of the large subunit are soluble under the conditions used, in agreement with the results of KAWASHIMA *et al.* (22). Despite this inherent limitation these authors were able to detect differences in the large subunit tryptic peptides of different *Nicotiana* species and explore some of the phylogenetic relationships between the species.

However the use of smaller fragments than the tryptic peptides will extend the usefulness of the peptide mapping technique for the large subunit of RuDP carboxylase from *Oenothera.*

ACKNOWLEDGEMENTS

I thank Professor DITER von WETTSTEIN for critical reading of the manuscript and Professor Dr. W. STUBBE, Botanisches Institut der Universität Düsseldorf for kindly providing the seed material. Support by grant GM-22051 of the USPHS National Institutes of Health to Professor D. von WETTSTEIN and by a Wellcome/Carlsberg Travelling Research Fellowship from the Wellcome Trust is gratefully acknowledged.

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