RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE FROM BARLEY (HORDEUM VULGARE).

ISOLATION, CHARACTERIZATION, AND PEPTIDE MAPPING STUDIES OF THE SUBUNITS

by

STEFFEN STRØBÆK

Department of Physiology – Carlsberg Laboratory
Gamle Carlsberg Vej 10 – DK-2500 Copenhagen Valby

and

GREGORY C. GIBBONS

Department of Biotechnology – Carlsberg Research Laboratory
Gamle Carlsberg Vej 10 – DK-2500 Copenhagen Valby

and

Institutes of Genetics and Biochemical Genetics, University of Copenhagen,
Øster Farimagsgade 2 A – DK-1353 Copenhagen K

Key words: Fraction I protein, rapid isolation, comparative amino acid composition.

This paper presents a simple and rapid procedure for the isolation of ribulose-1,5-diphosphate carboxylase from leaves. By column chromatography on Sephadex G-25 and Sepharose 6B combined with concentration by ultrafiltration high yields of undenatured protein are obtainable in less than one working day. RudP carboxylase purified from barley in this way has been characterized. The molecular size is similar to that of spinach, wheat and oat. The apparent molecular weight determined by column chromatography was found to be 510,000, approximately 3 % higher than that for the tobacco protein in the same systems. RudP carboxylase from barley consists of two different kinds of subunits with the same molecular weight properties as described for other plants. The amino acid composition of the native protein shows similarities with another monocotyledon, oat, both having lower contents of leucine and tyrosine than the dicotyledons, spinach and tobacco. The high content of tryptophan in barley RudP carboxylase gives a higher extinction at 280 nm than has been reported for other organisms ($E^{1\,0/00}_{280\,nm} = 2.06$). This paper also describes a mapping technique for the tryptic peptides of the subunits of RudP carboxylase by two-dimensional high voltage paper electrophoresis which is rapid, reproducible and gives well defined spots. The peptide mapping technique is well suited as a screening method for RudP carboxylase mutants.

1. INTRODUCTION

Ribulose-1,5-diphosphate carboxylase also named Fraction I is the major soluble protein of green plant tissues, where carbon fixation occurs essentially via the C_3 pathway of photosynthesis (the Calvin Cycle). Fraction I is exclusively located in the chloroplasts of such plants. Following the discovery of chloroplast-specific DNA, ribosomes, transfer RNA and all of the other components of an autonomous system for translation of genetic information, Fraction I protein, as the major soluble chloroplast protein, has received close attention (10,19,33).

The native protein can be separated into two different kinds of subunits. In inter-specific hybridisation studies of the genus *Nicotiana* by WILDMAN and his coworkers (6,8,22) properties of the small subunit exhibit biparental inheritance whilst properties of the large subunit appear to be maternally inherited. Our accessibility to a large number of mutants in barley (*Hordeum vulgare* L.) has prompted us to start an investigation into the inheritance of the two subunits of Fraction I protein in this monocotyledon. This first paper discribes the purification and properties of native Fraction I protein and its two subunits from 7 day old green leaves of barley, together with a screening technique for Fraction I mutants using tryptic digestion and two-dimensional electrophoretic peptide mapping.

2. MATERIALS AND METHODS

2.1. Plant Material

Seeds of barley (*Hordeum vulgare* L. var. Svalöf's Bonus) were germinated and grown at 20°C for 7 days in vermiculite under continuous illumination 3,000 lux – provided by two Philips TL 20/33 and one TL 20 W/32 fluorescent tubes. Leaves of tobacco (*Nicotiana tabacum* var. Turkish Samsun) were kindly provided by Dr. T. NILSSON-TILLGREN.

2.2. Chemicals and Proteins

All chemicals were analytical grade. D-ribulose-1,5-diphosphoric acid, tetrasodium salt (RuDP); sodium dodecyl sulfate (SDS); polyvinyl pyrrolidone (PVP-10); ribonuclease A, Type IA and bovine thyroglobulin, Type I was obtained from Sigma Chemical Co., Mo., U.S.A. Phosphotungstic acid was purchased from E. Merck, Darmstadt, Germany. (^{14}C)-sodium bicarbonate was obtained from Radio Chemical Centre, Amersham, England, Omnifluor R scintillation mixture from New England Nuclear, Dreieichenhain, Germany, chlorhexidine gluconate (Hibitane R) from Imperial Chemical Industries, Cheshire, England, trypsin TPCK (200 u/mg) from Worthington, New Jersey, U.S.A. and Combithek R calibration proteins (cytochrome c, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase and ferritin) from Boehringer, Manheim, Germany. The source for fibrinogen was Kabi, Stockholm, Sweden, for sperm whale myoglobin Koch-Light Laboratories, Colnbrook, England. Sephadex R G-100, Sephadex G-25 Medium, Sephadex G-200 and Sepharose R 6B from Pharmacia Fine Chemicals, Uppsala, Sweden were prepared following the instructions of the manufacturers. Bio-Gel R P-60 (100–200 mesh) and Bio-Glas R–500 (minus 325 mesh) from Bio-Rad Laboratories, Ca., U.S.A. were used following the instructions of the manufacturers.

2.3. RuDP Carboxylase Assay

The activity of ribulose-1,5-diphosphate carboxylase (E.C. 4.1.1.39) was measured in a reaction mixture (3 ml) containing: Tris/HCl, pH 7.8 – 600 μmoles, RuDP – 2.1 μmoles, $NaH^{14}CO_3$ – 150 μmoles (12 μCi), $MgCl_2$ – 30 μmoles, EDTA, pH 7.5 – 0.18 μmoles, dithiothreitol-30 μmoles. The assay was carried out as follows: Tris, dithiothreitol, EDTA, $NaH^{14}CO_3$, and the enzyme sample were added in that sequence to a reaction vessel equilibrated at 30°C. After 10 minutes incubation, RuDP was added and the reaction thus started. Samples of 0.5 ml were taken at 3, 6, 9, 12, and 20 minutes and added to 0.5 ml of 4N HCl. Duplicate subsamples of 0.1

Abbreviations: dansyl = 5-dimethylaminonaphtalene-1-sulfonyl, RuDP = ribulose-1,5-diphosphate, SDS = sodium dodecyl sulfate.

ml were immediately taken and dried under heating lamps on 2.5 cm circles of Whatman GF/C filter, which were suspended in air with the aid of pins. Radioactivity remaining on the filters was measured in Omnifluor scintillation mixture on a Nuclear Chicago Isocap 300 Scintillation Spectrometer. Control samples were taken from a reaction vessel in which destilled water had been added instead of RuDP. These control samples were used to correct for activity due to enzyme-bound RuDP or non-specific incorporation of $^{14}CO_2$.

2.4. Amino Acid Analysis

Hexaplicate samples containing 25 µg of protein were lyophilized and hydrolysed in 100 µl of 6N redistilled HCl containing 1 ‰ phenol. Hydrolysis was carried out at 110°C under reduced pressure (0.2 Torr) in an atmosphere of nitrogen for 24, 48, and 72 hours. Aliquots of the hydrolysates were analysed on a Durrum D-500 amino acid analyser equipped with 590/440 nm optics.

Cysteine was determined as cysteic acid or as carboxymethylcysteine.

Tryptophan was determined by the tyrosine/tryptophan ratio method of BENCZE and SCHMID (3), after 12 hours dialysis of the protein sample against distilled water and subsequent transfer into 0.1N NaOH.

2.4.1. Statistical Analysis of the Amino Acid Compositions

A value was calculated and used to express the difference between two proteins. The procedure suggested by SHAPIRO (32) was used. No distinction was made between aspartic acid and asparagine or between glutamic acid and glutamine and the values of cysteine and tryptophan are omitted. The values for the remaining 16 amino acids were transformed into number per 100 of these selected amino acid residues, and the Chi^2 value for two amino acid compositions in question was calculated on the basis of the resulting 16 × 2 matrix.

2.5. Titration of SH Groups

The sulfhydryl content (unblocked cysteine) was determined by titration with ELLMAN'S reagent (5,5'-dithiobis-(2-nitrobenzoic acid), 0.33 mM) as described by HABEEB (14) both with and without denaturing reagent (SDS, 2 %). The absorbance of identical samples without 5,5'-dithiobis-(2-nitrobenzoic acid) was measured at 280 nm, to determine the protein content (ca. 0.25 mg/ml).

2.6. Protein Crystallization

Fraction I protein from tobacco was crystallized by the method of CHAN *et al.* (7). Fraction I protein from barley was precipitated in 55 % saturated ammonium sulphate, and the precipitate progressively resuspended in 54 %, 53 % down to 42 % saturated ammonium sulphate until crystals were observed in the supernatant after centrifugation at 1000 × g for 5 minutes (17).

2.7. Electron Microscopy

Samples of purified Fraction I protein containing 1 µg/ml protein were mixed with an equal volume of 2 % w/v aqueous phosphotungstic acid pH 7.0 and allowed to stand on a carbon-coated formvar-treated grid for 5 to 10 minutes. After drying off excess liquid with a filter paper the samples were examined in a Siemens Elmiskop I electron microscope.

2.8. Tryptic Digestion

Samples containing 1 mg of either large subunit or small subunit separated by SDS Sephadex G-100 column chromatography were made 80 % v/v with respect to acetone and the thereby precipitated subunit collected by centrifugation. After drying under nitrogen the subunits were suspended to a protein concentration of 10 mg/ml in 0.1 M NH_4HCO_3 pH 8. Toluene-p-sulfonyl-L-phenylalaninechlorometyl ketone treated trypsin (1 mg/ml in 0.01 N HCl) was added in the ratio 1 trypsin: 50 protein (w/w) and incubated at 37°C. After 2 hours incubation an equal volume of 0.3 % v/v aqueous acetic acid containing 10 % v/v pyridine (high voltage electrophoresis buffer pH 6.5) was added and the digest centrifuged prior to high voltage paper electrophoresis.

2.9. High Voltage Paper Electrophoresis

High voltage paper electrophoresis was car-

ried out in Shandon model 500 panglas chromatography tanks fitted with cooling coils. The coolant for pH 6.5 electrophoresis was toluene containing 8 % v/v pyridine and that for pH 1.9 electrophoresis was varnolene. The power supply was from Locarte, London, England.
Buffers for high voltage paper electrophoresis were:

a) pH 6.5: 0.3 % v/v acetic acid in 10 % v/v aqueous pyridine.
b) pH 1.9: 8 % v/v acetic acid in 2 % v/v aqueous formic acid.

Whatman 3 MM paper (42 × 57 cm) was used in all high voltage paper electrophoresis runs.

2.10. Peptide Mapping

Samples (1 mg) of hydrolysed protein were spotted onto a full size sheet of 3 MM paper 20 cm from the anionic end of the sheet. When larger samples were run, the hydrolysate was streaked in a 6 cm band on the start line (20 cm from anionic end); after sharpening the protein concentration along the start line was 0.5 – 1.0 mg/cm. The visible markers methyl orange and methyl green were spotted onto both sides of the sheet 2 cm from the edge. After drying, a solution of dansyl arginine/dansyl acid (0.4 mM) was applied along the start line and the paper dried again. After wetting the paper and sharpening the sample zone in high voltage paper electrophoresis pH 6.5 buffer, electrophoresis was run at 1 kV for 10 minutes (to remove salts) followed by 3 kV for 35 minutes. The paper was removed, dried and examined under UV-light where the dansyl arginine and dansyl acid bands were traced in.
The dried paper was cut into either 4 cm longitudinal strips for runs with 1 mg protein or 1 – 2 cm strips in the larger scale runs. 42 cm of the strips were sewed 12 cm from the anionic end of a new 42 × 57 cm sheet of 3 MM paper. Markers and dansyl arginine/dansyl acid were applied as before. After wetting and sharpening in pH 1.9 high voltage paper electrophoresis buffer, electrophoresis was run at 3 kV for 30 minutes.

2.11. Location of Peptides

Duplicate peptide maps were stained for total peptides in cadmium-ninhydrin (15), and for arginine containing peptides in phenanthrenequinone reagent (43). Arginine peptides were located under ultraviolet light and the sheet was subsequently stained in acidic cadmium-ninhydrin (100 ml Cd-ninhydrin reagent + 10 ml glacial acetic acid).

2.12. Gel Electrophoresis

2.12.1. Phenol-Acetic Acid-Urea Gel Electrophoresis

Soaked acid gels containing phenol, glacial acetic acid and 8 M urea (2:1:1 w/v/v) were prepared according to MEISSNER and FLEISHER (27). Samples of purified Fraction I containing 1 mg protein/ml were diluted 10 × into fresh phenol-acetic acid-urea (2:1:1 w/v/v) containing pancreatic ribonuclease as an internal standard. The samples were reduced prior to electrophoresis by heating to 100° for 5 minutes after addition of 2 drops of β-mercaptoethanol/ml. Molecular weight determinations of the subunits of Fraction I were kindly run by Dr. N.C. NIELSEN, using the standards described by NIELSEN (29).

2.12.2. Sodium Dodecyl Sulphate Gel Electrophoresis

SDS gel electrophoresis using fluorescently marked proteins was carried out following the method of TALBOT and YPHANTIS (40) with the following modifications:
a) 10 % acrylamide gels containing 0.1 M NH_4HCO_3, pH 9.8 and 0.1 % SDS were used.
b) The electrode buffer was also 0.1 M NH_4HCO_3 containing 0.1 % SDS. Samples containing 0.2 mg of purified Fraction I were added to tubes containing 20 μl 10 % SDS, 50 μl 0.5 M NH_4HCO_3, and 20 μl 1 % dansyl chloride. The tubes were then sealed and heated in a boiling water bath for 5 minutes. 1 μl of β-mercaptoethanol was then added and the samples were heated for another 5 minutes at 100°. Gel electrophoresis was carried out at 2mA/5 mm gel tube for 10 minutes followed by 5 mA/tube for 3 hours.
The mobility of the proteins was then mea-

sured under UV-light and expressed as R (dansyl acid).

3. RESULTS

3.1. Isolation of Native Ribulose-1,5-diphosphate Carboxylase

Leaves of 7 day old light-grown barley plants were harvested and chopped into 5 mm pieces. The following steps were carried out in the cold. The chopped leaves (50 g) were placed in the chilled cup of a Sorvall omnimixer containing 100 ml ice-cold buffer (0.05 M Tris/HCl buffer pH 7.4, 1.0 M NaCl, 0.001 M EDTA, 0.002 M $MgCl_2$, 0.08 M β-mercaptoethanol, 0.2 % polyvinyl pyrrolidone, and 0.002 % chlorhexidine gluconate). After grinding 5 times for 30 seconds with a one minute cooling pause between each grind the homogenate was filtered through 3 layers of muslin and one layer of Miracloth. The material was centrifuged at 19,600 × g for 30 minutes in the GSA rotor of a Sorvall RC-2B centrifuge. The supernatant was filtered through glasswool to remove floating lipid material and directly applied to a 2.5 × 35 cm column of Sephadex G-25 Medium equilibrated in 0.025 M Tris/HCl buffer pH 7.4 containing 0.2 M NaCl, 0.0005 M EDTA and 0.002 % chlorhexidine gluconate, and eluted at a flow rate of 50 ml/min in the same buffer. The protein containing material eluting after the void volume was collected (70 ml). On columns of Sephadex G-25 Medium Fraction I together with membranous material was partially separated from smaller proteins, mercaptoethanol and polyphenols. Sephadex G-25 was preferred in this step because of its affinity for phenolic compounds (9). Figure 1 shows a typical separation on Sephadex G-25 as compared with Bio-Gel P-60 (100–200 mesh), a polyacrylamide molecular sieve which lacks this high affinity for phenols. In these runs the membrane particles have been removed by centrifugation at 69,100 × g for 60 minutes. The first peak in both runs is Fraction I protein. The second peak represents the so-called Fraction II proteins (41) and the shoulder on this second peak is β-mercaptoethanol. The third and fourth peak, in the case of Sephadex G-25, and the third peak, in the case of Bio-Gel P-60, represents the polyphenols. The major portion of the polyphenols was eluted from Sephadex after more than twice the total gel volume (V_t) had been passed through the column, and

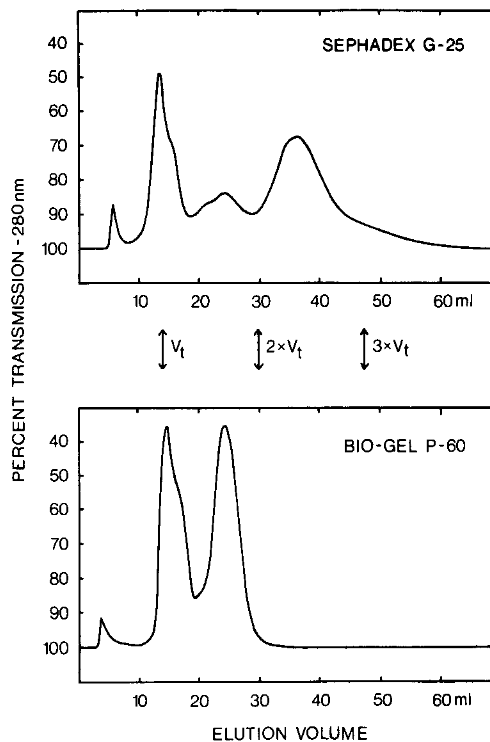


Figure 1. Chromatography of homogenate of barley leaves. Sephadex G-25 and Bio-Gel P-60 were prepared and packed in accordance with the manufacturers' specifications. Both columns (0.9 × 24.5 cm) were equilibrated with 0.025 M Tris buffer pH 7.4 containing 0.2 M NaCl, 0.0005 M EDTA and 0.002 % chlorhexidine gluconate at a flow rate of 13.5 ml/hr. The starting material was prepared as described in the text up to the stage of Sephadex G-25 chromatography followed by centrifugation at 69,100 × g for 60 minutes in the 50.1 rotor of a Beckman L-2 ultracentrifuge to remove membrane particles. Samples 0.5 ml (3 % of total volume – V_t) were loaded onto the columns and developed in the equilibrating buffer at the same speed. The elution pattern was measured in a 3 mm cell of an LKB Uvicord II running at 280 nm. V_t, 2 × V_t and 3 × V_t represent 1 ×, 2 × and 3 × the total column volume V_t = 15.7 ml. In both runs the first peak is Fraction I protein and the second peak Fraction II proteins and β-mercaptoethanol. The other peaks represent the polyphenols.

could be characterized by their broad adsorption at 350 nm (figure 2).

Fraction I containing eluate from Sephadex G-25 was concentrated to a volume of 15 ml by either hollow-fibre filtration in an Amicon CH_3 using an H1DX50 membrane, or precipitation with ammonium sulphate (37 % – 45 % saturation fraction) and subsequent dialysis, and loaded onto a 5 × 45 cm column of Sepharose 6B equilibrated in the Sephadex G-25 buffer. Fraction I protein, assayed as ribulose-1,5-diphosphate carboxylase activity was eluted at a flow rate of 1.67 ml/min in the equilibration buffer and the separation pattern is shown in figure 3. In figure 3 a Sephadex G-200 separation of identical starting material is shown and it can be clearly seen that Sepharose gives a superior separation of Fraction I protein. It should also be noted that Sepharose 6B can be run at a much higher flow rate than Sephadex G-200. The high absorbance of the first peak from the Sepharose 6B run is primarily due to light-scattering of the smaller membrane particles and aggregated protein in this fraction. The time course of the preparation when hollow fibre filtration is utilized is summarized in Table I and it should be noted that isolation of Fraction I protein using this technique can be carried out in less than one day.

Table I.

Time Course of Fraction I Preparation

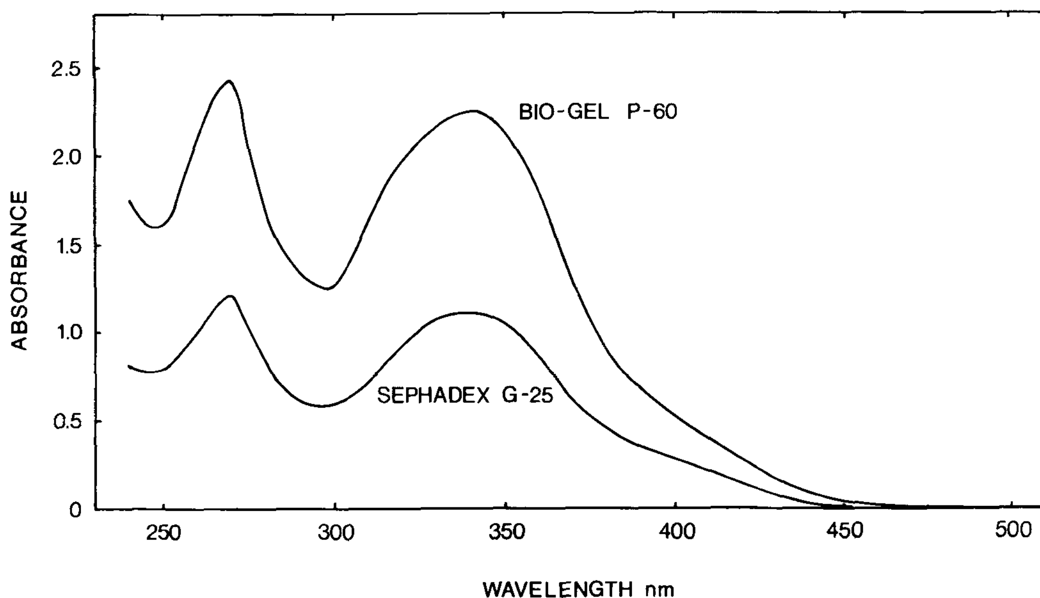
Time (min)	Preparation Step
0	Harvest fresh leaves
5	Cut to 5 mm
15	Grind
30	Filter – Miracloth
40	Centrifuge
90	Filter – glasswool
95	Sephadex G-25
115	Concentrate
145	Sepharose 6B
400	Fraction I

Figure 2. Spectrum of polyphenols from barley. The peak fractions occurring at 37 ml in the case of Sephadex G-25 and 27 ml in the case of Bio-Gel P-60 in the separations shown in figure 1 were examined spectroscopically in 1 cm quartz cuvettes on a Zeiss PMQ IV at 5 nm intervals. The blank contained column equilibration buffer.

3.2. Properties of the Native Protein

3.2.1. Molecular Weight

Molecular weight determination of Fraction I protein from barley showed that barley protein was larger than Fraction I protein isolated from tobacco. On Sepharose 6B barley Fraction I behaved as a globular pro-



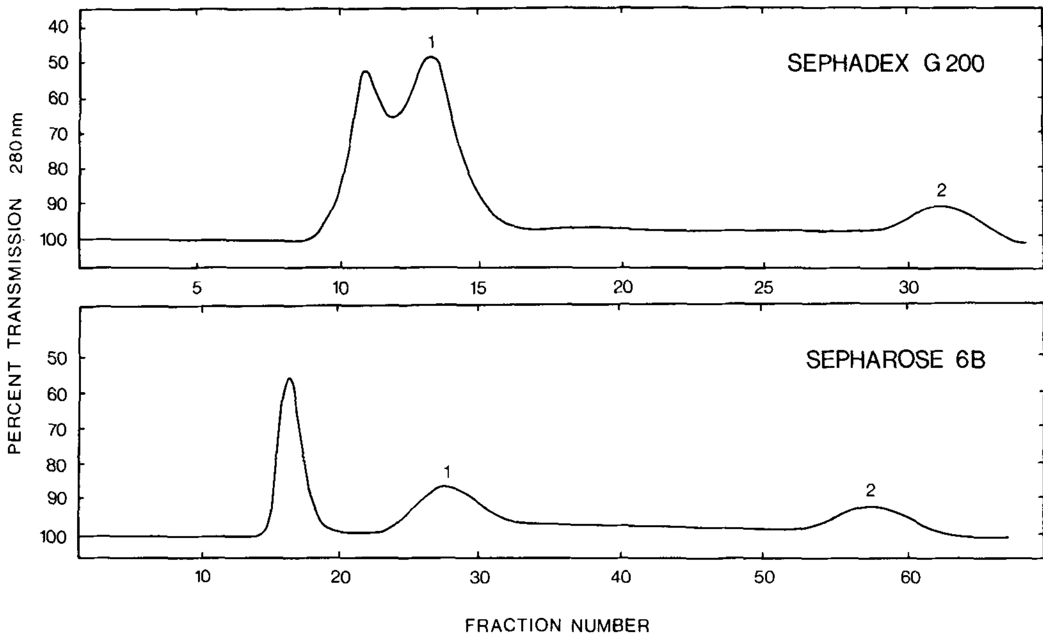


Figure 3. Separation of barley Fraction I protein on Sephadex G-200 and Sepharose 6B. Sephadex and Sepharose were prepared and packed as described by the manufacturers and equilibrated in 0.025 M Tris/HCl buffer pH 7.4 containing 0.2 M NaCl, 0.0005 M EDTA and 0.002 % Hibitane.

Starting material was prepared as follows: 207 g leaves were ground, filtered, centrifuged and filtered through glasswool as described in the text. The material was freed from polyphenols on a 5 × 30 cm column of Sephadex G-25 at a flow rate of 3000 ml/hr. The protein precipitating between 37 % (20 min) and 45 % (18 hrs) saturated ammonium sulphate was collected by centrifugation and dissolved by dialysis against the Sephadex G-200 equilibration buffer for 24 hours. The sample (now in a volume of 15 ml) was applied to a 5 × 41 cm Sephadex G-200 column (V_t = 810 ml) and the column developed in the equilibration buffer at a flow rate of 26 ml/hr. The elute was measured at 280 nm in a 3 mm cell of an Uvicord II and 26 ml fractions were collected.

The Sepharose 6B column (5 × 46 cm, V_t = 910 ml) was equilibrated in the same buffer as Sephadex G-200 at a flow rate of 99 ml/hr and 16.5 ml fractions were collected. The starting material treated exactly as described above for the Sephadex G-200 run.

tein of molecular weight of 511,000 daltons (± 5,000 (½ extreme range)), whereas the tobacco protein exhibited an apparent molecular weight of 498,000 (± 5,000 (½ extreme range)) daltons (figure 4). On Sephadex G-200 barley Fraction I behaved as a globular protein with molecular weight 510,000 (± 5,000 (½ extreme range)) whilst tobacco Fraction I gave 492,000 (±5,000 (½ extreme range)) daltons (figure 5). In both standard plots the apparent molecular weight of bovine serum albumin, catalase and ferritin, have been corrected in accordance with the results of Andrews (1). In similar experiments on Sepharose 6B barley Fraction I had a higher apparent molecular weight than Fraction I protein from spinach, in agreement with a recent suggestion by BÖRNER *et al.* (4).

3.2.2. Amino Acid Composition

The amino acid composition of barley Fraction I protein is presented in Table II. For the sake of comparison the data for Fraction I protein isolated from *Avena sativa* (oat), *Nicotiana tabacum* (tobacco), *Beta vulgaris* (beet), *Spinacia oleracea* (spinach), and *Chlamydomonas reinhardi* are also included. The data for Fraction I protein from *Rhodospi-*

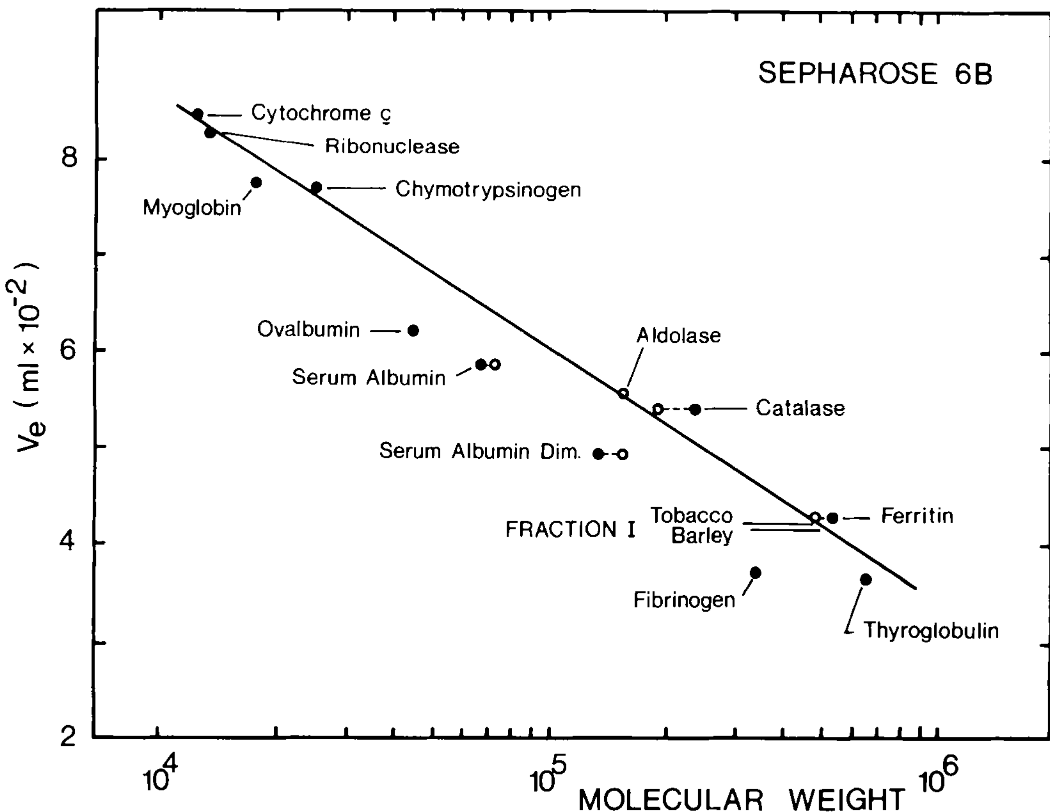
rillum rubrum, which consists of two identical 56,000 daltons subunits are given in Table III. The two monocotyledons, barley and oat, have a lower content of leucine and tyrosine than the dicotyledons spinach and tobacco and the green alga *C. reinhardi.*

When the amino acid compositions were compared according to SHAPIRO (32) using a Chi^2 analysis, the lowest value of 0.29 was obtained when barley and oat Fraction I protein were compared. The highest value of 2.31 was found in the comparison of spinach and *Chlamydomonas.*

When samples of Fraction I protein were analysed without prior dialysis against distilled water two extraneous peaks were observed between phenylalanine and lysine. These peaks could be shown to originate from Tris buffer itself (11).

The number of free sulfhydryl groups relative to phenylalanine was determined to be 0.40 in the denatured protein, corresponding to 94 cysteines in a molecule with 550,000 molecular weight. Without denaturation a level of 73 % of the number of cysteines was reached after 20 hours and remained constant until 44 hours, whereafter discoloration took place. This absence of cystine in the protein is in agreement with the results from spinach leaf carboxylase as determined by titration with p-chloromercuribenzoate (37), with those from *Chromatium* after titration with (^{14}C)p-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoic acid) (39) and with those from tobacco after titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (S. STRØBÆK, unpublished results). In *Hydrogenomonas* on the other hand only one third of the total cysteines can be titrated with 5,5'-dithiobis-(2-nitrobenzoic acid) in 8 M urea (23) and in *Rhodospirillum*

Figure 4. Plot of V_e (elution volume) against $\log_{10}$ (molecular weight) for proteins on a Sepharose 6B column (5 × 46 cm). Elution was carried out with 25 mM Tris/HCl buffer pH 7.4 containing 0.2 M NaCl, 0.0005 M EDTA and 0.002 % Hibitane, at a flow rate of 99 ml/hr. The open circles are corrected values as mentioned in the text.



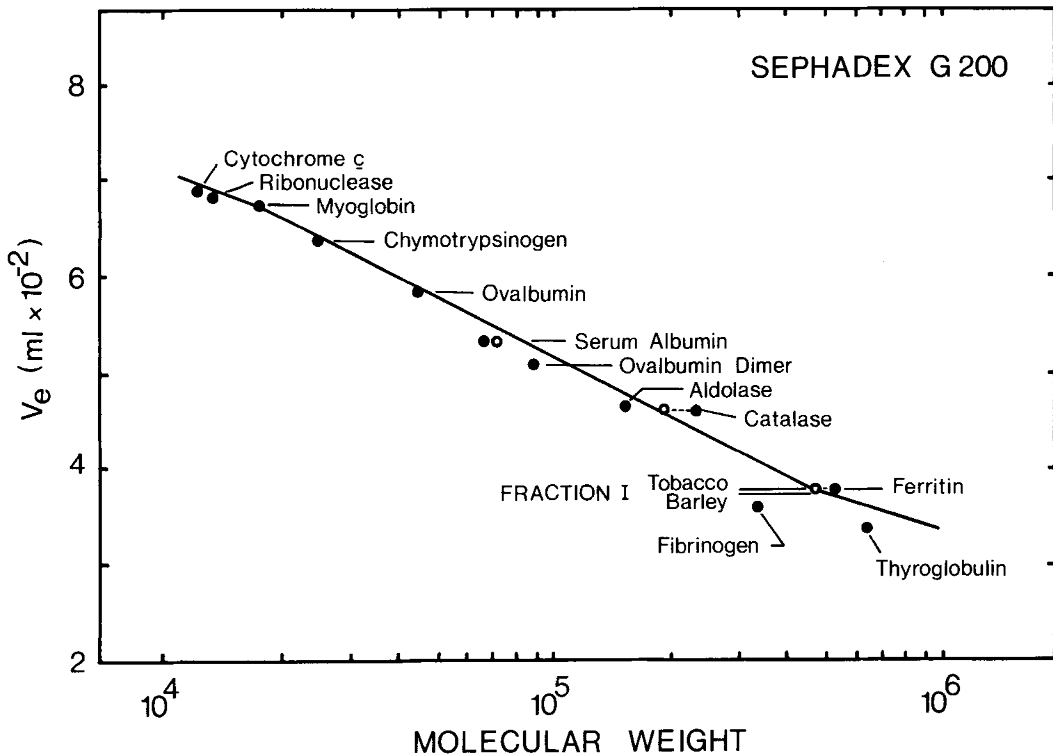


Figure 5. Plot of V_e (elution volume) against $\log_{10}$ (molecular weight) for proteins on a Sephadex G-200 column (5 × 41 cm). Elution was carried out with 25 mM Tris/HCl buffer pH 7.4 containing 0.2 M NaCl, 0.0005 M EDTA and 0.002 % Hibitane, at a flow rate of 26 ml/hr. The open circles are corrected values as mentioned in the text.

rubrum only one half of them with 5,5'-dithio-bis-(2-nitrobenzoic acid) in 8 M urea (38).
As has been reported for the tobacco protein (21) β-mercaptoethanol is not required for dissociation of the barley subunits and SDS separations could be obtained on columns of Bio-Glas-500 (minus 325 mesh) without prior reduction with β-mercaptoethanol.
From the amino acid analysis data and the molar absorbances of tyrosine and tryptophan at 280 nm at neutral pH (1197 and 5559 respectively (34)) the extinction coefficient of total Fraction I protein from barley could be determined to be $E^{1\text{0/00}}_{280\,\text{nm}} = 2.06$. This allows to determine the protein concentration from the absorbance A, using the formula: c(mg/ml) = 0.486 × $A^{1\,\text{cm}}_{280\,\text{nm}}$. The extinction coefficient for barley Fraction I is somewhat higher than has been reported for spinach: 1.64 (42) and tobacco: 1.43 (25). This corresponds to a higher content of tryptophan in barley Fraction I protein.

3.2.3. *Electron Microscopy*

Electron microscopic examination of negatively stained barley Fraction I protein (figure 6) revealed particles with a size range of 95 – 105 Å in diameter, and the structure resembled closely that of spinach (37) and wheat (36). The particles had a central hole as described by STEER *et al.* (35) in the case of oat Fraction I.

3.2.4. *Crystallization*

Although attempts to crystallize barley Fraction I using the method developed for the tobacco protein by CHAN *et al.* (7) met with no success, it was possible to obtain crystalline barley Fraction I protein by the ammonium sulphate method of JAKOBY (17). The crystals obtained using this technique had exactly the same morphology as crystalline tobacco Fraction I prepared in this laboratory and as described by KAWASHIMA and WILDMAN (20).

Table II.

Amino Acid Composition of Ribulose-1,5-diphosphate Carboxylase [a]

Amino Acid	Barley	Oat	Tobacco	Beet	Spinach	*Chlamydomonas*
Asx	1.82	1.90	1.82	1.77	2.20	2.64
Thr	1.21	1.23	1.26	1.43	1.63	1.46
Ser	0.94	0.84	0.95	1.11	0.69	1.24
Glx	2.29	2.26	2.55	2.09	2.36	2.53
Pro	1.24	1.45	1.17	1.16	1.20	1.47
Gly	1.99	2.06	2.25	1.96	2.09	2.56
Ala	1.85	1.98	2.02	1.80	1.92	2.67
Cys	0.42[b]	0.48[b]	0.53[b]	0.39[b]	0.50	0.67[b]
Val	1.38	1.41	1.60	1.80	1.57	1.91
Met	0.45	0.38	0.39	0.39[e]	0.40	0.95
Ile	0.89	1.08	0.97	1.00	0.87	1.16
Leu	1.67	1.67	2.05	1.89	2.09	1.90
Tyr	0.78	0.75	1.02	0.89	1.09	1.00
Phe	1.00	1.00	1.00	1.00	1.00	1.00
His	0.51	0.59	0.54	0.59	0.73	0.64
Lys	1.08	1.12	1.30	1.20	1.12	1.85
Arg	1.29	1.32	1.40	1.02	1.41	1.74
Trp	0.73[f]	0.28[g]	0.59[f]	0.30[g, h]	0.58[i]	n.d.
References	present work	(35)	(24)	(31)	(39)	(16)

a. These numbers are calculated as relative molar ratios of phenylalanine
b. Determined as cysteic acid
e. Determined as methionine sulfone
f. Determined by the method of BENCZE and SCHMID (3)
g. Determined by the method of BEAVEN and HOLIDAY (2)
h. Determined by the method of OPIENSKA-BLAUTH *et al.* (30)
i. Determined by the method of GOODWIN and MORTON (13)

n.d. not determined

3.3. Isolation of Subunits

Purified Fraction I protein was concentrated by either diafiltration on an Amicon model 402 ultrafiltration cell fitted with a PM30 Diaflo membrane, or by 50 % ammonium sulphate precipitation and subsequent dialysis. Samples containing 150 mg protein were dissociated in 15 ml 0.05 M Tris/HCl buffer pH 8.5 containing 0.5 % SDS and 0.14 M β-mercaptoethanol by incubation at 37°C for 1 hour under nitrogen. Separation of subunits was achieved at 25°C on a 5 × 82 cm column of Sephadex G-100 equilibrated with 0.05 M Tris buffer pH 8.5 containing 0.5 % SDS, and run at a flow rate of 32 ml/hr. Sample size was 1 % of the total column volume. The large subunit was eluted with K_{av} 0.08 – 0.11, whilst the small subunit was eluted with a K_{av} value of 0.22 – 0.25.

3.4. Properties of Subunits

3.4.1. Molecular Weight

The elution volumes of the large and small subunits on Sephadex G-100 in 0.05 M Tris buffer pH 8.5 containing 0.5 % SDS corresponded to apparent molecular weights of 55,000 ± 10 % (½ extreme range) and 13,000 ± 10 % (½ extreme range), respectively, when compared to the elution volumes of bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome c. SDS gel electrophoresis of the subunits resulted in molecular weight values of 50,000 (± 10 % (½ extreme range)) for the large subunit and 13,000 (± 10 % (½ extreme range)) for the small subunit (figure 7). On phenol: acetic acid: urea gels the large subunit exhibited on apparent molecular weight of

Table III.

Amino Acid Composition of the Large and Small Subunits of Ribulose-1,5-Diphosphate Carboxylase [a]

	Amino Acid	Barley	Tobacco	Beet	Spinach	*Chlamydomonas*	*Rhodospirillum*
Large	Asx	1.98	2.02	1.95	2.20	2.42	2.54
	Thr	1.32	1.26	1.40	1.73	1.39	1.40
	Ser	1.07	0.72	0.88	0.70	0.87	1.39
	Glx	2.26	2.26	2.30	2.28	2.40	2.44
	Pro	1.15	0.98	1.06	1.12	1.27	1.53
	Gly	2.20	2.25	2.23	2.33	2.62	2.77
	Ala	2.07	2.02	2.13	2.18	2.61	3.47
	Cys	0.28[c]	n.d.	0.35[c]	0.44[d]	0.93[b]	0.25[b]
	Val	1.57	1.69	1.57	1.67	1.80	1.62
	Met	0.30	0.37	0.33	0.39	0.66	0.65
	Ile	1.12	0.98	0.88	0.92	1.07	1.11
	Leu	1.90	2.03	1.91	2.16	2.02	1.96
	Tyr	0.70	0.88	0.76	0.94	0.96	0.61
	Phe	1.00	1.00	1.00	1.00	1.00	1.00
	His	0.61	0.63	0.62	0.77	0.63	0.54
	Lys	1.06	1.14	1.15	1.13	1.25	1.05
	Arg	1.25	1.45	1.26	1.48	1.62	1.54
	Trp	0.70[f]	n.d.	n.d.	0.48[i]	n.d.	0.47[i]
Small	Asx	1.58	1.68	1.44	2.20	2.14	k
	Thr	0.95	0.98	0.77	1.23	0.96	
	Ser	1.06	0.84	0.91	0.64	1.08	
	Glx	2.48	3.45	2.17	2.71	2.08	
	Pro	1.01	1.54	1.43	1.56	1.16	
	Gly	1.50	1.69	1.58	1.07	1.09	
	Ala	1.37	1.35	1.09	0.79	1.96	
	Cys	0.35[c]	n.d.	0.35[c]	0.64[d]	0.72[b]	
	Val	1.46	1.51	1.41	1.12	1.83	
	Met	0.37	0.38	0.28	0.45	0.65	
	Ile	0.87	1.00	0.70	0.66	0.74	
	Leu	1.49	1.95	1.54	1.78	1.19	
	Tyr	0.93	1.83	0.99	1.74	0.91	
	Phe	1.00	1.00	1.00	1.00	1.00	
	His	0.26	0.15	0.21	0.54	0.18	
	Lys	1.31	1.86	1.44	1.09	1.05	
	Arg	0.83	1.07	0.58	1.10	1.02	
	Trp	0.78[f]	n.d.	n.d.	0.96[i]	n.d.	
	References	present work	(18)	(28)	(39)	(16)	(38)

a. These numbers are calculated as relative molar ratios of phenylalanine
b. Determined as cysteic acid
c. Determined as CM-cysteine
d. Determined by alkylation of SH groups by (^{14}C) iodoacetamide in the presence of 8 M urea and dithiothreitol
f. Determined by the method of BENCZE and SCHMID (3)
i. Determined by the method of GOODWIN and MORTON (13)
k. *Rhodospirillum rubrum* carboxylase consists of two identical 56,000 daltons subunits
n.d. not determined

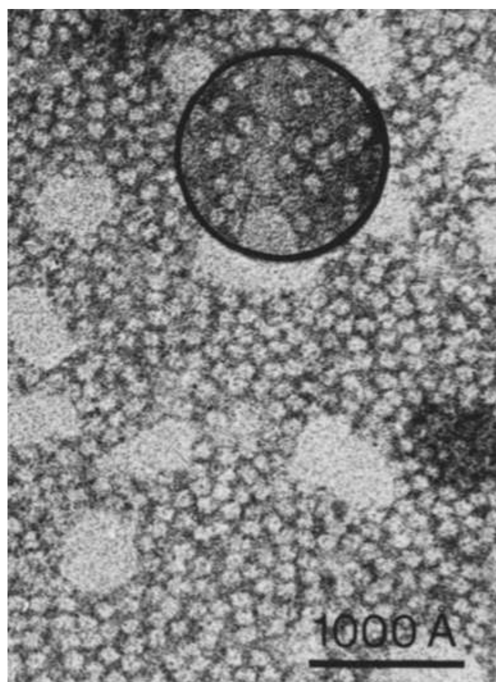


Figure 6. Electron micrograph of ribulose-1,5-diphosphate carboxylase from *Hordeum vulgare* L. var. Svalöf's Bonus, negatively stained with sodium phosphotungstate. The interted picture clearly shows the central hole as described by STEER *et al.* (35). Magnification is × 205,000.

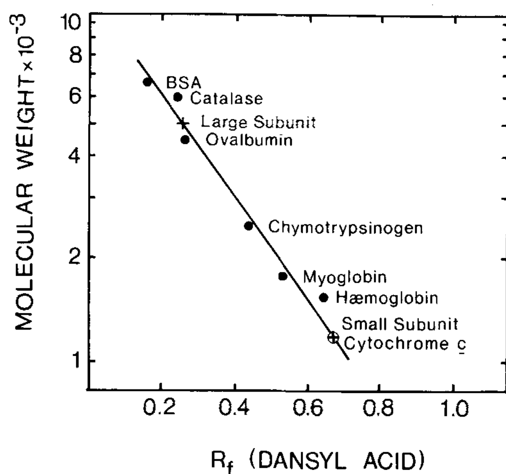


Figure 7. Plot of $\log_{10}$ (molecular weight) of proteins against the electrophoresis mobilities relative to dansyl acid on 10 % polyacrylamide gels containing 0.1 % SDS as described under methods. The mobilities of the subunits of ribulose-1,5-diphosphate carboxylase from *Hordeum vulgare var.* Svalöf's Bonus are marked with crosses on the line of best fit.

55,000 (± 4 % (standard deviation)). The apparent size of the small subunit was greater than that seen in the separations involving SDS, namely 18,000 (± 4 % (standard deviation)).

Preliminary column chromatography on Sephadex G-100 gels in 0.2 M phosphate buffer pH 11.2, using the method of KAWASHIMA and WILDMAN (21) revealed – in parallel to their results with tobacco Fraction I protein – a subunit with an apparent molecular weight of 25,000, a larger subunit with an apparent molecular weight of 55 – 60,000 and a peak of aggregated material at the front.

3.4.2. Amino Acid Composition

The amino acid composition of the subunits of barley Fraction I protein is shown in Table III, where the amino acid compositions of Fraction I subunits from tobacco, beet, spinach, *Chlamydomonas reinhardi* and *Rhodespirillum rubrum* are also presented for comparison.

In accordance to previous results the amino acid composition of the small subunit exhibits greater variation (Chi^2 values ranging from 1.27 to 7.98) than that of the large subunit (Chi^2 values ranging from 0.29 to 1.04, *R. rubrum* vs. the other species gave a value ranging between 1.44 and 3.11). The amino acid compositions of Fraction I protein from five species of *Nicotiana* were compared using the data of KAWASHIMA *et al.* (18). Chi^2 values of 0.13 – 0.38 were found when the amino acids of the larger subunit were compared, 0.35 – 1.93 when the amino acids of the small subunit were compared. The Chi^2 values are compared in figure 8. For unknown reasons, when these same data of KAWASHIMA *et al.* (18) were subjected to computer analysis (24,26) the Chi^2 values obtained were considerably larger. The trend of the results, however, was the same.

3.5. Peptide Mapping

Peptide maps of the large and small subunits of barley Fraction I protein (shown in figures 9 and 10, respectively) indicated the presence of 42 tryptic peptides in the case of the large subunit and 25 peptides in the case of the

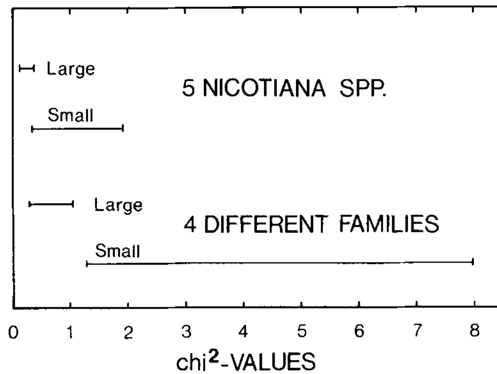


Figure 8. Comparison of Chi^2 values for the amino acid composition of the large and small subunits of ribulose-1,5-diphosphate carboxylase from four different families (calculated from the data in Table III) and five species within the same genus (*Nicotiana,* calculated from the data of KAWASHIMA *et al.* (18)).

small. In our hands the technique of two-dimensional high voltage paper electrophoresis was that which resulted in the maximum number of well defined and separated peptide spots. Although the "neutral" peptides (peptides with zero charge at pH 6.5) could further separate by overnight paper chromatography in butanol: acetic acid: water: pyridine (15:3:12:10 by vol.) no extra peptides were resolved. Tailing of neutral peptides during re-electrophoresis at pH 1.9 is a well described phenomenon (5).

The ninhydrin positive spot at the origin in the case of the large subunit (figure 9) represents insoluble protein and can be obviated either by subjecting the trypsin hydrolysate to high speed centrifugation prior to application onto the electrophoresis sheet, or by applying the tryptic hydrolysate onto the sheet as a broad band, as described under methods. Repeated tryptic digestion and subsequent peptide mapping of this insoluble fraction resulted in identical peptide maps as that shown in figure 9, indicating that this material does not represent an "indigestible core" of the large subunit.

The central retardation of the dansyl arginine band seen in figure 9 is a result of the insoluble protein at the origin, and could be avoided when the insoluble protein was removed as described above.

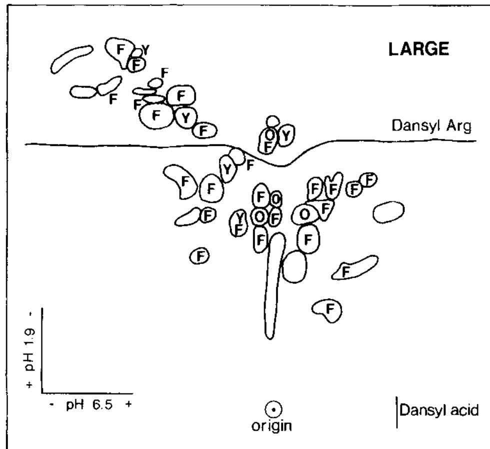


Figure 9. Two-dimensional peptide map of the large subunit of ribulose-1,5-diphosphate carboxylase from *Hordeum vulgare* L. var. Svalöf's Bonus after tryptic digestion and high voltage paper electrophoresis as described under methods. 42 Cd-ninhydrin positive spots are seen, those marked with a Y or an O appeared with a yellow or orange colour, respectively, indicating N-terminal glycine, asparagine, serine, threonine or proline, the other spots were red (5). Spots marked with an F are fluorescent after staining with phenanthrenequinone for arginine. The mobility of dansyl acid in the pH 6.5 dimension and the mobility of dansyl arginine in the pH 1.9 dimension are seen.

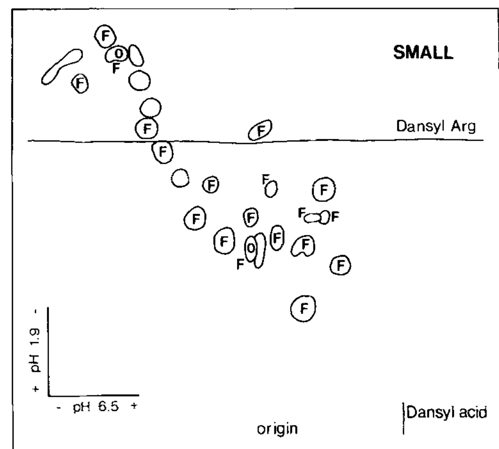


Figure 10. Two-dimensional peptide map of the small subunit of ribulose-1,5-diphosphate carboxylase from *Hordeum vulgare* L. var. Svalöf's Bonus after tryptic digestion and high voltage paper electrophoresis as described under methods. 25 Cd-ninhydrin positive spots are seen. For explanation of symbols, see figure 9.

4. DISCUSSION

The isolation procedure for Fraction I protein developed during the course of this work may be considered as one of the simplest and most rapid procedures so far described in the literature. High yields of undenaturated protein can be obtained in less than one working day, and if the purified Fraction I protein is collected directly from the column and concentrated by ultrafiltration, separation of subunits is possible overnight so that separated subunits are available on the morning of day 2.

Acetone precipitation of subunits and subsequent tryptic digestion followed by peptide mapping, using two-dimensional high voltage paper electrophoresis, as described under methods, can also be completed within 8 hours. This allows a complete peptide mapping analysis of Fraction I protein subunits to be carried out in a total of two days. The method has also been used with both tobacco and spinach leaves and has been found equally suitable for this material. Fraction I of tobacco prepared by our method is identical to three times crystallized protein prepared by the method of CHAN *et al.* (7) as judged by the criteria of size, amino acid composition and behavior on SDS gels.

Barley Fraction I protein appears to be slightly larger than tobacco Fraction I protein as judged by its behavior on Sepharose 6B and Sephadex G-200. This apparent size difference could reflect differences in the molecular weight of the native protein, possibly due to size differences in the small subunit (4). However, this size difference may be due to slight conformational differences in the two proteins, or due to a difference in the distribution of phenolic amino acids on the surface of the protein leading to a slight retardation of the barley protein on Sephadex and Sepharose. The amino acid composition of the native barley protein resembles Fraction I protein from other higher plants, in particular the protein isolated from another member of the Poaceae, *Avena sativa,* oats.

The two subunits of barley Fraction I protein can be separated by a variety of different methods including dissociation in SDS, alkali, and phenol-acetic acid-urea. The apparent size of the subunits is a function of the dissociating system used. β-mercaptoethanol is not required for dissociation of the subunits, we found, however, that in practice, addition of β-mercaptoethanol was useful by preventing aggregation when samples of native Fraction I were to be stored before subunit separation. β-mercaptoethanol also served as an excellent marker of the total column volume due to its high ultraviolet absorption at both 280 nm and 254 nm – the wavelengths commonly used for ultraviolet detection of column chromatography. The amino acid composition of the subunits of barley Fraction I protein demonstrated that the major difference observed between native barley Fraction I protein and the native protein isolated from other higher plants, can be attributed to differences in the amino acid composition of the small subunit. This tendency is also clearly seen in the comparison of tobacco species (24,26).

The peptide mapping technique described in this paper promises to be useful in screening for barley Fraction I mutants. The present method has several advantages: it is rapid; the spots are very distinct as a result of buffer sharpening of the starting zone in both directions prior to electrophoresis; the separation of peptides is based primarily on the defined physical parameters of size and charge in contrast to methods where paper chromatography is involved; the results are highly reproducible both with regard to the position of the peptide spots and their colour reactions.

The occurence of the crescent shaped spots on the upper left hand side of both maps represents free lysine – indicative of repetitive lysine sequences in both subunits. This observation is interesting in the light of the Lys-Lys-Lys sequence at position 9 – 10 – 11 in the N-terminal sequence of the small subunit of tobacco Fraction I (12).

ACKNOWLEDGEMENT

The work was supported in part by grant GM-22051 of the National Institutes of Health, United States Public Health Service.

The authors thank Professors D. VON WETTSTEIN and B. FOLTMANN for valuable discussions and suggestions. We thank Dr. HENNING JACOBSEN for the molecular weight determinations with SDS gel electrophoresis. We would also like to thank Dr. ALBERT KAHN and Mr. CARSTEN POULSEN together with the staff of the Institute of Genetics and the Institute of Biochemical Genetics for helpful discussions during the course of this work.

REFERENCES

1. ANDREWS, P.: The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595–606 (1965).
2. BEAVEN, G.H. & E.R. HOLIDAY: Ultraviolet absorption spectra of proteins and amino acids. Adv. in Protein Chemistry 7: 319–386 (1952).
3. BENCZE, W.L. & K. SCHMID: Determination of tyrosine and tryptophan in proteins. Anal. Chem. 29: 1193–1196 (1957).
4. BÖRNER, T., G. JAHN & R. HAGEMANN: Electrophoretic mobility of the subunits of Fraction I protein of higher plant species. Biochem. Physiol. Pflanzen 169: 179–181 (1976).
5. BROWN, J.R. & B.S. HARTLEY: Location of disulphide bridges by diagonal paper electrophoresis. Biochem. J. 101: 214–228 (1966).
6. CHAN, P.H.: The role of chloroplast and nuclear DNAs in coding for the large and small subunits of Fraction I isolated from *Nicotiana* species and reciprocal, interspecific hybrids. Ph.D. thesis. (University of California, Los Angeles, U.S.A.) 1972.
7. CHAN, P.H., K. SAKANO, S. SINGH & S.G. WILDMAN: Crystalline Fraction I protein: Preparation in large yield. Science 176: 1145–1146 (1972).
8. CHAN, P.H. & S.G. WILDMAN: Chloroplast DNA codes for the primary structure of the large subunit of Fraction I protein. Biochim. Biophys. Acta 277: 677–680 (1972).
9. DETERMANN, H. & I. WALTER: Source of aromatic affinity to 'Sephadex' dextran gels. Nature 219: 604–605 (1968).
10. ELLIS, R.J.: Fraction I protein. Commentaries in Plant Science 4: 29–38 (1973).
11. GIBBONS, G.C., S. STRØBÆK & E.Aa. GEERTSEN: Tris (hydroxymethyl) aminomethane buffer and amino acid analysis. Acta Chem. Scand. 27: 3868–3872 (1973).
12. GIBBONS, G.C., S. STRØBÆK, B. HASLETT & D. BOULTER: The N-terminal amino acid sequence of the small subunit of ribulose-1,5-diphosphate carboxylase from *Nicotiana tabacum*. Experientia 31: 1040–1041 (1975).
13. GOODWIN, T.W. & R.A. MORTON: The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40: 628–632 (1946).
14. HABEEB, A.F.S.A.: Reaction of protein sulfhydryl groups with ELLMAN'S reagent. *In:* C.H.W. HIRS, ed., Methods in Enzymology, Vol. XXV, pp. 457–464. Academic Press (New York and London) 1972.
15. HEILMANN, J., J. BARROLLIER & E. WATZKE: Beitrag zur Aminosäurebestimmung auf Papierchromatogrammen. HOPPE-SEYLER'S Z. Physiol. Chem. 309: 219–220 (1957).
16. IWANIJ, V., N.-H. CHUA & P. SIEKEVITZ: The purification and some properties of ribulosebisphosphate carboxylase and of its subunits from the green alga *Chlamydomonas reinhardtii.* Biochim. Biophys. Acta 358: 329–340 (1974).
17. JAKOBY, W.B.: Crystallization as a purification technique. *In:* W.B. JAKOBY, ed., Methods in Enzymology, Vol. XXII, pp. 248–252. Academic Press (New York and London) 1971.
18. KAWASHIMA, N., S.Y. KWOK & S.G. WILDMAN: Studies on Fraction-I protein. III. Comparison of the primary structure of the large and small subunits obtained from five species of *Nicotiana*. Biochim. Biophys. Acta 236: 578–586 (1971).
19. KAWASHIMA, N. & S.G. WILDMAN: Fraction I protein. Ann. Rev. Plant Physiol. 21: 325–358 (1970).
20. KAWASHIMA, N. & S.G. WILDMAN: Studies on Fraction-I protein. I. Effect of crystallization of Fraction-I protein from tobacco leaves on ribulose diphosphate carboxylase activity. Biochim. Biophys. Acta 229: 240–249 (1971).
21. KAWASHIMA, N. & S.G. WILDMAN: Studies on Fraction I protein. II. Comparison of physical, chemical, immunological and enzymatic properties between spinach and tobacco Fraction I proteins. Biochim. Biophys. Acta 229: 749–760 (1971).
22. KAWASHIMA, N. & S.G. WILDMAN: Studies on Fraction I protein. IV. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contains the code for a chloroplast protein. Biochim. Biophys. Acta 262: 42–49 (1972).
23. KUEHN, G.D. & B.A. MCFADDEN: Ribulose 1,5-diphosphate carboxylase from *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*. II. Molecular weight, subunits, composition, and sulfhydryl groups. Biochemistry 8: 2403–2408 (1969).
24. KWOK, S.Y.: Studies on the crystal structure and properties of D-ribulose-1,5-diphosphate carboxylase obtained from tobacco leaves.

Ph.D. thesis. (University of California, Los Angeles, U.S.A.) 1972.
25. KWOK, S.Y., N. KAWASHIMA & S.G. WILDMAN: Specific effect of ribulose-1,5-diphosphate on the solubility of tobacco Fraction I protein. Biochim. Biophys. Acta 234: 293–296 (1971).
26. KWOK, S.Y. & S.G. WILDMAN: Evolutionary divergence in the two kinds of subunits of ribulose diphosphate carboxylase isolated from different species of *Nicotiana*. J. MOL. Evol. 3: 103–108 (1974).
27. MEISSNER, G. & S. FLEISHER: Characterization of sarcoplasmic reticulum from skeletal muscle. Biochim. Biophys. Acta 241. 356–378 (1971).
28. MOON, K.E. & E.O.P. THOMPSON: Subunits from reduced and S-carboxymethylated ribulose diphosphate carboxylase (Fraction I protein). Aust. J. biol. Sci. 22: 463–470 (1969).
29. NIELSEN, N.C.: Electrophoretic characterization of membrane proteins during chloroplast development in barley. Eur. J. Biochem. 50: 611–623 (1975).
30. OPIENSKA-BLAUTH, J., M. CHAREZINSKI & H. BERBEC: A new, rapid method of determining tryptophan. Anal. Biochem. 6: 69–76 (1963).
31. RIDLEY, S.M., J.P. THORNBER & J.L. BAILEY: A study of the water-soluble proteins of spinach beet chloroplasts with particular reference to Fraction I protein. Biochim. Biophys. Acta 140: 62–79 (1967).
32. SHAPIRO, H.M.: A multivariate statistical method for comparing protein amino acid compositions: Studies of muscle actins and proteins derived from membranes and microtubular organelles. Biochim. Biophys. Acta 236: 725–738 (1971).
33. SIEGEL, M.I., M. WISHNICK & M.D. LANE: Ribulose-1,5-diphosphate carboxylase. *In:* P.D. BOYER, ed., The Enzymes, 3rd., Vol. VI, pp. 169–192. Academic Press (New York and London) 1972.
34. SOBER, H.A., ed.: Handbook of Biochemistry, 2nd ed., p. B-75. The Chemical Rubber Co. (Ohio, U.S.A.) 1970.
35. STEER, M.W., B.E.S. GUNNING, T.A. GRAHAM & D.J. CARR: Isolation, properties, and structure of Fraction I protein from *Avena sativa* L. Planta 79: 254–267 (1968).
36. SUGIYAMA, T. & T. AKAZAWA: Structure and function of chloroplast proteins. I. Subunit structure of wheat Fraction-I protein. J. Biochem. 62: 474–482 (1967).
37. SUGIYAMA, T., N. NAKAYAMA, M. OGAWA, T. AKAZAWA & T. ODA: Structure and function of chloroplast proteins. II. Effect of p-chloromercuribenzoate treatment on the ribulose 1,5-diphosphate carboxylase activity of spinach leaf Fraction I protein. Arch. Biochem. Biophys. 125: 98–106 (1968).
38. TABITA, F.R. & B.A. MCFADDEN: D-ribulose 1,5-diphosphate carboxylase from *Rhodospirillum rubrum*. II. Quaternary structure, composition, catalytic, and immunological properties. J. Biol. Chem. 249: 3459–3464 (1974).
39. TAKABE, T. & T. AKAZAWA: The role of sulfhydryl groups in the ribulose-1,5-bisphosphate carboxylase and oxygenase reactions. Arch Biochem. Biophys. 169: 686–694 (1975).
40. TALBOT, D.N. & D.A. YPHANTIS: Fluorescent monitoring of SDS gel electrophoresis. Anal. Biochem. 44: 246–253 (1971).
41. WILDMAN, S.G. & J. BONNER: The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. Arch. Biochem. 14: 381–413 (1947).
42. WISHNICK, M. & M.D. LANE: Ribulose diphosphate carboxylase from spinach leaves. *In:* A.S. PIETRO, ed., Methods in Enzymology, Vol. XXIII, pp. 570–577. Academic Press (New York and London) 1971.
43. YAMADA, S. & H.A. ITANO: Phenanthrenequinone as an analytical reagent for arginine and other monosubstituted guanidines. Biochim. Biophys. Acta 130: 538–540 (1966).