Theor Appl Genet (1986) 71:708–715

One large subunit of ribulose 1,5-bisphosphate carboxylase oxygenase in *Medicago, Spinacia* and *Nicotiana*

H. V. Daday [1], M. I. Whitecross [2] and D. C. Shaw [3]

[1] Division of Plant Industry, CSIRO, Canberra, ACT 2601, Australia
[2] Botany Department, Australian National University, Canberra, ACT 2601, Australia
[3] John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia

Accepted August 11, 1985
Communicated by H. F. Linskens

Summary. Isoelectric focusing of subunits of ribulose 1,5-bisphosphate carboxylase oxygenase of *Medicago, Spinacia* and *Nicotiana* were investigated, using a rapid isolation technique, without S-carboxymethylation. RuBPC-ase and its subunits were isolated by gel electrophoresis. Isoelectric focusing of RuBPC-ase of *M. sativa* and *M. falcata* showed that this enzyme consists of one large subunit (LSU) polypeptide and two or three small subunits (SSU), depending on the genotype. The pI of the LSU's was identical, but the pI of SSU's of the two genotypes was different. Amino acid composition and tryptic peptide maps further supported the concept of a conserved nature of LSU and heterogeneity of SSU polypeptides in *Medicago.* It was also found that *S. oleracea, N. tabacum, N. glutinosa* and *N. excelsior* have a single LSU polypeptide, but they differ in respect of pI values. The SSU polypeptides appeared to be variable. S-carboxymethylation affected the number as well as the pI values of LSU and SSU polypeptides. It is suggested that one LSU polypeptide is probably the general rule in higher plants, rather than the three LSU polypeptides demonstrated by Chen et al. (1977) and Wildman (1979).

Key words: Ribulose 1,5-bisphosphate carboxylase oxygenase-large subunit – *Medicago, Spinacia, Nicotiana*

Introduction

The photosynthetic enzyme, ribulose 1,5 bisphosphate carboxylase oxygenase (RuBPCase) is a high molecular weight enzyme which has been shown to consist of eight large subunits (LSU) and eight small subunits (SSU) (Kawashima and Wildman 1970). A physicochemical examination of RuBPC-ase from *Medicago sativa* was carried out by Noguchi et al. (1978). Analytical ultracentrifugation gave a $S^0_{20,W}$ value of 18.0, which corresponds to a molecular weight close to 536,000. X-ray diffraction analysis indicated that the molecules of RuBPC-ase are roughly $115 \times 115 \times 100$ Å in size. SDS-gel electrophoresis revealed two sizes of subunits. Molecular weights were estimated at 53 kd and 14 kd for LSU and SSU polypeptides, respectively.

The synthesis of RuBPC-ase is reported to be regulated by both nuclear and chloroplast genes in such a way that the synthesis of the SSU polypeptide is under nuclear control (Kawashima and Wildman 1972) and that of the LSU polypeptide is under chloroplast DNA control (Chan and Wildman 1972).

Phenotypic variation of RuBPC-ase in the plant kingdom was investigated by Chen et al. (1977) and Wildman (1979). The S-carboxymethylated RuBPC-ase of all plant species examined had three LSU polypeptides on IEF gels. The number of SSU was found to be variable, and they were all acidic. They also reported that *M. sativa* had three LSU polypeptides and one acidic SSU polypeptide, and *Spinacia* had three LSU polypeptides and two acidic SSU polypeptides. Wildman (1979) studied the evolution of RuBPC-ase in the genus *Nicotiana* comprising 66 species, and 20 different kinds of RuBPC-ase molecules were identified. With respect to LSU, the genus could be separated into four different groups which differ in the isoelectric point of their polypeptides. All species had three S-carboxymethylated LSU polypeptides. He also found a range of differences in the isoelectric points of the SSU polypeptides.

In contrast to the above investigations, one report of an analysis of the subunits of RuBPC-ase of *Triticum aestivum* by gel isoelectric focusing (O'Connell and Brady 1981) revealed a single LSU and one SSU. However, if carboxyamidomethylation of the RuBPC-ase was carried out before isoelectric focusing, three or more bands of LSU appeared. Preparative procedures involving aggregation of the RuBPC-ase also resulted in complex isoelectric focusing patterns. The simplest

patterns, with one type of LSU and one type of SSU, were obtained when the RuBPC-ase was isolated rapidly and gently by immunoprecipitation or preparative polyacrylamide gel electrophoresis and analysed by isoelectric focusing without alkylation of thiol groups.

Our aim in this investigation is to examine the LSU of *Medicago, Spinacia* and *Nicotiana* to determine whether the observations of Chen et al. (1977) and Wildman (1979) are valid concerning LSU multiple polypeptides or whether their findings are artifacts of preparation as suggested by the work of O'Connell and Brady (1981). A further aim is to examine the variability of the SSU polypeptides in the same species.

Materials and methods

Plant material

Plant materials utilized in various experiments were as follows.

M. sativa cv. 'Hunter River' (HR) and cv. 'Hairy Peruvian' (HP), were both commercial cultivars from South Australia; *M. falcata,* Sc-1765 a tetraploid (C.P.I. 96983) (MF) was obtained from the Canadian Department of Agriculture, Swift Current, Saskatchewan, Canada.

Nicotiana excelsior (7553), *N. tabacum* (Q 1232) and *N. glutinosa* (R 608-5) were provided by the Department of Primary Industries (Mareeba, Queensland).

Seed of spinach (*Spinacia oleracea*) was obtained from local commercial sources.

Plants of the above species were grown in 15 cm pots (using standard glasshouse soil) under natural light at 1,200–1,500 $\mu E \cdot m^{-2} s^{-1}$, in a heated glasshouse with the temperature maintained between 20° and 25 °C.

Isolation of RuBPC-ase

A 3 g sample of fresh leaf material was collected and homogenized in the presence of 3 ml extraction buffer (200 mM Tris, 5 mM Na_2 EDTA, 25 mM $MgSO_4 \cdot 7H_2O$, 1% polyvinylpyrrolidone, 10 mM 2-mercaptoethanol, 5 mM cysteine, adjusted to pH 8.5 with boric acid) in a precooled mortar at 4 °C. The resulting homogenate was squeezed through a layer of Miracloth and the filtrate was centrifuged at 108,000 g for 45 min.

Six percent polyacrylamide gel (Davis 1964), with a top layer of 1 cm 2.5% polyacrylamide concentrating gel, was prepared on standard glass electrophoresis plates (8 × 8 cm).

The gel was placed into an electrophoresis tank containing the following buffer: 45 mM Tris, 25 mM boric acid, and 0.7 mM Na_2-EDTA, pH 8.5. A 0.7 ml plant extract sample was applied and the gel was run at 30 mA and approximately 120 V for 3 h. The edges of the gel were cut off and stained with Coomassie blue (4 g Coomassie brilliant blue in one litre of absolute ethanol, 200 ml acetic acid, and 800 ml water). Destaining was done with a mixture of water, acetic acid and ethanol (6 : 1 : 3 by volume). The band previously shown to be RuBPC-ase (O'Connell and Brady 1981) was located in the gel and the strip of gel containing the protein was cut out.

The gel strips were homogenised in the above buffer and placed in electrophoresis tubes. Tubes were covered at one end with cellophane membrane and the protein in the gel was recovered by electroelution (5 mA per tube) overnight (O'Connell and Brady 1981).

Isolation of LSU and SSU

One ml of a fraction containing RuBPC-ase (4 mg protein) was supplemented with SDS (2% w/v) and 2-mercaptoethanol (5% v/v), heated at 50 °C for 10 min, and loaded on a 13% polyacrylamide (Davis 1964) slab gel. The electrophoresis buffer contained 45 mM Tris, 25 mM boric acid, 0.7 mM Na_2 EDTA and 0.1% SDS, pH 8.5. Electrophoresis was carried out for 2 h at 30 mA and approximately 120 V at room temperature. The edges of the gel were cut off and stained in Coomassie Blue. The LSU and SSU bands were located in the gel and the strips of gel containing the protein were cut out. Electroelution of subunits from the gel was as described for the RuBPC-ase.

Isoelectric focusing (IEF)

A description of the apparatus, preparation of the gel slab and samples, sample application, running conditions, gel staining, and chemicals used has been published previously (Daday and Whitecross 1983).

The stained gels were cut into 1 cm wide slices along the vertical length of the gel and scanned in a Gilford spectrophotometer 240. The wavelength was set at 600 nm and the absorbance control was 500.

Amino acid compositions and tryptic peptide maps

Amino acid compositions were determined using the method of Byers (1971).

After separation by PAGE, the LSU and SSU of RuBPC-ase were subjected to trypsin hydrolysis and the tryptic peptides resolved by two-dimensional paper electrophoresis and solvent chromatography by the technique described previously (Gray et al. 1978).

Results

Purity of LSU and SSU preparations

The RuBPC-ase, LSU and SSU extracted by the methods given in the previous section were tested for purity by applying them to a 13% polyacrylamide gel together with a sample of the whole RuBPC-ase enzyme. After electrophoresis, the gel was stained with Coomassie blue. RuBPC-ase separated into two bands one at 53 kd and the other 14 kd band (Fig. 1, lane 1). The LSU polypeptide (Fig. 1, lane 2) had a single band at 53 kd. The SSU polypeptide (Fig. 1, lane 3) had one band at 14 kd. Therefore, it can be concluded that the isolated LSU and SSU polypeptides as prepared were fully resolved and not significantly contaminated with each other or with other proteins.

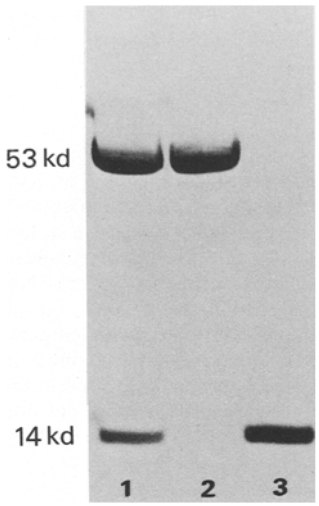

Fig. 1. SDS gel electrophoresis of RuBPC-ase, LSU and SSU proteins of *Medicago* HR genotype. *Lane 1:* RuBPC-ase; *lane 2:* LSU; *lane 3:* SSU; 53 kd = LSU, 14 kd = SSU

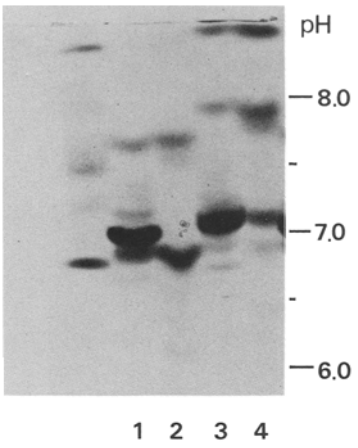

Fig. 2. Isoelectric focusing separation of RuBPC-ase and SSU of *Medicago* HR and MF genotypes. *Lane 1:* RuBPC-ase of HR genotype; *lane 2:* SSU polypeptide of HR genotype; *lane 3:* RuBPC-ase of MF genotype; *lane 4:* purified SSU polypeptide of MF genotype. The *left hand lane* represents marker molecules used for determination of pl values

Isoelectric focusing of RuBPC-ase and SSU of HR and MF genotypes

RuBPC-ase was extracted from a 6% polyacrylamide gel and SSU's from HR and MF genotypes were obtained from a 13% polyacrylamide gel following electrophoresis. Protein (40 μg) from each sample was applied to the isoelectric focusing (IEF) gel without any S-carboxymethylation. After a 9 h IEF run the gels were stained in bromophenol blue.

The results of an isoelectric focusing run are illustrated in Fig. 2. RuBPC-ase of HR genotype (lane 1) showed one major LSU, two minor SSU polypeptides and an additional minor band (pl 7.1), which is likely to be a degradation product of LSU. The LSU band is located at pl 7.0; one SSU band is just below the LSU (pl ca. 6.8) and the second SSU can be seen at pl ca. 7.7. The two bands of the isolated SSU HR polypeptide (lane 2) corresponded to the positions of SSU of RuBPC-ase in lane 1. The RuBPC-ase of MF genotype (lane 3) had one LSU band (pl 7.0) and apparently two SSU polypeptide bands at pl 8 and pl 8.5. However, the purified SSU of MF genotype had three polypeptide bands (lane 4 – at pl 7, pl 7.9, and pl 8.5); all three bands correspond to SSU of RuBPC-ase (Fig. 4). The SSU at pl 7 was masked by the LSU band of RuBPC-ase (lane 3).

Densitometric scans (600 nm) of isoelectric focusing gels of RuBPC-ase of HR and MF genotypes showed that the ratio of the total amount of LSU/SSU polypeptide is 3.91 : 1 and 3.77 : 1 for HR and MF genotypes, respectively. This result confirms the differences in number and position of SSU polypeptides of the HR and MF genotypes. It is also noted that the LSU/SSU polypeptide ratios are close to the expected (3.79 : 1) from the difference in molecular weight.

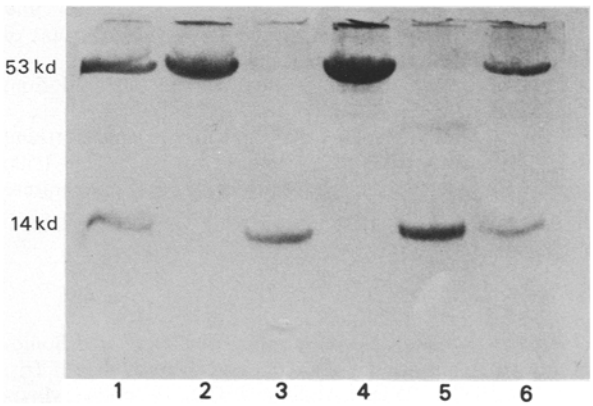

Fig. 3. SDS gel electrophoretograms of LSU and SSU extracted from an isoelectric focusing gel. *Lane 1:* Total RuBPC-ase of HR extracted from 6% Davis gel.; *lane 2:* LSU of HR; *lane 3:* SSU of HR; *lane 4:* LSU of MF; *lane 5:* SSU of MF; *lane 6:* RuBPC-ase of MF

In order to confirm the identity of the LSU and to determine that the SSU bands from the isoelectric focusing gel were indeed as proposed, the gel pieces of LSU and SSU bands were homogenized and applied to a 13% polyacrylamide SDS gel. Samples analysed were the following: RuBPC-ase of the HR genotype (Fig. 3,

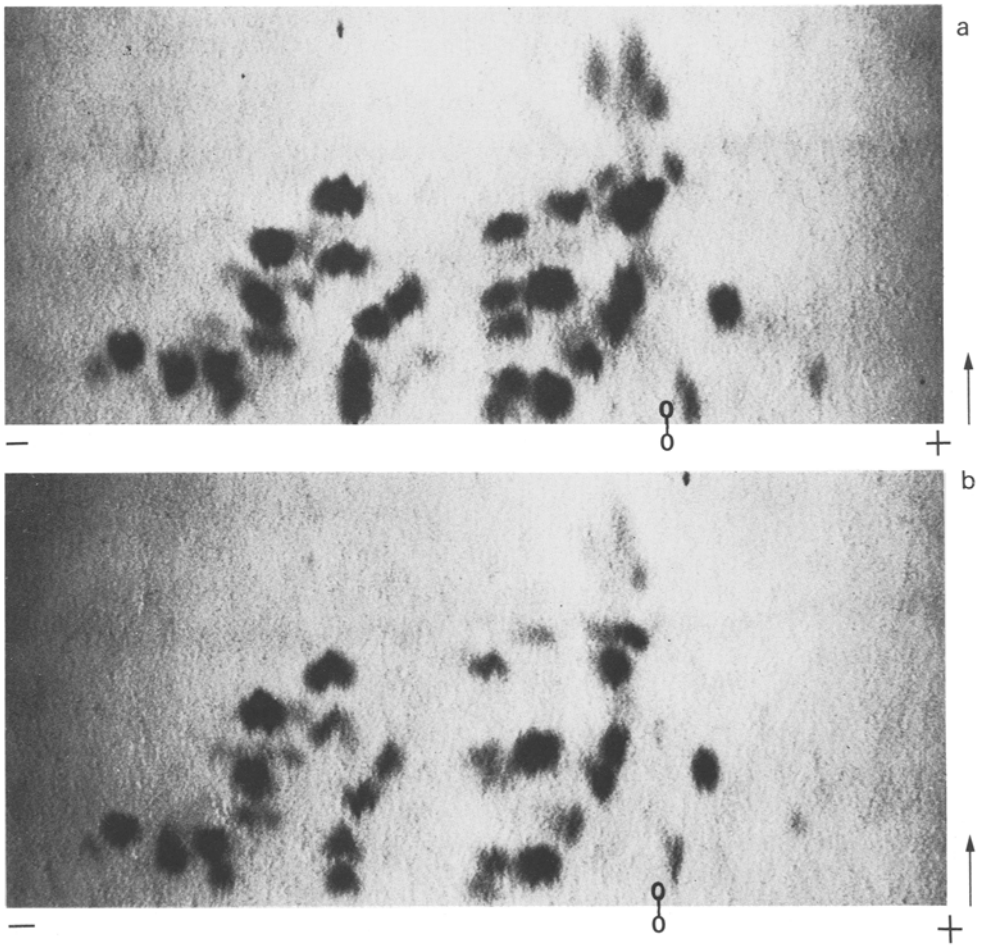

Fig. 4. Tryptic peptide maps of LSU polypeptide of HR (**a**), and MF (**b**) genotypes

lane 1); LSU and SSU of HR genotype (lanes 2 and 3); LSU and SSU of MF genotype (lanes 4 and 5); RuBPC-ase of the MF genotype (lane 6). All IEF-extracted samples proved to be pure LSU or SSU polypeptides.

Amino acid composition of RuBPC-ase, LSU and SSU of Medicago

Amino acid analyses were carried out on the RuBPC-ase LSU and SSU of the HR, HP and MF *Medicago* genotypes and the results are presented in Table 1. The amino acid composition of the LSU of the three genotypes differed very little. In contrast, the amino acid composition of the combined SSU's of each of the three genotypes varied considerably. The MF genotype differed from that of HR and HP, particularly in the proportions of lysine, arginine, aspartic acid, serine and glutamic acid. Statistical analysis confirmed a significantly greater variability among the SSU than among the LSU of these three genotypes, the variability of residues per 100 residues being 3.88 times greater ($P < 0.001$).

Tryptic peptide maps of LSU and SSU of HR and MF genotypes of Medicago

The tryptic peptide maps for LSU of HR and MF genotypes of *Medicago* are presented in Figs. 4 a, b. Separation of trypsin hydrolysates of the polypeptides by two dimensional electrophoresis and chromatography showed approximately 37 peptides in both LSU peptide maps. The maps are very similar; the main apparent differences are due to there being less material on the lower (MF) map. Although the extent of staining varies the lower map contains essentially the same distribution or pattern of peptide spots.

In the comparison of the SSU's the mixture of pl forms of HR and MF were digested with trypsin and those hydrolysates separated by paper electrophoresis and chromatography. While the maps have an overall

Table 1. Amino acid composition of RuBPC-ase from the *Medicago* HR genotype, and of LSU and SSU from HR, HP and MF genotypes

	RuBPC-ase	Large subunit (LSU) Residues per 100			Small subunit (SSU) Residues per 100		
		HR	HP	MF	HR	HP	MF
Lysine	6.97	6.07	5.91	6.17	9.17	8.27	7.65
Histidine	3.78	4.36	4.21	3.99	2.51	2.40	1.91
Arginine	8.94	8.68	7.77	8.46	8.45	7.23	6.70
Aspartic acid	9.32	9.78	10.10	9.48	6.68	6.83	7.57
Threonine	5.85	5.85	5.59	5.23	3.99	4.15	4.54
Serine	3.53	2.55	2.57	2.86	3.54	3.60	4.26
Glutamic acid	11.97	10.08	10.18	10.32	14.81	14.81	13.53
Proline	2.82	3.61	3.52	3.74	5.83	5.80	5.92
Glycine	5.49	5.19	5.89	5.90	3.79	3.85	4.03
Alanine	6.39	7.43	7.19	7.50	3.35	3.35	3.73
Valine	5.83	6.78	6.70	6.30	5.08	5.46	5.51
Methionine	2.22	2.44	2.36	2.31	0.95	1.02	1.20
Isoleucine	5.04	3.93	5.11	5.30	5.91	6.01	6.17
Leucine	9.73	9.76	10.55	10.44	10.15	10.42	10.54
Tyrosine	5.69	5.07	5.32	5.12	8.19	9.21	8.71
Phenylalanine	6.43	6.79	7.09	6.84	7.61	7.59	8.03

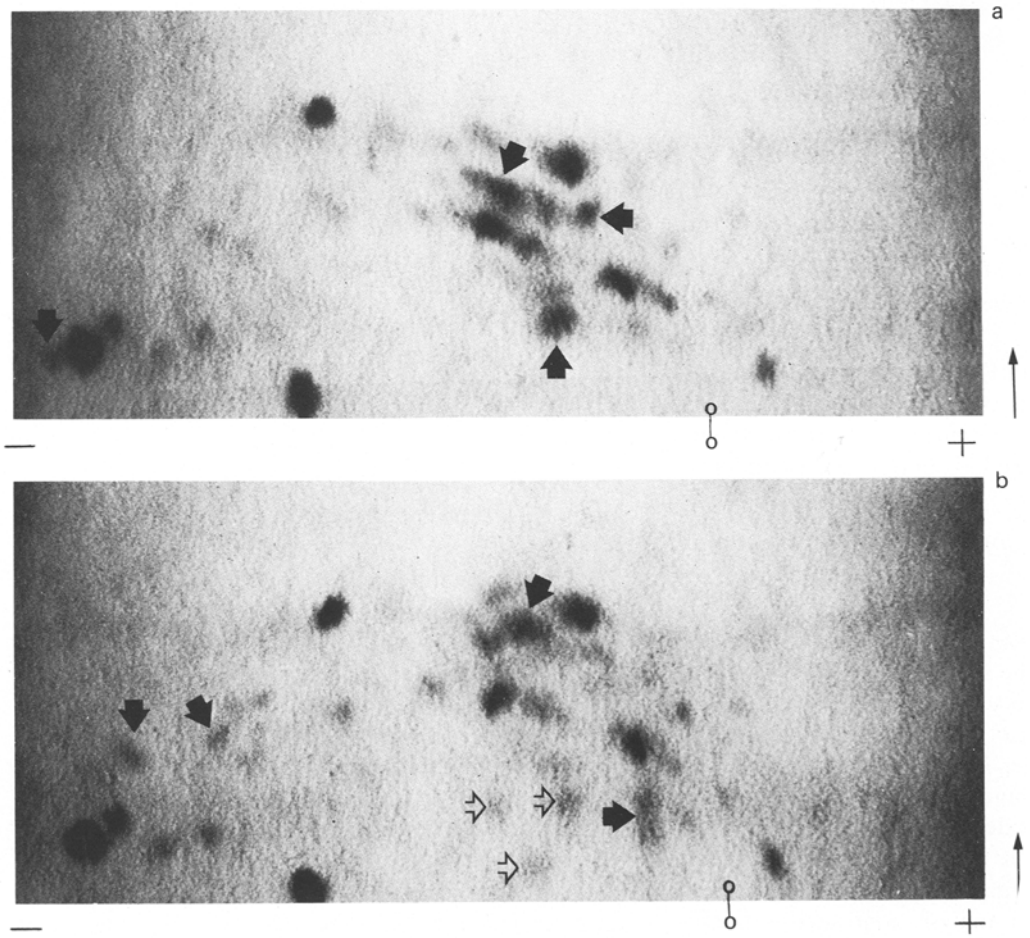

Fig. 5. Tryptic peptide map of SSU polypeptides of HR (**a**), and MF (**b**) genotypes. Unique peptides are indicated by *arrows*; strongly staining ones by *solid arrows*, weakly staining ones by *open arrows*

similarity and some peptides are common to both, there are real differences in the pattern – particularly in the neutral region. Some of these differences are indicated by arrows. The top map (Fig. 5 a) of HR SSU's, which consists mainly of the pl 6.8 form gave about 19 strong and a few other weakly staining peptide spots. The bottom map (Fig. 5 b) of the MF SSU's gave fewer strong but a greater number of weakly staining spots than was evident for HR. This observation is consistent with the map of MF SSU's being derived from a mixture of 3 forms (pl's 7.0, 7.9 and 8.5) which occur in roughly equal proportions (cf. Fig. 3.1).

Isoelectric focusing of RuBPC-ase of Spinacia and Nicotiana

Samples of RuBPC-ase of *Medicago* (HR genotype) *Spinacia,* and *Nicotiana* were extracted from a 6% polyacrylamide gel and applied to an isoelectric focusing gel (Fig. 6). The HR genotype (lane 1) had one LSU (heavy band) and two alkaline SSU polypeptides. *Spinacia* (lane 2) had one LSU (heavy band) and one acid SSU polypeptide. *Nicotiana* (lane 3) had one LSU (heavy band) and two acid SSU polypeptides which migrated to the more acidic region of the gel.

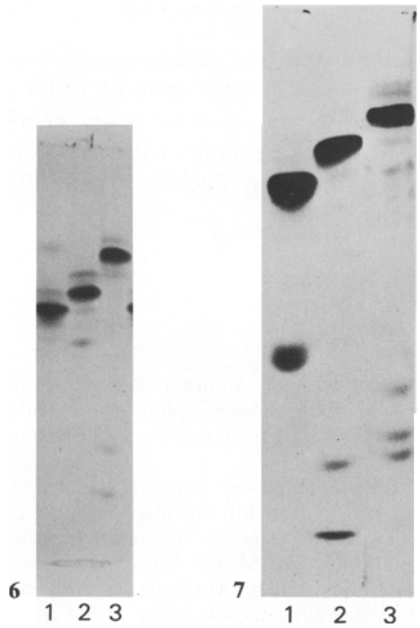

Fig. 6. Isoelectric focusing separation of RuBPC-ase of *Medicago sativa, Spinacia oleracea* and *Nicotiana tabacum. Lane 1:* RuPBC-ase of *Medicago, lane 2:* RuPBC-ase of *Spinacia, lane 3:* RuPBC-ase of *Nicotiana*

Fig. 7. Isoelectric focusing separation of RuPBC-ase of *N. glutinosa, N. tabacum,* and *N. excelsior. Lane 1:* RuPBC-ase of *N. glutinosa, lane 2:* RuPBC-ase of *N. tabacum, lane 3:* RuPBC-ase of *N. excelsior*

The polypeptide composition of three *Nicotiana* species is illustrated in Fig. 7. *N. glutinosa* (lane 1) has one LSU (heavy band) and two acidic SSU which migrated very close to each other. *N. tabacum* (lane 2) also shows one LSU and two well separated acidic SSU polypeptides. *N. excelsior* (lane 3) shows also one LSU and four acidic SSU polypeptides. The LSU (heavy bands) of the three species are separated by 0.1 unit of pl.

Discussion

The results of these experiments demonstrated that the species of *Medicago, Spinacia* and *Nicotiana* each have a single LSU polypeptide, in contrast to the observed heterogeneity of the SSU. The combined evidence of IEF and SDS PAGE make the proposition of there being a single LSU beyond reasonable doubt.

The demonstration in this study that RuBPCase has a single LSU polypeptide in three widely separated genera *Medicago, Spinacia* and *Nicotiana* is in agreement with the finding of O'Connell and Brady (1981) for *Triticum* but contrasts with other previous reports for *Spinacia* and *Medicago* (Chen et al. 1977) and for *Nicotiana* (Wildman 1979; Kung 1984). It now seems plausible that a single LSU is the rule in most taxa and that reports of three or even more LSU polypeptide bands probably result from artifacts. O'Connell and Brady (1981) attributed the appearance of multiple bands in wheat to S-carboxymethylation of the preparations prior to isoelectric focusing. Our findings support this. There still appear reports of multiple bands for LSU however; indeed the major review by Kung (1984) does not even mention the possibility that multiple bands might be artifactual.

Our results are also in accordance with those of Gray et al. (1978) in that they failed to demonstrate reproducible differences in fingerprinting of trypsin hydrolysates of the three LSU polypeptides. The lack of clearcut differences in amino acid composition of the three LSU bands of the same species further supports the notion that the three bands are the same gene product separated on analysis by an induced chemical artifact. The finding that only one gene for LSU exists in the chloroplast genome in *Pisum sativum* (Bedbrook et al. 1979) lends further support for the expectation that a single LSU exists in most species.

Similarity of LSU's from HR and MF genotypes of *Medicago* was shown by pl values, amino acid composition and peptide maps, indicating a conserved nature of the LSU polypeptides. Such a conserved nature is also reflected in the nucleic acid sequence as determined for other plants. The chloroplast DNA in maize, coding for the LSU of RuBPC-ase has been sequenced in its entirety (including the flanking regions) by McIntosh et al. (1980), using a cloned restriction fragment carrying this gene. Amino acid sequences from LSU of barley and spinach were subsequently shown to align with the complete maize sequence by Poulsen (1981). The deduced sequence of the 475 amino acids of spinach LSU was also shown to have only 10% divergence from that of the maize LSU (Zurawski et al. 1981). The sequences in LSU of spinach published by Erion (1983) differed by only two nucleotides from that of Zurawski et al. (1981). A comparison of the sequence of the untranslated 3′ end of the LSU gene of

Petunia hybrida with that of *Nicotiana tabacum* also revealed a striking similarity (Bovenberg et al. 1984). Amiri et al. (1984) found that the amino acid sequence of the LSU of RuBPCase of *N. tabacum*, as found by tryptic digestion, was in agreement with the one deduced from gene sequencing. In fact the greatest divergence so far found in amino acid sequencing is 13–14% found when *Chlamyodomonas reinhardii* was compared with maize or spinach. (Dron et al. 1982).

In the present study, no differences were found in pl values of the LSU polypeptides of *Medicago* species, though heterogeneity of LSU in *Nicotiana*, similar to that reported by Wildman (1979), was demonstrated. Although four different clusters of LSU polypeptides were demonstrated among *Nicotiana* species, separated on the basis of pl values, only small differences in amino acid composition were seen to account for this (Wildman 1979). The wide range of pl heterogeneity found by Chen et al. (1977) for numerous genera and species, could also have resulted from relatively few amino acid differences.

The isoelectric focusing experiments, using a rapid preparation procedure and modified apparatus showed two or three SSU polypeptides of *Medicago* depending on the species in the alkaline region of the gel. This was the first evidence that "alkaline" SSU polypeptides occur in any plant species. In contrast to these results, Chen et al. (1977) reported that *M. sativa* had a single "acidic" SSU polypeptide. The reason for this discrepancy is obscure though S-carboxymethylation can be ruled out. In our experiments, this step did not result in changed pl values for SSU *in M. sativa*. Heterogeneity of SSU polypeptides in *Medicago* was demonstrated further by amino acid analysis and tryptic peptide mapping. S-carboxymethylation of RuBPC-ase did alter the SSU polypeptide number and pl value of *Spinacia*. S-carboxylmethylated RuBPC ase of *Spinacia* was shown to have two SSU polypeptides (Chen et al. 1977). In contrast, in the absence of S-carboxymethylation, *Spinacia* had one SSU in the present study. The isoelectric focusing patterns of SSU polypeptides in *Nicotiana glutinosa, N. tabacum* and *N. excelsior* in the present investigation were essentially the same as those of Wildman (1979).

Allelic 'polymorphism' has been observed previously in a comparison of the N-terminal amino acid sequences (110 to 120 amino acids) of the SSU in *Oenothera biennis*, barley, pea, bean, and tobacco (review by Wettstein et al. 1978). Haslett et al. (1976) investigated the amino acid sequence of the SSU of RuBPC-ase from *Pisum sativum* and *Vicia faba*. Pea and broad bean sequences were very similar, with only two differences in the first 25 positions. Sequenced DNA encoding for the precurser of the SSU of *Pisum sativum* showed that the molecule consists of 123 amino acids and has a considerable homology with that of spinach and 89 residues were the same in both species (Bedbrook et al. 1980). The mature SSU of RuBPCase from soybeans consists of 123 amino acids, and contains 30 and 34 amino acid replacements relative to the amino acid sequences from pea and spinach respectively (Berry-Lowe et al. 1982). Complete amino acid sequence of the SSU from *Nicotiana tabacum* disclosed a polypeptide chain of 123 amino acids and it is homologous (70–75%) with the respective polypeptide chains from spinach and pea (Mueller et al. 1983). Finally, the N-terminal sequences (40 amino acids) of SSU were used for the study of plant phylogeny in trees (Martin and Dowd 1984). As the isoelectric focusing patterns of the SSU polypeptides of *Medicago* are different, it is not unreasonable to assume that these differences would be reflected in the SSU polypeptide sequences as well.

The relatively less conserved nature of the amino acid sequence of SSU polypeptides of RuBPC-ase from pea, spinach, petunia and wheat was investigated by S.M. Smith (personal communication) who showed that 69% sequence conservation was observed, whereas LSU sequence conservation was 90%.

Genetic information and origin of SSU was considered by Cammaerts and Jacobs (1981) who found that the two SSU polypeptides in *Lycopersicum* is contained on heterologous chromosomes and gene mutations could be the origin of the SSU polypeptide composition.

The greater variability of amino acid composition of SSU of the two genotypes in contrast to those of LSU could also result from several nuclear genes being responsible for the SSU (e.g. Dunsmuir et al. 1983) thus having a greater total mutation frequency than the single gene for the LSU.

References

Amiri I, Salnikow J, Vater J (1984) Amino acid sequence of the large subunit of d ribulose bisphosphate carboxylase oxygenase EC – 4.1.1.39 from *Nicotiana tabacum*. Biochim Biophys Acta 784:116–123

Bedbrook JR, Coen DM, Beaton AR, Bogorad L, Rich A (1979) Location of single gene for the large subunit of ribulose bisphosphate carboxylase on the maize chloroplast chromosome. J Biol Chem 254:905–910

Bedbrook JR, Smith SM, Ellis J (1980) Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase. Nature 287:692–697

Berry-Lowe SL, McKnight TD, Shah DM, Meagher RB (1982) The nucleotide sequence, expression and evolution of one member of a multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean. J Mol Appl Genet 1:483–498

Bovenberg WA, Koes RE, Kool AJ, Nijkamp HJJ (1984) Physical mapping nucleotide sequencing and expression in *Escherichia coli mini* cells of the gene for the large subunit

of ribulose bisphosphate carboxylase from *Petunia hybrida*. Curr Genet 8:231–241

Byers M (1971) Amino acid composition of some leaf protein preparations. In: Pirie NW (ed) Leaf protein: its agronomy, preparation, quality and use. IBP Blackwell, Oxford Edinburgh, pp 95–114

Cammaerts D, Jacobs M (1981) Genetic control of small subunit polypeptides of Fraction I protein in the genus *Lycopersicum*. Plant Sci Lett 22:147–154

Chan PH, Wildman SG (1972) Chloroplast DNA codes for the primary structure of the large subunit of Fraction I protein. Biochim Biophys Acta 277:677–680

Chen K, Johal S, Wildman SG (1977) Phenotypic markers for chloroplast DNA genes in higher plants and their use in biochemical genetics. In: Bogorad L, Weil JH (eds) Nucleic acid and protein synthesis in plants. Plenum Press, New York London, pp 183–194

Daday HV, Whitecross MI (1983) Isoelectric focusing of ribulose bisphosphate carboxylase from lucerne and spinach. Sci Tools 30:11–14

Davis BJ (1964) Disc electrophoresis. 11. Method and application to human serum proteins. Ann NY Acad Sci 121: 404–427

Dron M, Rahire M, Rochaix JD (1982) Sequence of the chloroplast DNA region of *Chlamydomonas – Reinhardii* containing the gene of the large subunit of ribulose bisphosphate carboxylase and parts of its flanking genes. J Mol Biol 162:775–794

Dunsmuir P, Smith S, Bedbrook J (1983) A number of different nuclear genes for the small subunit of RuBPCase are transcribed in petunia. Nucleic Acids Res 11: 4177–4183

Erion JL, Tarnowski J, Peacock S, Caldwell P, Redfield B, Brot N, Weissbach H (1983) Synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in an in vitro partially defined *E. coli* system. Plant Mol Biol 2:279–290

Gray JC, Kung SD, Wildman SG (1978) Polypeptide chains of the large and small subunits of Fraction I protein from tobacco. Arch Biochem Biophys 185:272–281

Haslett BG, Yarwood A, Evans IM, Boulter D (1976) Studies on the small subunit of Fraction I protein from *Pisum sativum* L. and *Vicia faba* L. Biochim Biophys Acta 420: 122–132

Kawashima N, Wildman SG (1970) A model of the subunit structure of Fraction I protein. Biochem Biophys Res Commun 41:1463–1468

Kawashima N, Wildman SG (1972) Studies on Fraction I protein. 4. Mode of inheritance of primary structure in relation to whether choroplast or nuclear DNA contains the code for chloroplast protein Biochim Biophys Acta 262:42–49

Kung SD (1984) Genetic analysis of tobacco RuBPCase. CRC Crit Rev Plant Sci 1:227–267

Martin PG, Dowd JM (1984) The study of plant phylogeny using amino-acid sequences of ribulose-1,5-bisphosphate carboxylase. 3. Addition of *Malvaceae* and *Ranunculaceae* to phylogenetic tree. Aust J Bot 32:283–290

McIntosh L, Poulsen C, Bogorad L (1980) Chloroplast gene sequence for the large subunit of ribulose bisphosphate carboxylase of maize. Nature 288:556–560

Mueller KD, Salnikow J, Vater J (1983) Amino acid sequence of small subunit of d ribulose bisphosphate carboxylase EC-4. 1.1.39 oxygenase from *Nicotiana tabacum*. Biochim Biophys Acta 742:78–83

Noguchi H, Maekawa T, Fujimoto S, Stake I, Sakakibara M (1978) Physicochemical studies on Fraction 1 protein from alfalfa. Agric Biol Chem 42:1553–1558

O'Connell PBH, Brady CJ (1981) Multiple forms of the large subunit of wheat ribulose bisphosphate carboxylase generated by excess iodoacetamide. Biochim Biophys Acta 670:355–361

Poulsen C (1981) Comments on the structure and function of the large subunit of the enzyme ribulose bisphosphate carboxylase oxygenase EC 4.1.1.39. Carlsberg Res Commun 46:259–278

Smith SM, Speirs J, Dunsmuir P, Bedbrook J (in preparation) An invariant hexadecapeptide in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from four species of higher plant

Wettstein D von, Poulsen C, Holder AA (1978) Ribulose-1,5-bisphosphate carboxylase as a nuclear and chloroplast marker. Theor Appl Genet 53:193–197

Wildman SG (1979) Aspects of Fraction 1 protein evolution. Arch Biochem Biophys 196:598–610

Zurawski G, Perrot B, Bottomley W, Whitfeld PR (1981) The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res 9:3251–3269