The Isolation and Partial Characterization of Fraction I Protein from Spinach-Beet Chloroplasts

By J. P. THORNER, S. M. RIDLEY and J. L. BAILEY
Twyford Laboratories, Twyford Abbey Road, London, N.W. 10

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Fraction I protein ($S_{20,w}$ 17–18 s) is the major soluble nitrogenous component of green leaves (Wildman & Bonner, 1947) and appears to be associated with the stroma of the chloroplasts (Lyttleton & Ts'o, 1958). Investigations on the enzymic nature of the protein have shown ribulose diphosphate-carboxylase, phosphoribulokinase and ribose phosphate-isomerase activities (Van Noort, Hudson & Wildman, 1961; Mendiola & Akazawa, 1964). Van Noort & Wildman (1964) demonstrated that the ribulose diphosphate carboxylase could be separated from the two other enzymes by precipitation of Fraction I protein with specific antibody. These authors suggested that the latter two activities were probably associated with the Fraction II proteins ($S_{20,w}$ 3–4 s) of green leaves (Wildman & Bonner, 1947). Trown (1965) has obtained ribulose diphosphate-carboxylase preparations exhibiting no significant phosphoribulokinase and ribose phosphate-isomerase activities by careful $(\text{NH}_4)_2\text{SO}_4$ fractionation and repeated gel filtration on Sephadex G-200. This author presented evidence that ribulose diphosphate carboxylase and Fraction I protein were synonymous.

In the present communication a method for the purification of Fraction I protein is described, new analytical data are presented, and the associated enzymic activity is confirmed to be only that of ribulose diphosphate carboxylase.

Isolation of Fraction I protein. Chloroplasts were prepared from spinach-beet (*Beta vulgaris*) leaves (160 g.) by differential centrifugation (Bailey & Whyborn, 1963) of a homogenate in 400 ml. of 0.5 M-sucrose–0.05 M- KHCO_3 adjusted to pH 7.5 with CO_2 . The isolated chloroplasts were suspended in water (total volume about 50 ml.) in a Potter-Elvehjem homogenizer, and centrifuged at 100 000 g for 20 min. The supernatant was removed and the procedure repeated.

The proteins in the chloroplast washes (about 80 ml.) were run into a DEAE-cellulose column equilibrated in 0.025 M- NaH_2PO_4 – Na_2HPO_4 buffer, pH 7.0. The column was then washed with the same buffer (300 ml.) and the proteins were eluted by using a salt gradient (Fig. 1a). Protein zones A and B were examined by analytical ultracentrifugation, and by electrophoresis on polyacrylamide gel in 0.03 M-tris–0.01 M-cysteine–HCl buffer, pH 8.0, in

the apparatus described by Ornstein & Davis (1962). Zone A contained Fraction I protein together with traces of Fraction II components, whereas zone B contained a larger proportion of the Fraction II proteins and a 26 s component as well as Fraction I protein. The isolated zones A and B were dialysed against 0.03 M- H_3BO_3 adjusted to pH 8.9 with N-NaOH and concentrated by freeze-drying in the presence of sorbitol (protein–sorbitol, 1:5, w/w). The Fraction I protein was further purified by gel filtration on Sephadex G-200 (Fig. 1b). All operations during the isolation of the protein were carried out at below 2°.

Analyses of Fraction I protein. The results reported below apply to the protein obtained from zone A by gel filtration (Fig. 1b).

(a) Analytical ultracentrifugation. The purified component exhibited only one boundary when examined in the buffer systems 0.1 M- NaH_2PO_4 – Na_2HPO_4 –0.01 M-cysteine–HCl, pH 7.9, or 0.03 M- H_3BO_3 –0.01 M-cysteine–HCl adjusted to pH 7.6 with N-NaOH. After application of experimentally determined correction factors for the viscosity and density of the buffer, and by using $\bar{v} = 0.737$ calculated from the amino acid analysis, it was found that the $S_{20,w}$ value increased with dilution of the protein in both buffers; the $S_{20,w}^0$ was 18.29 s in both solvents. The simplified approach-to-equilibrium method of Ehrenberg (1957) was used to determine the molecular weight; values of 5.83×10^5 and 5.47×10^5 were obtained for two preparations.

(b) Polyacrylamide-gel zone electrophoresis. After purification, Fraction I protein exhibited only a single band (stained with Amido Black 10B) in 0.03 M-tris–0.01 M-cysteine–HCl buffer, pH 8.0, and pH 8.9 (adjusted with N-NaOH). The single band gave a positive stain reaction with Lipid Crimson, and a positive reaction for ketose in the cysteine-carbazole reaction (Dische & Borenfreund, 1951).

In the absence of cysteine, additional components, thought to be aggregates, were observed on electrophoresis and ultracentrifugation. The 26 s component of zone B could not be dissociated by cysteine; this component was reported to be a dimer of Fraction I protein (Trown, 1965).

(c) Amino acid analysis. Various preparations of the protein were hydrolysed with constant-boiling

Fig. 1. (a) Elution pattern of an aqueous extract of chloroplasts from a DEAE-cellulose column (10 cm. $\times$ 2 cm.). An exponential salt gradient [0.025 M- NaH_2PO_4 - Na_2HPO_4 +0.2 M- NaCl , pH 7.0 (500 ml.), was run into a reservoir containing 0.025 M- NaH_2PO_4 - Na_2HPO_4 , pH 7.0 (500 ml.)] was applied at fraction no. 1. After 500 ml. (i.e. at fraction no. 47) had passed through the column 0.025 M- NaH_2PO_4 - Na_2HPO_4 +1.0 M- NaCl , pH 7.0 (500 ml.), was run into the solution in the reservoir. Contents of tubes, each containing 10 ml., were pooled as indicated between arrows to give zones A and B. (b) Elution patterns of concentrated zone A on a Sephadex G-200 column (60 cm. $\times$ 2 cm.) equilibrated and eluted with 0.03 M- H_3BO_3 adjusted to pH 8.8 with N-NaOH. Contents of tubes, each containing 1.7 ml., were combined as indicated to give the purified protein solution. $\circ$, $E_{280 \text{ m}\mu}$; $\blacktriangle$, ribulose diphosphate-carboxylase activity (counts/min. fixed/ml.); $\blacksquare$, phosphoribulokinase ($\mu\text{moles of NADH oxidized/min. ml. at } 20^\circ$); $\square$, ribose phosphate isomerase ($\mu\text{moles of ribulose 5-phosphate formed/min. ml. at } 37^\circ$).

HCl at 110° under N_2 in tubes sealed under partial vacuum, and their amino acid compositions determined with an EEL automatic analyser. The molar ratios from nine analyses (two samples each after 12, 24, 48 and 72 hr. hydrolysis and one oxidized sample after 48 hr. hydrolysis) were: cystine (half), 1.32; aspartic acid, 5.92; methionine, 1.32; threonine, 4.80; serine, 3.17; glutamic acid, 6.89; proline, 3.85; glycine, 6.56; alanine, 6.05; valine, 5.92; isoleucine, 3.35; leucine, 6.35; tyrosine, 2.99; phenylalanine, 3.38; lysine, 4.05; histidine, 1.97; arginine, 3.47. The molar ratio of tyrosine to tryptophan was determined as 2.99 (mean value of three observations) by the method of Beaven & Holiday (1952). Hence a comparable molar ratio for tryptophan of 1.00 was obtained. The percentage of tryptophan in the protein obtained by direct estimation (Opieńska-Blauth, Chareziński & Berbeć, 1963) was 2.57% (mean value for three samples); this is in good agreement with the value (2.33%) calculated from the values given above.

(d) Enzymic activities. After gel filtration (Fig. 1b) the contents of each tube were assayed for ribose phosphate-isomerase and phosphoribulokinase activities (Hurwitz, Weissbach, Horecker & Smyrnotis, 1956), and ribulose diphosphate-carboxylase activity by a method based on that of Rabin & Trown (1964), differing in that each incubation mixture contained $\text{NaH}^{14}\text{CO}_3$ (0.05 μmole ,

1.2 μC) and ribulose diphosphate (0.19 μmole). The ribulose diphosphate-carboxylase activity coincided with the main peak, whereas the other enzymes were eluted from the column after the main peak.

Analysis of the Fraction I protein isolated from zone B (Fig. 1a) showed that its $S_{20, w}$, electrophoretic mobility, amino acid analysis and enzymic activity were identical with those of the protein isolated from zone A. It therefore appears that the major components in both zones were identical, and the trailing portion of zone A (Fig. 1a) was removed by the stronger salt gradient to produce zone B.

The binding to Fraction I protein of extraneous cell components during the isolation procedure (Trown, 1965) has tended to hinder the assessment of true analytical data. By using the method of purification described, there was no binding of ribose phosphate isomerase or phosphoribulokinase. Nucleic acid, reported by Eggman, Singer & Wildman (1953) to be contained in Fraction I protein, was not found, by using the method of Warburg & Christian (1941), in either of the protein solutions eluted from the DEAE-cellulose column. However, the presence of lipid and carbohydrate in our preparations may have arisen during the isolation procedure.

The average molecular weight of our Fraction I

protein (5.64×10^5) prepared from isolated chloroplasts is much greater than the value of 3.75×10^5 reported by Eggman *et al.* (1953) for Fraction I protein derived from a cell-free protoplasmic extract of spinach leaves (*Spinacia* sp.), but is in closer agreement with the value of 5.15×10^5 for ribulose diphosphate carboxylase from the chloroplasts of spinach leaves (Trown, 1965).

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Purification of Pig Renin

By W. S. PEART, A. M. LLOYD, G. N. THATCHER, N. PAYNE, N. STONE and A. F. LEVER
Medical Unit, St Mary's Hospital, London, W. 2

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The major previous work on purification of pig renin was that of Haas, Lamfrom & Goldblatt (1953), who used methods mainly dependent on selective denaturation and salt precipitation. In the present work newer methods of protein purification have been used in an attempt to isolate the pure enzyme. Some of these have previously been applied as single procedures (Peart, 1959; Passananti, 1959; Nairn, Chadwick & Fraser, 1960; Lever & Peart, 1962). The steps in the preparation are shown in Table 1; a standard renin and method of assay were needed to follow changes in specific activity. The standard renin consisted of a large batch of renin taken through to the post-DEAE-cellulose stage and kept frozen (-10°) in 5ml. volumes. In these circumstances it was stable for at least 2 years without deterioration. An arbitrary unit was used based on the biological assay and which has no relation to any other renin unit reported from this Laboratory (Lever, Robertson & Tree, 1964) for the measurement of renin in body fluid. For the direct assay a solution of 80 units/ml. was used, and the pressor effect of the unknown was compared with the standard by intravenous injection into the anaesthetized rat (Peart, 1957).

One unit usually raised the blood pressure by 10–20 mm. Hg. The other assay method was by an enzyme velocity measurement (Lever *et al.* 1964), where standard renin or unknown was incubated with a standard substrate and the rate of production of angiotensin was measured. The final pressor assay was again performed in the rat. The protein concentration of solutions was measured by the technique of Lowry, Rosebrough, Farr & Randall (1951). In the preparation of a single batch, 18 kg. of fresh pig kidney was minced and then extracted with 30l. of cold (4°) ethanol overnight; the ethanol was removed by centrifugation and the residue again extracted overnight with 30l. of cold (4°) 0.9% NaCl. The supernatant containing the renin was recovered by centrifugation and the renin was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (40g./100ml.). After settling overnight, the precipitate was filtered off on Whatman no. 50 filter paper. It was dissolved in a minimum amount of cold tap water and dialysed in Visking cellophane sacs (36/32) against daily changes of tap water for 3 days until free of NH_3 . The resultant precipitate was centrifuged off, solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to a concentration of 3% (w/v),