

Isolation purification and characterisation of rubisco



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Carbon is essential for life. Unfortunately, carbon in the earth and atmosphere is locked in highly oxidized forms, such as carbon dioxide. In order to be useful, this oxidized carbon must be “ fixed” into organic forms. Plants perform this task by ‘ carbon-fixation’ - through photosynthesis. There is an enzyme inside plant cells, called Ribulose biphosphate carboxylase/oxygenase (Rubisco). It takes carbon dioxide and attaches it to ribulose biphosphate.

In spite of its central role, rubisco is a very slow catalyst, when compared to other enzymes. Typical enzymes can process a thousand molecules per second, but rubisco fixes only about three carbon dioxide molecules per second. This slow rate is compensated by its increased production. Rubisco comprises of half of the protein in the chloroplasts making it the most plentiful single enzyme on the Earth.

Rubisco also shows lack of specificity. in rubisco, an oxygen molecule can bind comfortably in the site designed to bind to carbon dioxide. Rubisco then
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attaches the oxygen to the sugar chain, forming a faulty oxygenated product. The plant cell then performs a costly series of salvage reactions to correct this.

Studies on the enzyme by Manuel J et al, in higher plants, revealed the presence of 8 small (S) chains with a MW of 13 kDa each and 8 large (L) chains with a MW of 55 kDa each. Assembly of all these chains occurs in the chloroplast stroma, building the whole holoenzyme L8S8 also called ' Form I'.

[Assessment of D-Ribulose-1, 5-Bisphosphate Carboxylase / Oxygenase (Rubisco) Enzymatic Activity – Handbook of Plant Ecophysiology Techniques, chapter 23, Springer Netherlands]. J. E. Musgrove et al found that the newly synthesized Rubisco large subunits made from isolated intact chloroplasts from *Pisum sativum* are bound non-covalently to large subunit binding protein. They found that the binding protein purified from *Pisum sativum* was in the form of an oligomer of relative molecular mass (Mr) about 720000.

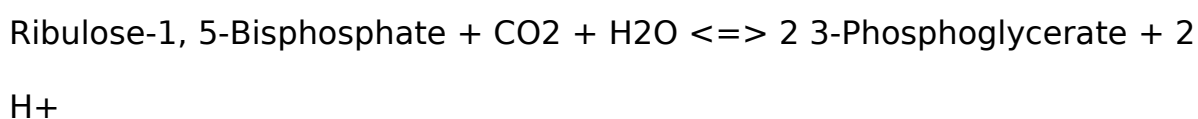
Analysis on polyacrylamide gels containing sodium dodecyl sulphate revealed equal amounts of two different types of subunit, termed alpha (Mr about 61000) and beta (Mr about 60000); thus the oligomer has the composition $\alpha_6\beta_6$ [The Rubisco Large Subunit Binding Protein, by © 1986 The Royal Society].

The post-translational modification the Rubisco was studied extensively by Mulligan R. M., et al and Houtz R. L., et al. Their study revealed that it undergoes at least three different types of post-translational modifications inside the cell. The larger subunit of the enzyme is coded by a plastid gene and is translated into Rubisco holoenzyme. Mass spectral and amino acid sequence analysis of peptides prepared from Rubisco had demonstrated that <https://assignbuster.com/isolation-purification-and-characterisation-of-rubisco/>

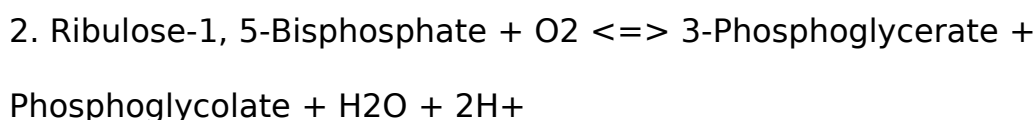
this subunit is processed to the mature form by removal of the N-terminal Met-1 and Ser-2 residues and acetylation of Pro-3 [Proc. Natl. Acad. Sci. U. S. A. 85: 1513-1517, (1989) Proc. Natl. Acad. Sci. U. S. A. 86: 1855-1859 respectively]. In 1989, Houtz R. L., et al found that the LS from many species contained a trimethyllysyl residue at Lys-14 [Proc. Natl. Acad. Sci. U. S. A. 86: 1855-1859, Houtz R. L., et al (1991) Plant Physiol. 97: 913-920, Houtz R. L., et al (1992) Plant Physiol. 98: 1170-1174]. The small subunit (SS) of Rubisco is also post-translationally modified. This polypeptide is post-translationally imported into chloroplasts and processed by a stromal processing peptidase that removes the targeting presequence. The resultant N-terminal methionine residue of the processed SS is subjected to N-methylation (Grimm R., et al (1997) FEBS Lett. 408: 350-354) prior to assembly with the LS into the holoenzyme.

The reactions of Calvin cycle is as shown below:

1. Rubisco is the enzyme catalyzing the following reaction:



The enzyme also has an unusual oxygenase activity, shown below:



At high concentrations CO₂ the reaction with O₂ is suppressed.

Phosphoglycolate is then dephosphorylated and passed into peroxisomes

where it is further oxidized, glyoxylate is amidated, and glycine is produced.
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This process is referred to as photorespiration and it occurs under conditions where the oxygen concentration is high.

Aim:

The principal objective of the experiment was to isolate, characterise the Rubisco from fresh pea leaves and estimate its specific activity. The isolation and extraction was done using ammonium sulphate precipitation at different concentrations. The enzyme fraction was separated using column chromatography with Sephacryl S-300 and confirmed with SDS-PAGE and native gel bands. The presence of the enzyme band was confirmed by comparison with that of a standard purified enzyme from spinach. The total protein and enzyme assay was done using standardised protocols.

Methods:

All procedures were performed at/or close to 10 °C.

Extraction: Fresh pea leaves, with veins removed, were taken from light-adapted actively-photosynthesised plants, which were previously put in sunlight for 1 hr prior to harvest. About 12g of leaf laminae were blended with cold extraction buffer [0.1 M Kphosphate, 1 mM EDTA, pH 7.2] and squeezed through wet Miracloth. BSA was quickly added to a concentration of 1 mg/ml and centrifuged at 20,000g for 15 min. An aliquot of 100 µL was stored for enzyme analysis and the rest was used for fractionation with ammonium sulphate.

Ammonium sulphate precipitation: Solid $[\text{NH}_4]_2\text{SO}_4$ at 30% saturation at pH 7.8 (adjusted with ammonia solution) was added and after 10 min, it was

centrifuged at 10, 000g for 10 min. The pellet was stored and to the supernatant again solid $[\text{NH}_4]_2\text{SO}_4$ at 45 % saturation at pH 7. 8, was added and centrifuged as before. The supernatant was poured off, and the precipitate was suspended in 15 ml of fresh 55% ammonium sulphate solution [2 mM EDTA, pH 7. 5] and was stored. The supernatant was brought to 90% ammonium sulphate and adjusted to pH 7. 8 as before. It was again centrifuged as before. The precipitates from 30% and 90% ammonium sulphate procedures were redissolved in 10mL of extraction buffer [0. 1M Kphospate, 1 mM EDTA, pH 7. 2] and stirred gently with glass rod. Both fractions were assayed for protein (Bradford method) and Rubisco activity.

The stored precipitate from 55% AS was centrifuged for 10 min at 10, 000g and dissolved gently in 4 ml of extraction buffer. This was again centrifuged at 26, 000 g for 10 min and the supernatant which was clear, pale yellow in color was kept.

Gel filtration: 3 ml of a sub sample from above was desalted by passing through Biorad Econopac-10 column with phosphate buffer [Accessed 28-Apr-2010] [50mM Kphosphate, 1 mM EDTA, pH 7. 5]. The colored compounds were absorbed and were separated from proteins.

3 ml of salt-free sample solution was loaded into the Sephacryl S-300 column, which was equilibrated with Hepes buffer [25mM Hepes, 0. 1 M NaCl, 1 mM EDTA, 1mM DTT, 25mM MgCl_2 , 25mM NaHCO_3 , pH 7. 8] at RT. The sample was allowed to run at 25 ml/sq. cm cross section per hour with Hepes buffer with a flow rate of 0. 5 mL/min. The first 10 mL was collected in a measuring cylinder and then fractions of 1. 5 mL were collected in

microfuge tubes. The protein was measured at 280 nm. The carboxylase was eluted as the first major peak of the protein in the elution profile. The protein samples were stored till the enzyme was identified. Then all the fractions containing the enzyme were pooled and its protein content was measured using Bradford assay.

The specific activity of the purified enzyme preparation from above was compared with that of purified RUBISCO from spinach. The enzyme preparation was diluted suitably for the assay.

PAGE gel: The protein content of the fractions collected from the column was determined and a suitable concentration of it was loaded in the SDS-PAGE and native gels as described by the method of Laemmli [Nature 227 (5259): 680-685]. They were then fixed, stained and destained for visualising the bands. The molecular weight of Rubisco was determined by the method of Shapiro et al [Biochem Biophys Res Commun. 28 (5): 815-820]

Enzyme assay: enzyme assay was done spectrophotometrically using coupled enzyme system. The 2, 3PG formed by the enzyme was phosphorylated using ATP and the resulting 2, 3 bisPG was coupled with G-3-PDH and NADH. ADP generated reacts with Creatine-phosphate to yield ATP and Creatine. The carboxylase activity was followed by the oxidation of NADH at 340 nm and 25 °C.

The substrate/buffer solution [82mM Na HEPES, 20mM MgCl₂, 1 mM ATP, 0.1 mg/ml BSA, 0.22 mM NADH, 10 mM Creatine-phosphate, 50 mM NaHCO₃] the coupling enzymes were phosphoglycerate kinase (380 U/ml), G-3-PDH (270 U/ml) and creatine kinase (200 U/ml). Pure carboxylase from spinach <https://assignbuster.com/isolation-purification-and-characterisation-of-rubisco/>

was added at 0.5 mg/ml concentration in phosphate buffer with 21 mM Ribulose biphosphate in sterile, filtered water.

Protein estimation: This was done by the method of Bradford M. M. [Anal. Biochem. 72: 248-254.]

Results:

Crude extract contained the maximum total protein and the enzyme concentration as usual. While the total enzyme units was high in the crude extract the specific activity of the enzyme was high in the 0-30% AS step. Also, the total protein extracted with AS was less with 30-45% stage but increased with 0-30% & 45-90% step significantly. The enzyme concentration, specific activity and total enzyme units was maximum at 0-30% fraction, indicating the relative purity to be the best at this fraction.

A calibration graph was constructed. From the graph, the O. D of 0.152 gave the concentration of the protein in the unknown sample as $180 \frac{1}{4} \text{g} / \text{mL}$.

The above gel of 2008 shows the presence of at three bands in most lanes except in lane 6 & 8. Accordingly, the thicker band corresponds to that of the larger subunit and the last band to that of smaller subunit of the enzyme with their respective molecular weights as calculated from the graph.

The lane 3 is my lane and does not show a thick band for LS of the enzyme. Still the SS is seen as a faint band when compared to that of lane 10- pure enzyme from spinach.

The native gel pattern also shows a faint band for the LS with SS subunit band almost absent. The gel pattern doesn't appear to be good with distorted bands in lanes 4, 5 6, inspite of the conspicuous presence of the LS in them.

(iv) Calculation of MW of Rubisco from standard molecular weight markers:

Protein

The band on the gel for the small subunit pea Rubisco's MW (MW 49. 6 kDa) was found to be between that of BSA and ovalbumin. For large subunit of the enzyme (MW 15. 16 kDa) it was between lysozyme and soybean trypsin inhibitor.

Discussion:

The principal objective is to extract, isolate and characterise the Rubisco from fresh pea leaves. As per the conventional methods of extraction and isolation, ammonium Sulfate at different concentrations was used to isolate all proteins from the fresh pea leaves. Each fraction showed different protein content, total enzyme activity and specific activity. Column (Sephacryl S-300) chromatography was employed to separate out all proteins with an isoelectric point of pH 8 or lower. The fraction with maximum concentration of the enzyme, which was from 0-30% AS step was pooled and assayed for total and specific activity as described in the methods. Bradford protein assay was used to determine the concentration of the protein in each sample in order to determine the specific activity of each fraction of the enzyme from the column. The specific activity was also found to be maximum at 0-30 % AS step.

The sample extract was run through column with positively charged matrix. Knowing that Rubisco's Isoelectric point is pH 4.2, a buffer with a pH of 8 is run through the column ensuring that Rubisco will stick to the matrix. Protein that remains in matrix is eluted using different salt concentrations in buffer and collected in fractions of 1.5 ml. Because Rubisco is known to be the most abundant protein in fresh pea leaves, the fractions containing the highest protein concentration are kept for each different salt concentration.

The proteins were separated using SDS-PAGE electrophoresis. The sample in my lane 3 contained 2 bands (with a faint LS) with a molecular weight of 49.6 kDa and 15.16 kDa. According to Creighton, et al [Encyclopedia of Molecular Biology, 4th ed. (New York: John Wiley and Sons, Inc.), 1999.]

Rubisco is made of 2 subunits, viz., large subunit: 50-55 kDa and a small subunit: 12-18 kDa. The specific activity was maximum with 0-30% AS step and decreased with increasing AS%, indicating that it was getting extracted at the earlier stage of the AS precipitation itself. There was a loss of activity as well as the relative purity of the enzyme with increasing AS% .

Though the PAGE electrophoretic patterns doesn't conspicuously confirm the presence of the enzyme, the assay from the fraction proved so. The probable reason of the faint band may be due to insufficient protein being loaded in the gel or may be due to over destaining of the band or less staining. On the whole Rubisco was successfully isolated.