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Isolation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase from Young Leaves of *Phytolacca dioica* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AMADG, NL and SR managed the purification and analyses of the study. Author ADM designed the study, wrote the protocol and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) catalyses a key reaction by which inorganic carbon is assimilated into organic carbon found in the biosphere. The present study was aimed to isolate this enzyme from leaves of *Phytolacca dioica* L.
Study Design: In this work, first crude extracts from leaves at different stages of development were assayed to isolate this enzyme, then young leaves of *P. dioica* were used considering high value of specific activity.
Methodology: Classical methods for protein isolation have been used to characterise RubisCO from *P. dioica* leaves.
Results: RubisCO was isolated from young leaves by gel-filtration. The pure RubisCO showed two predominant bands (56- and 15-kDa) by SDS-PAGE. N-terminal sequences data on large (56 kDa) and Small (15 kDa) subunits obtained by automatic Edman degradation show a high percentage of identity with large and small subunit of other RubisCo enzymes. Moreover, the N-

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terminal amino acid sequence obtained by Edman degradation of the expressed large subunit (56 kDa) corresponds to the traduced one found by the analysis of the chloroplast genome of *P. dioica* (access number AFU65422).
Conclusion: The data on RubisCO from young leaves of *Phytolacca dioica* L. (RubisCO-Pd), obtained in the present work, could be used as the starting point for biological characterization of this enzyme.

Keywords: Edman degradation; Phytolacca dioica L.; protein purification; RuBisCO.

1. INTRODUCTION

Ribulose 1,5-bisphosphate (RuBP) carboxylase/ oxygenase (RubisCO) catalyzes the condensation of one molecule of CO_2, H_2O and ribulose 1,5-bisphosphate (RuBP) to form two molecules of 3-phospho-D-glycerate and initiates net CO_2 fixation in autotrophs [1]. The most abundant enzyme on Earth, RubisCO, comprises around 40% of the total proteins of the plant [2]. RubisCO, however, is a bifunctional enzyme that also possesses an oxygenation activity. In this reaction, the sugar substrate is oxygenated to produce one molecule of 3-PGA and one molecule of 2-phosphoglycolate. The latter product is the substrate for photorespiration [3]. The ratio of carboxylation to oxygenation at any specified concentrations of CO_2 and O_2 is referred to as the CO_2/O_2 specificity factor [4].

RubisCO has been purified and characterized from a variety of sources. On the basis of amino acid sequences, four known forms of RubisCO, forms I, II, III and IV, were found in nature [5]. The form I has been isolated from plants, algae, proteobacteria and cyanobacteria, whereas the form II from proteobacteria, dinoflagellates. Moreover, the forms III and IV have been found in archaea or in proteobacteria, cyanobacteria, archaea and algae, respectively.

The most abundant form of RubisCO is the form I of holoenzyme, which consists of 55- and 15-kDa subunits (named L and S subunit, respectively). In higher plants the large subunit is encoded by the chloroplast genome, whereas the small subunit is encoded by the nuclear genome [6]. The molecular weight of this holoenzyme is typically 550,000. The structure presumably consists of eight of each of the two types of subunits $(LS)_8$ [6]. The large RuBisCO subunits display a considerable sequence homology approximatly of 70-90%. Large subunits of all three enzyme types are approximatly 30-35% identical in amino acid sequence, but show highly conserved active sites. The small subunits in $(LS)_8$ are required for maximal catalytic rates [7].

RuBisCO is usually only active during the day as ribulose 1,5-bisphosphate and it is not regenerated in the dark. This is due to the regulation of several other enzymes in the Calvin cycle. Moreover, the RubisCO activity involves the interaction with various molecules as ions, phosphate, ATP/ADP and stromal reduction/oxidation state as well as RuBisCO activase [8,9]. In particular, RubisCO activase is the primary enzyme responsible for RubisCO's catalytic efficiency and it is heat labile. Moreover, this enzyme has even been suggested as the primary cause for reduced photosynthetic performance in response to moderate heat [10].

Phytolacca dioica L. (commonly known as ”Ombú” or “Bella Sombra”) is considered from our research group an important system model for the characterization of peptides, proteins and enzymes [11-14]. Therefore, in the present work, a Ribulose-1,5-bisphosphate Carboxylase/ Oxygenase (hereafter RuBisCO-Pd) has been isolated from young leaves of *P. dioica*. The purification and partial characterization of RuBisCO-Pd from *P. dioica* are the essential steps to establish a basis for further studies.

2. MATERIALS AND METHODS

2.1 Chemicals, Reagents and Buffers

Young leaves of *P. dioica* were harvested at the end of March from a single female tree in the Botanical Garden of Naples, Italy (Fig. S1).

3-phospho-D-glycerate and D-ribulose-1,5-bisphosphate were procured from Sigma-Aldrich (Milan, Italy). The silica gel 60-coated (with specific surface area = 500 cm^2/g; pore volume = 0.75cm^3/g; pore diameter = 60 Å) micro TLC plates were purchased from Merck (Merck, Darmstadt, Germany). The solvents of highest purity “analytical grade” were purchased from Carlo Erba reagent (Milan, Italy). Coomassie Brilliant Blue R-250 was purchased from Fluka (Milan, Italy). Standard proteins for SDS-PAGE was purchased from Sigma-Aldrich. All other reagents and chemicals were of analytical grade.

Reagents for automated Edman degradation were supplied by Life Technologies (Monza, Italy).

The following buffers have been used: buffer A: 25 mM Tris●Cl, 1 mM EDTA, and 1 mM dithiothreitol (DTT), pH 7.6; buffer B: 25 mM Tris●Cl containing NaCl 0.3 M, pH 8.2. buffer C: 200 mM bicine solution, pH 8.2, containing 0.4 mM EDTA and 1 mM DTT.

2.2 Fast RubisCO-Pd Purification

Young leaves (10 g) were homogenized in 50 mL of buffer A by 20-s bursts at full power using a Waring Blendor (Waring Products), at 4°C. The homogenate was then filtered through Miracloth paper (Inalco, Milan, Italy) and centrifuged at 12,400*g* in a JA-14 rotor (Centrifuge Avanti J-25, Beckman Coulter, CA, USA) for 60 min, at 4°C. The homogenate was further concentrated in Centriprep YM-10 (Millipore Corporation, Bedford, MA, USA). This concentrated fraction was first centrifuged as above and then gel-filtered on a Hi-load 26/60 Superdex 75 (GE Healthcare, Milan, Italy; separation range 70-3 kDa), equilibrated and eluted with buffer B, at a flow rate of 2.5 mL/min, using the AKTA prime 100 FPLC (GE Healthcare). Fractions with RubisCO activity were pooled, concentrated and stored at -20°C.

2.3 Protein Electrophoresis

Homogeneity of isolated proteins was determined by SDS-PAGE with a Mini-Protean II mini-gel apparatus (Bio-Rad; Rome, Italy), using 6% (w/v) stacking polyacrylamide gel and 15% (w/v) separation gel [15].

2.4 *N*-terminal Amino Acid Sequencing by Edman Degradation

Native RubisCO-Pd was separated by SDS-PAGE in reduction condition. After electrophoresis, protein was transferred to PVDF membrane (Life Technologies, Monza, Italy) by electroblotting with the mini trans-blot cell (Bio-Rad, Rome, Italy), according to the manufacturer's instructions in 10 mM CAPS, pH 11.0, containing 10% methanol. PVDF membranes were then stained for 1 min with Coomassie Blue R-250, destained with the washing solution (50% methanol) [16], dried and directly analysed by Edman degradation on a Procise Model 491C sequencer (Applied Byosystem Inc., Foster City, CA, USA) [17].

2.5 Homology Studies

Amino acid sequences of other RubisCO enzymes were retrieved and aligned using the BLASTp software on the NCBI taxonomy browser. The program was available at the NBCI web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment was performed by using the on line program ClustalW, available at http://www.ebi.ac.uk/Tools/msa/clustalw2/.

2.6 Enzymatic Activity

RubiscCO activity was measured using a nonradioactive assay method based on thin-layer chromatographic separation of 3-phospho-D-glycerate (3-PGA) and D-ribulose-1,5-bisphosphate (RuBP) as previously reported [18]. Briefly, the substrate RuBP, was incubated at 37ºC into the reaction tube in buffer C. Then, the reaction was initiated by adding RubisCO enzyme. The mixture was incubated at 37°C for a fixed period in the presence CO_2 gas. For activity determination, usually, single-time point estimates were made at 15-min time intervals. Finally, the samples were withdrawn and subjected to TLC analysis. The TLC plate was allowed to stand in a glass through the solvent system 2-methylpropanoic acid/1 M NH_3/0.1M EDTA (125:75:2 v:v:v). After 3h, the TLC plate was withdrawn and air-dried. In this solvent system at 25°C, different relative migration distances (Rf) were observed for RuBP and 3-PGA after that plates were developed by staining the whole plate with 5% ammonium molybdate, pH 8.6, or the ammonium molybdate reagent to detect sugar phosphate esters.

3. RESULTS AND DISCUSSION

3.1 RubisCO-Pd Isolation

The presence of RubisCO in *P. dioica* has not been investigated so far. Based on that, extracts from young leaves of *P. dioica* were assayed (data not shown). The preliminary data showed a high RubisCO activity from young leaves of *P. dioica*. Therefore, a purification procedure using young leaves was undertaken in order to isolate potential novel RubisCO. Fractionation of the concentrated portion of the homogenate was carried out by gel-filtration chromatography. Detection of the biological activity was carried out as described above. Fractions showing RubisCO activity were analysed by SDS-PAGE (data not shown) and the homogeneous samples (fraction numbers 39-43) were pooled (Peak 1, Fig. 1).

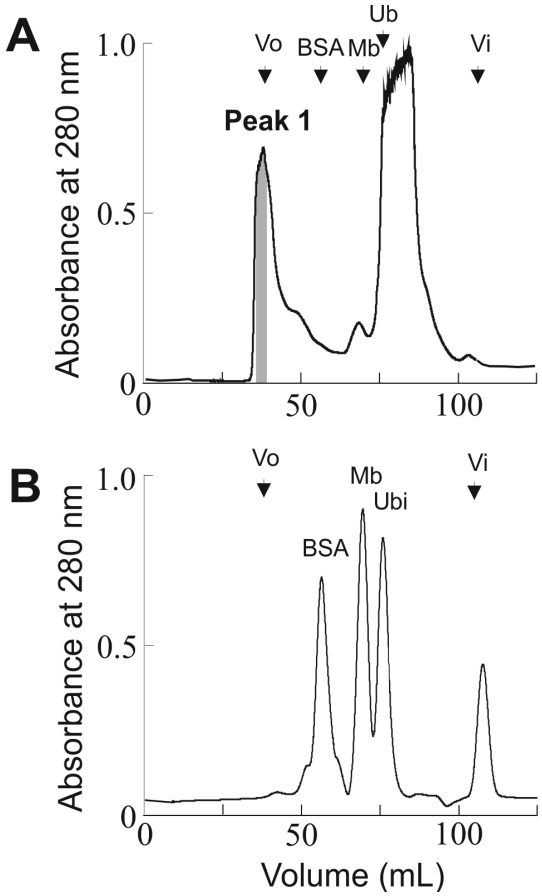

Fig. 1A. elution profile of the gel-filtration chromatography of the crude extract from young leaves of *P. dioica*. Fractions containing RubisCO activity are shaded (Peak 1). B, the chromatographic elution profile of standard proteins is also reported (BSA, bovine serum albumin, 66 kDa; Mb, horse myoglobin, 17 kDa; Ubi, ubiquitin, 8.5 kDa)

Peak 1, showing RubisCO activity (hereafter was named RubisCO-Pd) was concentrated and subjected to further analysis. Subjection of isolated RubisCO-Pd to SDS-PAGE (in the presence of β-mercaptoethanol), revealed two bands of 56 and 15 kDa (Fig. 2A, lanes 1-2). On the other hand, when characterized by gel-filtration, a higher value of more than 100kDa was obtained. These findings suggest that the native RubisCO-Pd is organised as an oligomeric, in agreement with other forms I of RubisCO [6]. In fact, it is known that RubisCO proteins are stable as holoenzyme enzymes, supporting the prominent connection between the stability of the proteins and their enzymatic activity.

Finally, in order to characterize the enzymatic activity, RubisCO-Pd, was incubated in buffer C, at 37°C for a fixed period in the presence of CO_2 gas, as previously reported [18]. Then, the sample was withdrawn and subjected to TLC analysis. A typical thin layer chromatogram (TLC) for the determination of RubisCO (carboxylation) activity is showed in Fig. 2B. As showed in this figure, the amount of 3-PGA in the assay increases over time, vice versa the amount of RuBP decreases, confirming the carboxylation reaction of RubisCO-Pd.

3.2 Protein Identification and Homology Studies

To obtain N-terminal sequence data on RubisCO-Pd, the bands of lane 2 (see Fig. 2A), were transferred to PVDF membrane and directly subjected to automatic Edman degradation, as described in the Materials and Methods section. The analysis of band 1 and band 2, revealed two different sequences; 1-AGVKDYKLTY YTPQYKP-17 and 1-QVWPDLLGKK FFETLSY-17, respectively (Fig. 3). Subsequently, to obtain further sequence information on the two subunits of RubisCO-Pd, a search for sequence similarities, was performed with the BLAST program. It revealed that the both N-terminal sequences of the band 1 and band 2 of RubisCO-Pd share a high percentage of identity with large subunit and small subunit of other RubisCO enzymes.

A multiple alignment of RubisCO-Pd subunits with other RubisCO sequences is presented in Fig. 3. Moreover, the analysis as previously reported, shows that the large subunits present highest identity with other large RubisCO subunits (100-94% of identity), whereas, for the small subunits is lower (82% of identity).

On the other hand, the N-terminal amino acid sequence obtained by Edman degradation of the expressed large subunit (56 kDa) corresponds to the traduced one found by the analysis of the chloroplast genome of *P. dioica* (access number AFU65422).

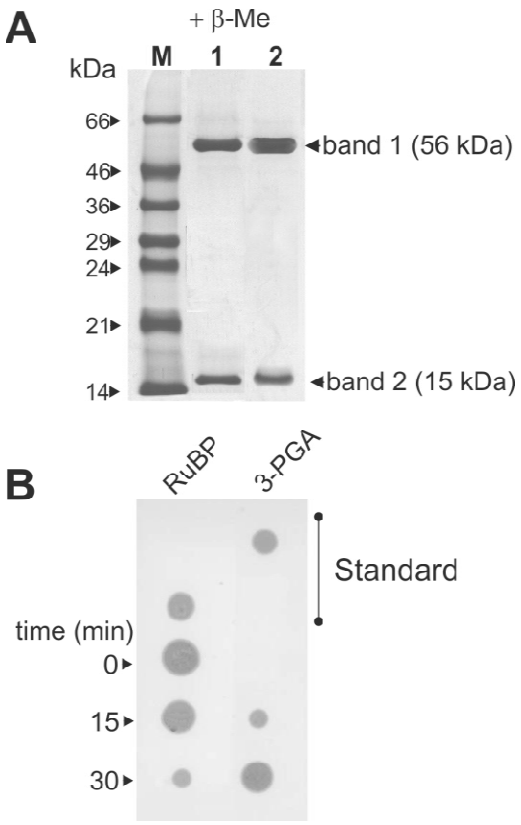

Fig. 2A. Analysis by SDS-PAGE in presence of β-mercaptoethanol of RubisCO-Pd from young leaves of *P. dioica*. M, marker proteins; lanes 1-2, isolated RubisCO-Pd, (2 and 3µg, respectively). B, A thin layer chromatogram (TLC) for the determination of RubisCO-Pd (carboxylation) activity. The D-ribulose-1,5-bisphosphate (RuBP) and 3-phospho-D-glycerate (3-PGA) as standard are indicated. The assay mixture contained RuBP and purified RubisCO-Pd were incubated for the indicated time interval and analyzed by TLC

A, band of ~56 kDa (RubisCO large subunit)

Plant	Accession	Sequence	Identity (%)
P. dioica		1-AGVKDYKLTY YTPQYKP-17	(100)
Rivina humilis L.	AFI21564	1-AGVKDYKLTY YTPQYKP-17	100
Comesperma hispidulum L.	CAJ81067	1-AGVKDYKLTY YTPQYKP-17	100
Phytolacca icosandra L.	AFI21562	1-AGVKDYKLTY YTPEYKP-17	94
[a]		********** ***:***	

B, band of ~15 kDa (RubisCO small subunit)

Plant	**Accession**	**Sequence**	**Identity (%)**
P. dioica		1-QVWPDLLGKK FFETLSY-17	(100)
Ricinus communis L.	XP_002521232	1-QVWP-PLGKK KFETLSY-17	82
Arachis duranensis	AIF75302	1-QVWP-PLGKK KFETLSY-17	82
Litchi chinensis	ADT78691	1-QVWP-PVGKK FFETLSY-17	82
[a]		**** .:*** ******	

Fig. 3. Alignment of the N-terminal sequences of the large and small subunits of RubisCO-Pd with those of other RubisCO. [a], Identical residues *, conserved substitutions (:) and semiconserved substitutions (.) are reported

4. CONCLUSION

A novel RubisCO, named RubisCO-Pd, was isolated from young leaves of *P. dioica* by gel filtration. Analysis of RubisCO-Pd by SDS-PAGE showed two predominant bands. The molecular weight of large and small bands are 56 kDa and 16 kDa, respectively. A molecular weight of more than 100 was obtained by gel-filtration. N-terminal sequences of both large and small subunits of RubisCO-Pd showed a high percentage of identity with those of other plants RubisCO enzymes. Further investigations on RubisCO-Pd primary structure are still in progress to extend our knowledge on the structure/function relationships of this enzyme.

Finally, it should be emphasized that primary structure data, obtained in the present work, could be used as the starting point for molecular biology studies aimed at cloning of the RubisCO-Pd gene.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

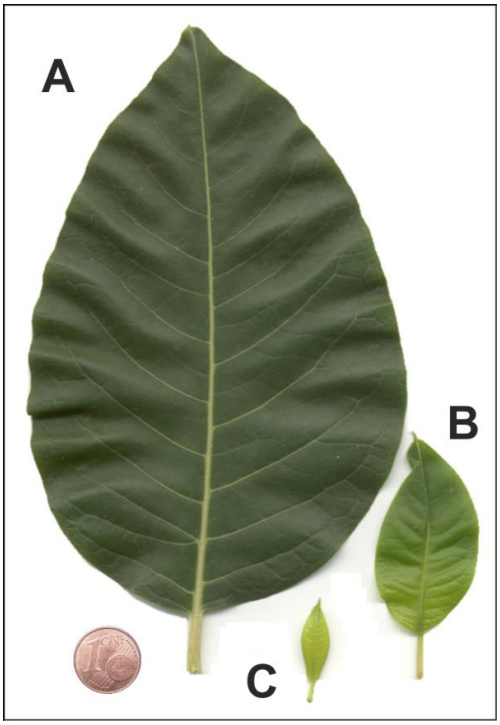

Fig. S1. Fully expanded (A), in development (B), and young leaves from an adult, 100-year-old plant, growing in the Botanical Garden of the University of Naples "Federico II". The leaves were harvested between April and June 2013. Only young leaves were collected in March

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