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Automated flow-based anion-exchange method for high-throughput isolation and real-time monitoring of RuBisCO in plant extracts

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ABSTRACT

In this work, a miniaturized, completely enclosed multisyringe-flow system is proposed for high-throughput purification of RuBisCO from *Triticum aestivum* extracts. The automated method capitalizes on the uptake of the target protein at 4 °C onto Q-Sepharose Fast Flow strong anion-exchanger packed in a cylindrical microcolumn (105 × 4 mm) followed by a stepwise ionic-strength gradient elution (0–0.8 mol/L NaCl) to eliminate concomitant extract components and retrieve highly purified RuBisCO. The manifold is furnished downstream with a flow-through diode-array UV/vis spectrophotometer for real-time monitoring of the column effluent at the protein-specific wavelength of 280 nm to detect the elution of RuBisCO. Quantitation of RuBisCO and total soluble proteins in the eluate fractions were undertaken using polyacrylamide gel electrophoresis (PAGE) and the spectrophotometric Bradford assay, respectively. A comprehensive investigation of the effect of distinct concentration gradients on the isolation of RuBisCO and experimental conditions (namely, type of resin, column dimensions and mobile-phase flow rate) upon column capacity and analyte breakthrough was effected. The assembled set-up was aimed to critically ascertain the efficiency of preliminary batchwise pre-treatments of crude plant extracts (*viz.*, polyethylenglycol (PEG) precipitation, ammonium sulphate precipitation and sucrose gradient centrifugation) in terms of RuBisCO purification and absolute recovery prior to automated anion-exchange column separation. Under the optimum physical and chemical conditions, the flow-through column system is able to admit crude plant extracts and gives rise to RuBisCO purification yields better than 75%, which might be increased up to 96 ± 9% with a prior PEG fractionation followed by sucrose gradient step.

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the most abundant protein in the biosphere constituting up to half of the soluble protein in plant leaves [1]. RuBisCO catalyses the fixation of atmospheric CO_2 to ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglycerate. By doing this function, RuBisCO constitutes the unique conversion point of inorganic to organic carbon and its activity amounts to more than 10^{11} tons of CO_2 per year [1]. In spite of its biological importance, RuBisCO presents a number of catalytic inefficiencies, the most important of which is its failure to distinguish between CO_2 and O_2. In addition, RuBisCO is a notoriously slow enzyme [2], which obligates plants to divert huge amounts of nitrogen to RuBisCO in order to achieve acceptable rates of photosynthesis. The low affinity for CO_2 and the production of miss-products during catalysis are other well known limitations of the enzyme [3]. All these inefficiencies greatly limit the photosynthetic capacity of plants, decreasing the efficiencies by which water and nitrogen are used in agriculture. It is therefore not surprising that RuBisCO has been historically targeted as a major gateway to increase plant productivity, and therefore food and energy production [4,5]. The expectative to improve the catalytic performance of RuBisCO has been enhanced by both the discovery of remarkably different versions of the enzyme among higher plants [6,7] and the progress in the production of transplantomic lines where the native RuBisCO is replaced by foreign forms [8,9]. Yet, to sustain the pos-

Abbreviations: Bicine, N,N-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; CC, communication channel; DIECA, diethyldithiocarbamate; DLLs, dynamic link libraries; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FC, fraction collector; FI, flow injection; HC, holding coil; LSU, large subunits of RuBisCO; MPV, multiposition selection valve; MSFI, multisyringe flow injection; MSP, multisyringe piston pump; P, purification yields; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylenglycol; PMMA, poly(methyl methacrylate); PMSF, phenylmethylsulphonyl fluoride; PS-DVB, poly(styrenedivinylbenzene); PTFE, poly(tetrafluoroethylene); PVC, poly (vinyl chloride); PVP, poly(vinylpyrrolidine); R, absolute recoveries; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS, sodium dodecylsulphate; SI, sequential injection; SSU, small subunits of RuBisCO; Tris, tris(hydroxymethyl)aminomethane.

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sibilities for an improved RuBisCO, it is necessary to gain improved knowledge upon the structure and regulatory processes of the enzyme and to deeply explore the variability of the kinetic traits in nature [5,10]. Increasing the knowledge on the enzyme would also potentially benefit other related subjects, namely, improvement of plant responses to raising atmospheric CO_2 concentration [11], and open new prospects for artificial photosynthesis and biofuel production [12].

RuBisCO needs to be purified from extracts for appropriate biochemical characterization. The degree of required purity depends on the specific analysis, with crystallization and measurements of some kinetic constants, such as the specificity factor, demanding high degrees of purification [7,13]. A survey of the literature revealed that a number of purification protocols and variants thereof for RuBisCO purification from several plant sources have been reported including fractionation with ammonium sulphate [14–16] or polyethylenglycol (PEG) [17–19], immunoprecipitation and immunoadsorption [20], sucrose gradient centrifugation [21,22] size-exclusion or dialysis [8,23], anion-exchange chromatography [7,8,16,24,25] and a plethora of combinations thereof [8,16,18,22,23,25]. However, the experimental results and conclusions drawn are in several instances controversial and debatable, and no universal analytical procedure for isolation of RuBisCO from plant sources is available as of yet.

This work is aimed at the development of an automated, entirely enclosed multisyringe flow injection (MSFI)-based analytical method for high-throughput purification of RuBisCO from plant extracts. The flow network capitalizes on the uptake of the negatively charged protein at pH ≥ 8.0 and 4 °C onto anion-exchange beads followed by stepwise ionic-strength elution as precisely controlled by flow programming. The flow manifold is equipped with a flow-through diode-array spectrophotometer and fraction collector for real-time monitoring of column effluent at the protein-specific wavelength of 280 nm and automated retrieval of highly purified RuBisCO fractions, respectively. The inherent versatility of the assembled flow device is exploited upon optimization to endorse a simplified automated procedure capable of admitting sample extracts regardless of the preceding sample processing steps (direct crude extracts, precipitation with PEG or ammonium sulphate or sucrose gradient centrifugation), and of giving rise to acceptable recoveries and purification yields of RuBisCO.

The first two generations of flow analysis, that is, flow injection (FI) and sequential injection (SI), and microfluidic devices have drawn much attention as platforms for automated assays of soluble proteins [26] and individual determinations of albumin [27–29] and/or creatinine [30,31] by resorting to highly sensitive dye-binding spectrophotometric assays (e.g., Eosin Y or tetrabromophenolphthalein ethyl ester for albumin and Jaffe's reaction for creatinine), beside the exploitation of SI-affinity chromatography for monitoring the binding of drugs on albumin [30]. To the best of our knowledge, however, no FI or SI method has been reported so far for retrieval and quantitation of RuBisCO in plant extracts.

2. Experimental

2.1. Plant material

Wheat (*Triticum aestivum* L., *T. aestivum* in the following) was selected as has been largely used as a model plant for RuBisCO characterization [7,32]. Wheat seeds were germinated and plants grown under controlled conditions (20–25 °C night–day temperatures, 16 h photoperiod and 700 μmol photons m^{-2} s^{-1}).

2.2. Reagents and solutions

All chemicals and reagents used in this work were of analytical reagent grade and used as purchased. A stock standard of highly purified RuBisCO from *T. aestivum* for method development was obtained according to the batchwise purification protocol by Galmés et al. [7]. The high purity of the stock RuBisCO from *T. aestivum* was corroborated by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), revealing only the two bands corresponding to the large (LSU) and small subunits (SSU) of RuBisCO. Doubly de-ionised water (resistivity = 18.2 MΩ·cm) obtained from a Milli-Q system (Milllipore Synthesis A10, Millipore Corporation, Billerica, MA, USA) was used throughout.

Q-Sepharose Fast Flow (GE Healthcare, Bio-Sciences AB, Sweden), with a particle size range of 40–165 μm, was used in the flow network as strong anion exchanger, with no need for any additional swelling protocol. Q-Sepharose Fast Flow is composed of a highly cross-linked, bead-formed 6% agarose matrix furnished with diethyl-(2-hydroxypropyl)aminoethyl as functional moiety.

A tris(hydroxymethyl)aminomethane (Tris) column buffer at pH 8.0, used as a carrier solution as well, contained 10 mmol/L Tris–HCl (acid salt) + NaOH, 10 mmol/L $MgCl_2$, 10 mmol/L $NaHCO_3$, 1 mmol/L Na_2EDTA, 1 mmol/L KH_2PO_4. $MgCl_2$, $NaHCO_3$ and KH_2PO_4 maintained RuBisCO activated [33,34], whilst the role of EDTA was to decrease the activity of proteases by chelation of transition metals [35].

2.3. Extraction and purification of RuBisCO

Three different RuBisCO purification procedures involving PEG precipitation, ammonium sulphate precipitation and sucrose gradient separation besides direct analysis of crude extracts have been evaluated prior to anion-exchange chromatography in terms of RuBisCO yield and purity, and sample throughput as well. The analytical procedures for extraction and preliminary processing of plant extracts performed in triplicate are summarized below and illustrated in Fig. 1.

2.3.1. Preparation of crude extract

All the extraction and purification steps were carried out at 4 °C. 60 g of leaf material was collected under illuminated conditions, ground to a powder in a mortar and immediately extracted with 250 mL of protein extraction buffer (pH 8.2) composed of 100 mmol/L N,N-Bis(2-hydroxyethyl)glycine (bicine), 6% (w/v) PEG-4000, 2 mmol/L $MgCl_2$, 1 mmol/L Na_2EDTA, 10 mmol/L $NaHCO_3$, 10 mmol/L sodium diethyldithiocarbamate (Na-DIECA), 1 mmol/L benzamidine, 1 mmol/L ε-amino-n-caproic acid, 50 mmol/L 2-mercaptoethanol, 10 mmol/L dithiothreitol (DTT), 2 mmol/L phenylmethylsulphonyl fluoride (PMSF) and 3% (w/v) poly(vinylpyrrolidine) (PVP) as per the procedure reported by Galmés et al. [7]. DTT and 2-mercaptoethanol worked as reducing agents, PVP was used for precipitation of polyphenolic compounds and benzamidine, ε-amino-n-caproic acid, Na-DIECA and PMSF were used as protease inhibitors. The plant extract was filtered through 2 layers of butter muslin and then centrifuged at 22,100 × *g* for 20 min. The supernatant liquid was sieved through 50 μm mesh nylon and injected into the MSFI-anion exchange separation system without further purification.

2.3.2. Purification based on selective precipitation using PEG

In this protocol, the crude extract resulting from the abovementioned sample treatment was subjected to a selective precipitation with PEG. It should be noted that the PEG concentration should be thoroughly selected for selective precipitation of macromolecules on the basis of molecular weight. To this end, 60% (w/v) PEG-4000 was added to the supernatant to give rise to a final concentration of