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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

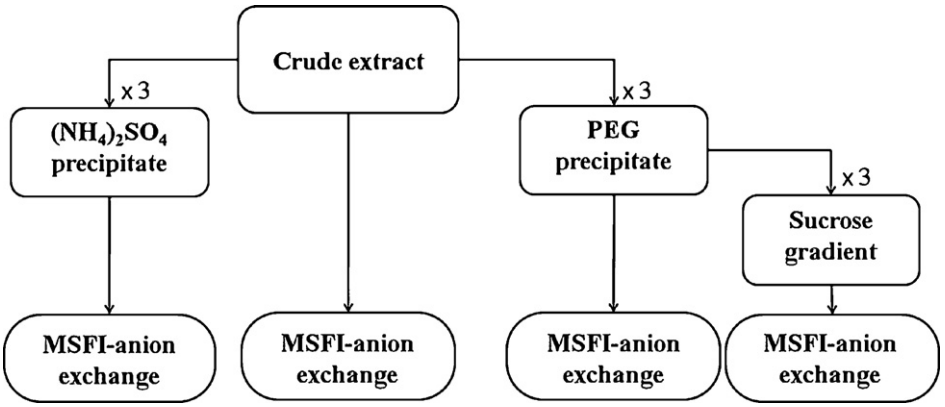

Fig. 1. Schematic diagram of the analytical procedures investigated for RuBisCO purification. MSFI: multi-syringe flow injection.

20% (w/v) for RuBisCO precipitation. In addition, 1 mol/L $MgCl_2$ was added dropwise to a final concentration of 20 mmol/L followed by gentle mixing for 10 min. Thereafter, the mixture was centrifuged at 22,100 × *g* for 20 min. The pellet was resuspended in 10 mL of column buffer (pH 8.0) containing 1 mmol/L each of PMSF, benzamidine and ε-amino-n-caproic acid. The suspension was clarified by centrifugation to remove insoluble material at 48,400 × *g* for 20 min, prior to anion-exchange separation.

2.3.3. Purification based on sucrose gradient

This protocol is exploited as a further extension of PEG precipitation. A metered volume of supernatant liquid, *namely*, 6 mL, was layered onto a linear-step density gradient from 0.2 to 1.2 mol/L sucrose of 2.5 mL each, in 0.1 mol/L bicine, 20 mmol/L $MgCl_2$, 50 mmol/L 2-mercaptoethanol and 11 mmol/L Na-DIECA and centrifuged at 103,900 × *g* for 14 h at 4 °C. Each 2.5-mL fraction of the sucrose gradient was subjected to Bradford assay (see below) to detect the layers containing the highest concentration of total soluble protein, which were pooled prior loading onto the anion-exchange column.

2.3.4. Purification based on selective precipitation using ammonium sulphate

Sample processing using ammonium sulphate as a molecular fractionation reagent was performed following RuBisCO extraction with bicine buffer. The supernatant was made up to 35% saturated (at 0 °C) with respect to ammonium sulphate, corresponding to 16.4% (w/v), for selective precipitation of macromolecules of higher molecular size than RuBisCO. The mixture was gentle stirred for 20 min whereupon was again clarified by centrifugation at 22,100 × *g* for 20 min. The resulting supernatant was further treated with ammonium sulphate to collect the material precipitating between 35 and 55% saturation, corresponding to 16.4–25.7% (w/v). After 20 min, the suspension was again centrifuged at 22,100 × *g* for 20 min. The pellet containing RuBisCO was resuspended in 10 mL of column buffer (pH 8.0) containing 1 mmol/L each of PMSF, benzamidine and ε-amino acid-n-caproic. Removal of insoluble material was accomplished by centrifugation at 48,400 × *g* for 20 min, to afford a supernatant solution ready for injection into the MSFI-chromatographic setup.

2.4. Automatic flow setup

A multisyringe piston pump with programmable speed (MSP, MicroBu 2030, Crison Instruments, Alella, Barcelona, Spain) was used as a liquid driver for automation of the anion-exchange column separation method. It was equipped with four high-precision bidirectional syringes (Hamilton, Bonaduz, Switzerland), S1–S4, with a capacity of 2.5 mL each and connected in block to a 16,000-step motor. A schematic diagram of the MSFI setup is illustrated in Fig. 2. S1, S2, S3 and S4 contained column buffer, 0.1 mol/L NaCl; 0.17 mol/L NaCl; and 0.8 mol/L NaCl, respectively, for stepwise ionic-strength gradient elution. A three-way solenoid valve (N-Research, Caldwell, NJ) was placed at the head of each syringe enabling automatic connection with either the liquid reservoirs (OFF) or the flow network (ON). The MSP module was coupled to an 8-port multiposition selection valve (MPV, Crison) operating as sample-processing unit. This valve encompasses a central port and a communication channel (CC) that can be programmed to address each of the peripheral ports. It was connected via a 2.0 mL-holding coil (HC, 1.5 mm i.d. PTFE tubing) to S1 for fluidic handling of sample and solutions for column rinsing and regeneration. The flow manifold was constructed from PTFE tubing (0.5 mm i.d.) using poly(vinyl chloride) (PVC) or poly(methyl methacrylate) (PMMA) fittings.

A PMMA cylindrical column (105 × 4 mm) packed with 194 ± 4 mg Q-Sepharose Fast Flow (void volume of 300 μL) was exploited for in-line anion-exchange separation of RuBisCO. Glass wool was used for bead trapping in lieu of porous frits because of decrease of pressure drop. The outlet of the column was coupled via a 12-cm long PTFE tubing (0.5 mm i.d.) to a flow-through diode-array spectrophotometer (Hewlett-Packard HP8452A, Vancouver, WA, USA) equipped with a quartz cell (18 μL, 10 mm path length) for real-time monitoring of column effluent and recording of the fiagram at the protein selective wavelength of 280 nm using 320 nm as a reference wavelength. Peak area was used as analytical signal for quantitation of eluted RuBisCO. Automated collection of protein containing fractions was accomplished by resorting to a 45-position rack fraction collector (FC, Crison). The entire flow setup (namely, MSP, MSV, column and FC) was refrigerated at 4 °C (Marecos Olitrem refrigerator, Tremês, Portugal) for undertaking RuBisCO purification at 4 °C.

Instrumental control, data acquisition, and processing of the chromatographic readouts were accomplished by means of the software package AutoAnalysis 5.0 (Sciware, Palma de Mallorca, Spain) [36]. The software based on dynamic link libraries (DLLs) is composed of a main protocol wherein the DLLs of MSP, MPV, FC and detector are in this work attached.

2.5. Analytical procedure

The MSFI-based anion-exchange separation procedure was utilized for further purification of the extracts resulting from the preliminary sample processing protocols detailed above. The entire procedure was monitored at real-time by in-line recording of spectra within the wavelength range of 190–500 nm and the fiagram at

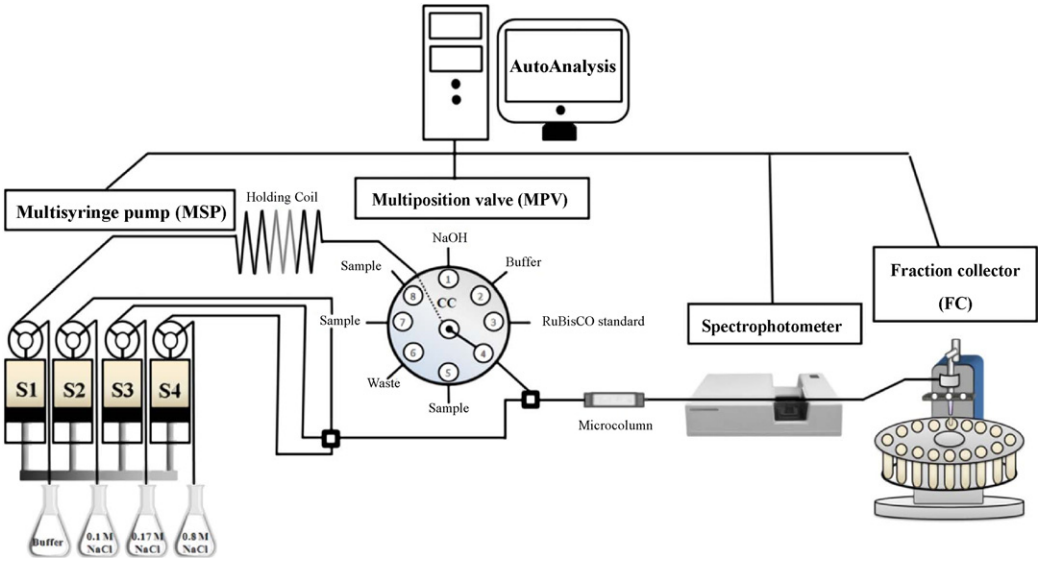

Fig. 2. Schematic illustration of the miniaturised flow-based anion-exchange separation system for automatic purification of RuBisCO in plant extracts. CC: communication channel; S: Syringe; Saline solutions: 0.1 M NaCl, 0.17 M NaCl, and 0.8 M NaCl.

280 nm against 320 nm all at 1 Hz. The overall analytical sequence encompassing the volumes and flow rates of handled solutions and positions of MPV and FC is compiled in Table 1 (shown for PEG and sucrose gradient procedures) and summarized as follows:

The anion exchanger was first conditioned by dispensing 2.5 mL of column buffer at 1.0 mL/min, followed by perfusing the column with 500 μL of RuBisCO standard or plant extract containing ca. 1000 μg of soluble protein as determined by Bradford's method (see below) previously loaded into the holding coil. Removal of non-retained matrix ingredients or components of the extraction buffer was accomplished by flushing the anion exchanger with 5.0 mL of column buffer at 1.0 mL/min. The stepwise gradient elution protocol of increasing ionic strength involves two preliminary steps, namely, the delivery of 2.0 mL of 0.1 mol/L NaCl followed by 2.0 mL of 0.17 mol/L NaCl both at 1.0 mL/min, in order to wash away weakly retained pigments and proteins prior to RuBisCO elution at 0.4 mol/L NaCl by concurrent activation of S1 and S4. As soon as the RuBisCO peak was in-line detected (a volume of 1250 μL of eluent is needed) the MSP and FC are synchronized to collect 4 individual fractions of 350 μL eluate each for further exploration of RuBisCO content and retrieval of highly purified protein fraction(s). The eluted column fractions were stored at −80 °C after quick freezing in liquid nitrogen. To eliminate potential interfering species that might become strongly trapped onto the beads, two final gradient steps were programmed involving the subsequent perfusion of 3.5 mL of 0.49 mol/L NaCl (simultaneous activation of S3 and S4) and 750 μL of 0.8 mol/L NaCl through the column followed by conditioning of the anion exchanger with 5.0 mL of column buffer prior to next chromatographic run. Each extract was assayed in triplicate.

In the analytical protocol for purification of plant extracts following sulphate ammonium precipitation, the anion-exchanger was rinsed with 5 mL of 1 mol/L NaOH prior to the next assay aimed to wash away precipitated or denatured proteins accumulated onto the beads. This regeneration protocol was repeated 2-fold when handling crude extracts. Remains of sodium hydroxide were removed by flushing the column with 25-fold column void volume of column buffer and monitoring the conductivity of the effluent until matching that of the buffer solution.

Table 1
Analytical procedure for automated MSFI-anion exchange separation of RuBisCO from plant extracts.[a]

Step	Instrumentation	Operation	Flow rate (mL min^{-1})	S1	S2	S3	S4
	FC	Move to position 1 (waste)					
	MPV	Move to port 4					
Conditioning of anion-exchanger	MSP	Dispense 2.5 mL	1.0	On	Off	Off	Off
	MPV	Move to port 8					
Loading of sample	MSP	Aspirate 500 μL	1.0	On	Off	Off	Off
Data acquisition	Detector	Get spectral range from 190 to 500 nm at 1 Hz					
Start gradient	MPV	Move to port 4					
	MSP	Dispense 5.0 mL	1.0	On	Off	Off	Off
	MSP	Dispense 2.0 mL	1.0	Off	On	Off	Off
	MSP	Dispense 2.0 mL	1.0	Off	Off	On	Off
	MSP	Dispense 1.25 mL	1.0	On	Off	Off	On
		Start loop					
	FC	Move to next position (collector)					
Collection of RuBisCO	MSP	Dispense 350 μL	1.0	On	Off	Off	On
		End loop: repeat 4 times					
	FC	Move to position 1 (waste)					
	MSP	Dispense 2.35 mL	1.0	On	Off	Off	On
	MSP	Dispense 3.50 mL	1.0	Off	Off	On	On
	MSP	Dispense 750 mL	1.0	Off	Off	Off	On
	Detector	Stop measurement					

MSP, multisyringe pump; MPV, multiposition valve, FC, fraction collector.
Additional steps for refilling of syringes are not shown in the table.
[a] Used in combination with PEG and PEG + sucrose gradient pre-treatment protocols.

2.6. Determination of total soluble proteins

The Bradford protein assay is the most currently used spectroscopic analytical procedure for measurement of the concentration of soluble proteins [37]. This method is based on the absorbance shift observed in an acidic solution of Coomassie Brillant Blue G-250 when added to a solution of proteins. The chromogenic reagent interacts primarily with arginine residues, but also binds to a lesser degree to hystidine, lysine, tyrosine, tryptophan and phenylalanine [38]. The dye has been assumed to bind to proteins via electrostatic interactions between dye's sulphonic groups and cationic moieties in proteins. The absorbance peak of the acidic dye solution shifts from 465 to 595 nm when binding to protein occurs. Therefore, measuring the absorbance of the protein–dye complex at 595 nm fosters quantitation of the protein content. This assay is very reproducible and rapid with the dye binding process virtually complete in 4 min.

Quantitation of total soluble protein using Bradford's method is usually effected via external calibration against standards of bovine serum albumin (BSA) [24,39]. Yet, in our study, we have evaluated external calibration against BSA and RuBisCO standards to ascertain the reliability of the conventional procedure. A six-level calibration plot based on least-squares linear regression was established within the range of 0–10 μg RuBisCO or BSA using 500 μL of commercially available Coomassie Brillant Blue G-250 (Sigma–Aldrich, Steinheim, Germany) in a final volume of 1000 μL for subsequent quantitation of total protein content in plant extracts and eluates of the automated anion-exchange separation method. Absorbance readouts of protein–dye associates under steady-state were recorded at 595 nm by resorting to a Beckman DU-730 UV/vis spectrophotometer.

2.7. Polyacrylamide gel electrophoresis

Quantitation of RuBisCO in the extracts and eluates was performed using SDS-PAGE. The samples were appropriately diluted with 0.5 mol/L Tris (basic form) + HCl (pH 6.8) buffer containing 5% (w/v) sodium dodecylsulphate (SDS), 1% (v/v) bromophenol blue used as a marker dye, 5% (v/v) 2-mercaptoethanol for protein denaturation and 10% (w/v) sucrose. The sample preparations were loaded onto slab gels (10 mm × 8.2 mm × 0.75 mm) containing 5% (w/v) polyacrylamide stacking gel in 0.5 mol/L Tris buffer at pH 6.8 with a 12.5% (w/v) polyacrylamide resolving gel prepared in 3 mol/L Tris at pH 8.8. Samples were electrophoresed at 200 V for 1 h using 0.012 mol/L Tris in glycine buffer (pH 8.3) containing 0.1% (w/v) SDS. Different amounts of RuBisCO standard ranging from 75 to 675 ng were loaded on five lanes serving for external calibration purposes. Proteins in the gel were fixed by 33% (v/v) concentrated acetic acid in methanol for 1 h (first 15 min at 65 °C) followed by staining in Coomassie Brilliant Blue R-250 (EZBlue™ Gel Staining, Sigma, Steinheim, Germany). Gels were destained with distilled water for 1 h and then scanned using image analysis (Odyssey infrared imaging system, Li-COR Biotechnology, Lincoln, NE, US). Identification and quantitation of RuBisCO was performed by densitometric analysis of the gel on the basis of the enzyme large subunit with the aid of the TotalLab software (Newcastle upon Tyne, UK).

3. Results and discussion

3.1. Sorbent material and column dimensions

This work is aimed at high-throughput retrieval of highly purified RuBisCO in plant extracts. To expedite conventional anion exchange chromatographic methods using large-sized columns (e.g., 880 × 4 mm) packed with Q-Sepharose [7] and lasting as long as 16 h, downscaling of the separation procedure in a flow-based format was explored using small sized packed columns (namely, 50 × 4 mm or 105 × 4 mm). The former was inappropriate because of limited sorbent bed capacity for RuBisCO uptake in harsh matrices (e.g., crude extracts). On the other hand, the latter was proven suitable for in-line processing of up to ca 1000 μg RuBisCO in less than 1 h without analyte breakthrough nor column saturation, and thus selected for the remainder of the work.

A survey of the literature revealed that anion exchangers bearing poly(styrenedivinylbenzene) (PS-DVB) [40] or polysaccharide-based [7,8] matrixes have been so far exploited for batchwise separation of RuBisCO, proteins or organic anions. Preliminary investigations resorting to AG 1-X8 (PS-DVB; Bio-Rad Laboratories) for in-line separations revealed a poor distribution constant for RuBisCO, which coeluted in the void volume. This is most likely a consequence of the slow transfer kinetics of the target protein from aqueous media onto the anion exchanger of hydrophobic polymer matrix. On the contrary, narrow peak widths for RuBisCO were attained at increasing ionic strength of the mobile phase whenever AG 1-X8 was replaced by Q-Sepharose Fast Flow. This observation is in good agreement with recent literature on RuBisCO purification by resorting to anion-exchange chromatography, where Q-Sepharose type exchangers are almost exclusively employed [7,8,25]. A matrix of cross-linked agarose features improved hydrodynamic conditions for fast anion exchange and negligible swelling/shrinking upon changing of the ionic strength of perfused solutions.

3.2. Gradient elution

A survey of the literature has revealed that RuBisCO is retrieved from anion-exchangers at electrolyte concentrations ≥0.3 mol/L NaCl [16,24] in column buffer. Preliminary studies were conducted to determine the minimum concentration of salt for quantitative elution of the target protein preceded by removal of weakly retained proteins, pigments and other matrix ingredients. Two stepwise gradient elution protocols involving automatic column perfusion of NaCl concentrations of 0.10 mol/L, 0.23 mol/L, 0.35 mol/L, 0.45 mol/L and 0.80 mol/L; or 0.10 mol/L, 0.30 mol/L, 0.40 mol/L, 0.60 mol/L and 0.80 mol/L, by synchronous activation of solenoid valves of MSP, were initially explored. In either case, experimental results revealed lack of quantitative elution of RuBisCO along with broad asymmetric peaks at 0.30–0.35 mol/L NaCl. In fact, as much as 21% (w/w) of injected RuBisCO was eluted in the subsequent step of 0.4 mol/L NaCl in the latter gradient procedure. Aimed to concentrate the target protein in a small number of highly purified eluate fractions, a 5-step ionic-strength gradient elution affording sharp RuBisCO peaks at 0.4 mol/L NaCl and involving increasing concentrations of NaCl in the order of 0.1 mol/L, 0.17 mol/L, 0.40 mol/L, 0.49 mol/L and 0.80 mol/L was selected for the remainder of the work.

The effect of column perfusion flow rate on the separation efficiency of RuBisCO was investigated within the range of 0.5–2.0 mL/min for 500 μL of 1.6 mg/mL RuBisCO standard. Undue pressure drop was observed at nominal flow rates ≥1.5 mL/min with the consequent pre-elution of the target protein well below 0.4 mol/L NaCl. Hence, we do suggest the use of a perfusate flow rate of 1.0 mL/min within the overall column separation procedure for reproducible elution profiles of RuBisCO without appreciable build-up of flow backpressure.

3.3. Analytical figures of merit

Analytical performance of the flow-through anion-exchange separation method was explored on the basis of column capac-

ity under dynamic conditions and potential analyte breakthrough by frontal analysis. To this end, increasing volumes of 1.6 mg/mL RuBisCO standard were loaded onto the packed anion-exchanger and peak area of the chromatogram recorded. A linear correlation of peak area against mass of RuBisCO (Abs = 0.06 × [RuBisCO (μg)] − 11.03, R^2 = 0.9934, p = 0.051 at the 95% confidence level) with repeatability < 6% (n = 8) within the overall dynamic range of 348–930 μg RuBisCO was encountered. For real-life sample assays, the soluble protein content as determined by Bradford's method should not exceed 1000 μg per run to prevent RuBisCO pre-elution in the early stages of the ionic-strength gradient.

A common practice in the literature to determine total soluble protein via Bradford's method involves the use of least-squares linear regression plots of absorbance against BSA standards [24,39]. Given the different aminoacid composition among vegetal and animal proteins, the potential bias of the analytical method should be estimated. Hence, mass calibration plots within the range of 2–10 μg protein were constructed using commercially available BSA and powdered, highly purified RuBisCO [7]. Estimated concentrations of RuBisCO standards on a weight basis were corroborated by resorting to absorbance measurements at 280 nm [39]. Significant differences at the 95% probability level were found between the sensitivities of both methods [41]. An improvement of ca. 25% in sensitivity of BSA-based calibration was actually detected, which in turn would give rise to underestimation of RuBisCO concentrations. To cope with this observation yet using inexpensive BSA for external calibration, a correlation was established between analytical signals (Abs) obtained in the analysis of a given number of pairs of BSA and RuBisCO standards (n = 9) of identical concentration. The resulting linear correlation ($Abs_{RuBisCO} = 0.761 \times Abs_{BSA}$; R^2 = 0.9930, p = 0.35 at the 95% confidence level) fosters unbiased calculation of RuBisCO concentrations on the basis of BSA regression plots.

3.4. Critical assessment of sample processing methods for analysis of plant extracts

The role of common batchwise sample pre-treatment procedures involving PEG or ammonium sulphate fractionation, or sucrose density gradient prior to in-line MSFI anion-exchange isolation of RuBisCO on the purification yields (P) and absolute recoveries (R) has been ascertained using *T. aestivum* extracts. Both terms are defined as following:

$$P = \frac{\text{mg RuBisCO}}{\text{mg soluble protein}} \times 100 \quad (1)$$

$$R = \frac{(\text{mg RuBisCO})_{\text{step}}}{(\text{mg RuBisCO})_{\text{crude}}} \times 100 \quad (2)$$

wherein P is calculated in each single purification step on the basis of SDS-PAGE results for quantitation of RuBisCO and Bradford's method for estimation of the soluble protein content, and R is calculated as the amount of RuBisCO in each step as compared to the initial concentration of RuBisCO in the crude extract both on the basis of SDS-PAGE measurements.

The use of PEG precipitation followed by MSFI-anion exchange separation (see Tables 2 and 3) afforded an increase of RuBisCO purity from 58 ± 3% up to 72 ± 5% with recoveries of ca. 30%. A sharp RuBisCO peak was recorded at 0.4 mol/L NaCl with resolution ≥1.5 as compared with interfering species (see Fig. 3C). Absence of RuBisCO in late-eluting fractions was corroborated using both real-time spectrum monitoring and SDS-PAGE of collected fractions. The overall analytical procedure lasted 3 h but embracing a chromatographic run of merely 35 min. A relevant asset of this protocol is the simple rejuvenation of the anion-exchanger using 5 mL-column buffer and reuse for ≥12 assays with repeatability ≤10% without additional rinsing steps with sodium hydroxide.

Table 2
Purity yields and absolute recoveries of RuBisCO from *T. aestivum* as obtained from preliminary batchwise sample processing procedures.[a]

STEP	Protein (mg)[b]	RuBisCO (mg)[c]	Purity yield (%)	Absolute recovery (%)[d]
Crude	2524 ± 37	1039 ± 65	41 ± 3	100 ± 6
Ammonium sulphate	395 ± 29	211 ± 25	54 ± 2	20 ± 2
PEG	893 ± 50	516 ± 44	58 ± 3	50 ± 4
PEG + sucrose gradient	396 ± 42	304 ± 25	77 ± 12	29 ± 2

[a] Results are expressed as the mean of three replicates of the analytical process ± standard deviation.
[b] Determined using Bradford's method.
[c] Determined using SDS-PAGE.
[d] Refer to RuBisCO in crude extracts.

Experimental results compiled in Tables 2 and 3 and Fig. 3D revealed that there is a significant improvement in purity yield up to 96 ± 9% when performing an additional purification step with sucrose gradient preceded by PEG precipitation. Yet, absolute recoveries dropped down to 15% and overall running times exceeded 17 h whenever using PEG and sucrose gradient procedures in series.

Chromatograms recorded in the analysis of leaf extracts following fractionation with ammonium sulphate (see Fig. 3B) revealed that a large number of concomitant interfering species remain in the sample medium, yet the experimental MSFI protocol described above is proven suitable for retrieval of RuBisCO fractions with purification yields ≥80%. Absolute recoveries were however down to 10% (see Table 3) as compared to 29 ± 2% with PEG as a consequence of two-fold sample centrifugation. Increasing of volumes of column buffer and 0.1 mol/L NaCl to 7.5 mL and 5.0 mL, respectively, was proven imperative for appropriate isolation of RuBisCO peak. Regeneration of the anion-exchanger for column reuse for ≥12 cycle assays with repeatability ≤10% was herein effected with 5 mL of 1.0 mol/L NaOH as described under Section 2.

Similar experimental procedure was utilized for RuBisCO purification in crude extracts with slight increase in duration of the initial gradient steps (7.5 mL of 0.1 mol/L NaCl followed by 5.0 mL of 0.17 mol/L NaCl) for efficient removal of concomitant interfering proteins and pigments and recovery of baseline (see Fig. 3A). Acceptable purification yields of the target protein (77 ± 1%) were obtained with markedly short overall analytical times (<2 h compared to 16 h in batchwise purification [7]), which make the proposed flow-setup suitable for high-throughput assays of RuBisCO. Absolute recoveries were estimated as 35 ± 2% (see Table 3) because of RuBisCO peak delimitation to highly purified fractions. A two-fold sodium hydroxide-based regeneration protocol (see above) was in this case needed for a column lifetime up to 10 injections of crude extract with repeatability ≤5%.

The efficiency of the varied purification procedures in terms of intensity and number of bands for isolation of RuBisCO from *T.*

Table 3
Purity yields and absolute recoveries of RuBisCO from *T. aestivum* as obtained from in-line MSFI-anion exchange separation preceded by various batchwise sample processing procedures.[a]

STEP	Purity yield (%)	Absolute recovery (%)[b]
Crude	77 ± 1	35 ± 2
Ammonium sulphate	84 ± 5	10 ± 1
PEG	72 ± 5	29 ± 2
PEG + sucrose gradient	96 ± 9	15 ± 1

[a] Results are expressed as the mean of three replicates of the analytical process ± standard deviation (3 injections each).
[b] Refer to RuBisCO in crude extracts.

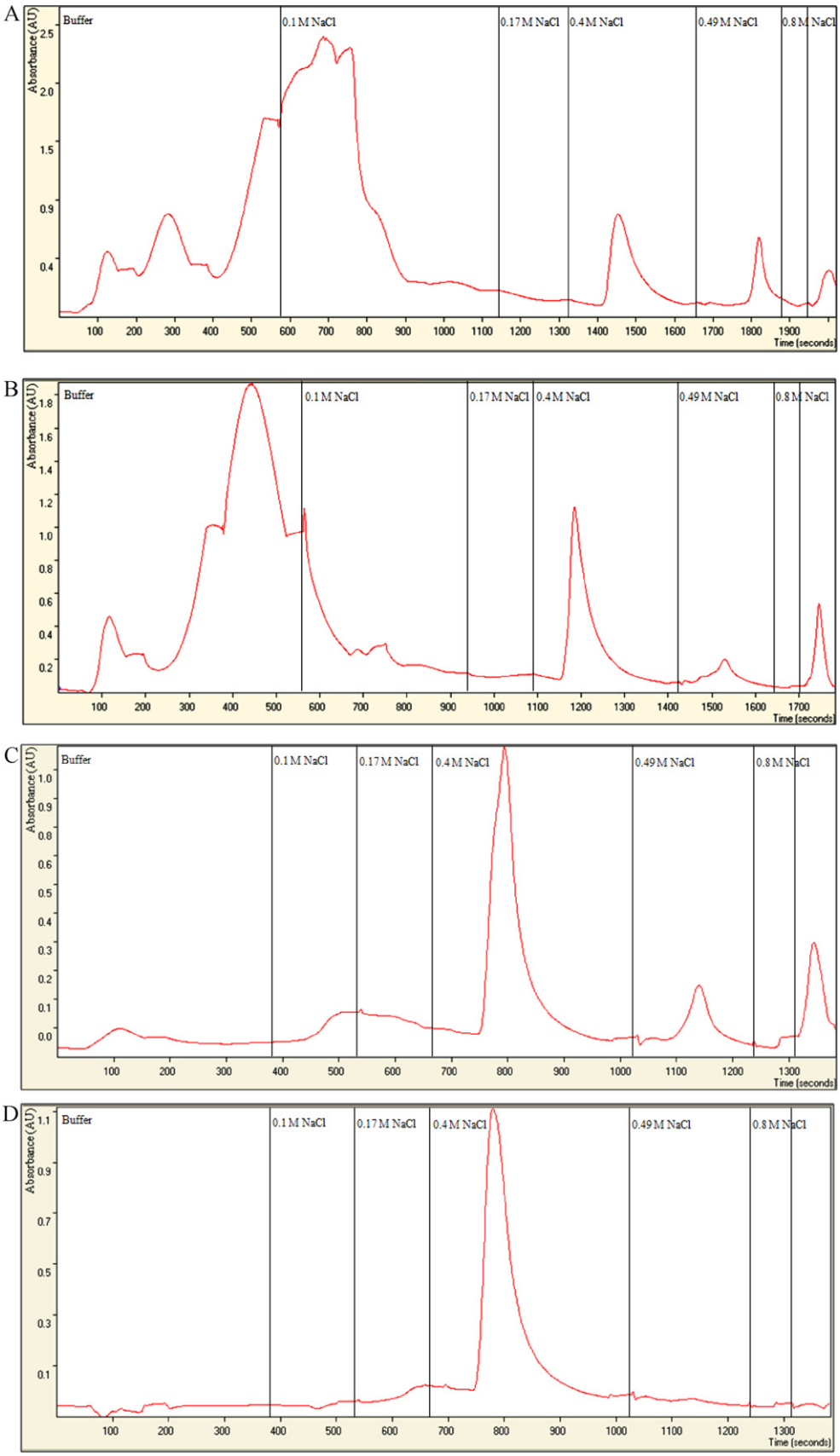

Fig. 3. (A) Close-up of the chromatogram of *Triticum aestivum* as obtained by direct injection of crude extract into the MSFI-anion exchange manifold. (B) Close-up of the chromatogram of *Triticum aestivum* extract as obtained by MSFI-anion exchange preceded by precipitation with ammonium sulphate. (C) Close-up of the chromatogram of *Triticum aestivum* extract as obtained by MSFI-anion exchange preceded by precipitation with PEG. (D) Close-up of the chromatogram of *Triticum aestivum* extract as obtained by MSFI-anion exchange preceded by precipitation with PEG and sucrose gradient separation. Injected samples contained ca. 1000 mg of soluble protein as determined by Bradford's method. RuBisCO peak eluted at 0.4 mol/L NaCl.

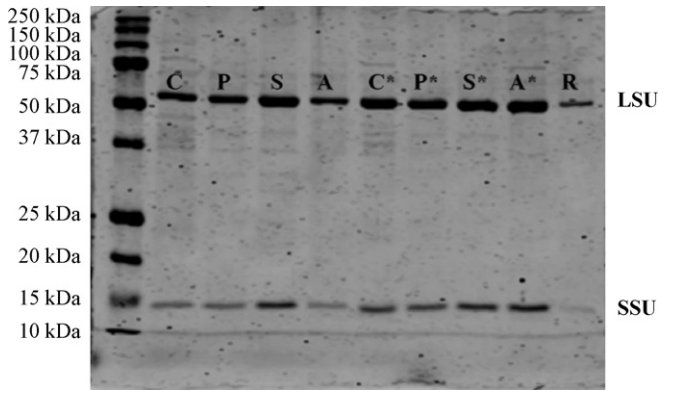

Fig. 4. SDS-PAGE image of different steps of purification of RuBisCO from *Triticum aestivum*. Molecular weight standards (kDa) used as marker (Precision Plus Protein Standards, Bio-Rad) are given on the left. LSU and SSU represent RuBisCO large and small subunits, respectively. Lanes C and C* were loaded with crude extract and crude extract after MSFI-anion exchange separation, respectively. Lanes P and P* were loaded with extracts obtained from PEG precipitation, and PEG precipitation followed by MSFI-anion exchange separation, respectively. Lanes S and S* were loaded with extracts obtained from sucrose gradient, and sucrose gradient followed by MSFI-anion exchange separation, respectively. Lanes A and A* were loaded with extracts obtained from ammonium sulphate precipitation, and ammonium sulphate precipitation followed by MSFI-anion exchange separation, respectively. Lane R was loaded with RuBisCO standard. Electrophoresis was carried out as described under Section 2. Soluble protein in the extracts ranged from 600 to 700 ng.

aestivum is illustrated in the close up of SDS-PAGE gel in Fig. 4. The high molecular weight bands revealed by the gel above large subunits of RuBisCO (LSU) are most likely LSU-binding proteins [42] eluting at the chromatograms (see Fig. 3A–C) at ≥0.49 mol/L NaCl.

4. Conclusion

An automated and versatile multisyringe-flow anion-exchange separation assembly is herein proposed for expeditious isolation and real-time monitoring of RuBisCO in plant extracts with purification yields within the range of 72–96%. Handling of crude extracts as well as pre-treated samples following PEG and ammonium sulphate precipitation is proven feasible without manifold reconfiguration by optimization of the stepwise ionic-strength gradient elution and regeneration protocols. As compared with robotic stations for protein purification dedicated to routine separations (e.g., Äktapurifier, GE Healthcare) the MSFI-set up does not merely offer a more open architecture and flexibility for method development relying upon flow programming but should be regarded as a cost-effective technique with 5-fold reduced equipment costs.

The approach presented in this work represents significant progress towards the development of a rugged and automatic flow-based manifold for high-throughput purification of RuBisCO as essential tool for further exploration of the variability of kinetic traits of RuBisCO in crops and identification of more enzymatically efficient forms to be exploited for genetic transformations of crop plants to increase productivity.

Current efforts in the authors' lab are focused on method scaling up using tandem-column anion-exchange separation, and orthogonal anion-exchange × size-exclusion chromatography for preparative separations emcompassing in-line desalting of eluate fractions.

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