CHEMICAL ENGINEERING TRANSACTIONS
VOL. 65, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Eliseo Ranzi, Mario Costa

ISBN 978-88-95608- 62-4; **ISSN** 2283-9216

Bio-Layer Interferometry Method for Quantification of Rubisco in Plant Extracts

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Biolayer interferometry (BLI) with biosensors has been tested for measuring the concentration of a protein in different media. Interferometry is based on the interference model of the light reflected by a surface. When the surface of an optical fibre is modified by a bounded compound, interference model changes as a function of the increase of the path length. This technique of protein quantification represents an alternative to the classical methods such as electrophoresis, spectrophotometry, immune-enzymatic tests (ELISA) or HPLC. The main advantages of this method are its specificity, its sensitivity and the promptness of the analysis. In this work, a measuring method for Ribulose-1,5-bisphosphate Carboxylase Oxydase (Rubisco) from different media has been developed. The most important conclusion is that Rubisco can be efficiently determined by this technique. Moreover, experimental results have shown the strong influence of pH on the measurements.

1. Introduction

The use of biomass for production of energy and chemicals is the object of a lot of studies as an answer to the problem of global warming. Rubisco is the most abundant vegetal protein in the world, because it is present in leafs of all the green plants. As an enzyme, Rubisco plays a fundamental role during photosynthesis because it allows the fixation of CO_2 in green biomass during the Calvin cycle. Since its reaction rate is very slow, its behaviour has been the object of numerous studies. Investigations have been conducted on subjects such as Rubisco molecular structure, functionality and stability, carboxylation reaction kinetics, molecular engineering of Rubisco, variability of Rubisco concentrations in the plant according to the growth cycles (Feller et al., 2008). Applications in biotechnologies are also expected in order to develop, through genetic modification, micro-organisms that could control CO_2 emissions, for example during fermentation (Guadalupe-Medina et al. 2013). Recent studies have also pointed out the industrial interest for its functional properties (van de Velde et al., 2011), while allergists are using this protein as a model of non-allergen vegetal protein. The development of a rapid, sensitive and selective method for Rubisco quantification, especially in real complex media, is therefore a challenge.

Different classical methods were used for Rubisco quantifying in synthetic media. One of the simplest and oldest methods used was the spectrophotometric analysis at 280 nm in a well-adapted medium (Lilley and Walker, 1974). This method was also used by Lan and Mott (1991) for measuring highly purified activated and non-activated Rubisco and Rubisco activase. Calibration was done by comparing the obtained values with the maximal enzymatic activity. Nevertheless, considering that enzymatic activity depends on environmental conditions during sampling, the enzyme-linked immunosorbent assay (ELISA) method was developed and used for measuring Rubisco content in leaf extracts of potato plants (Catt and Millard, 1988) and in different varieties of barley (Metodiev and Demirevska-Kepova, 1992). Based on the selective interaction between Rubisco antibody and the protein, this method can be considered to be specific and accurate enough for the determination of Rubisco and was successfully used in plant extracts.

Most recently, Kerfai et al. (2011a, 2011b) have developed a size-exclusion HPLC method for the identification and quantification of Rubisco in plant extracts, with an UV-Vis detector measuring at 280 nm. This method allows the determination of the protein in different complex media, such as the green juices. However, the time of analysis is long (about 50 min) when real media are analysed because a perfect cleaning of the column is necessary. In a study devoted to the Rubisco extraction and purification from plants by ion

exchange chromatography at laboratory scale, Rubisco analysis was carried out by electrophoresis using a polyacrylamide gel (PAGE) (Suarez et al, 2011). Gels were then studied by densitometric analysis and image analysis for Rubisco determination on the basis of the enzyme large subunit.
Biolayer interferometry is an analytical technique based on the measurement of the interferences of two light beams. The interference is generated by two different distances to be crossed by the beams in a biosensor with an antibody immobilised at the tip (Pall Fortebio, 2013). When immersed in a solution containing the antigen (protein) the bound antigen–antibody will promote an increase of the biosensor thickness at the tip and will modify the interferometric pattern (Sultana and Lee, 2015). Thus, it is possible to correlate the number of immobilized molecules (concentration) with a shift in the interferometric profile, the response being obtained in real time (Delis, 2016). Figure 1 schematises the basis of the technique. In addition to the quickness of the analysis (approximately 3 minutes per measurement), this technique has the advantage of being applicable in complex media because of the specific binding reaction. Regardless of this specificity, the matrix of the calibration solutions must correspond to that of the sample containing the analyte because of sensibility of proteins to their chemical environment.
The aim of this work is to apply this principle in order to develop a selective quantitative method to determine Rubisco concentrations in different media. Rubisco solutions were prepared from pure Rubisco powder, refined from alfalfa green juice and purified by size-exclusion chromatography (Sephacriyl S-300, GE Healthcare). To verify the influence of media, four different matrices were used namely at different pH values.

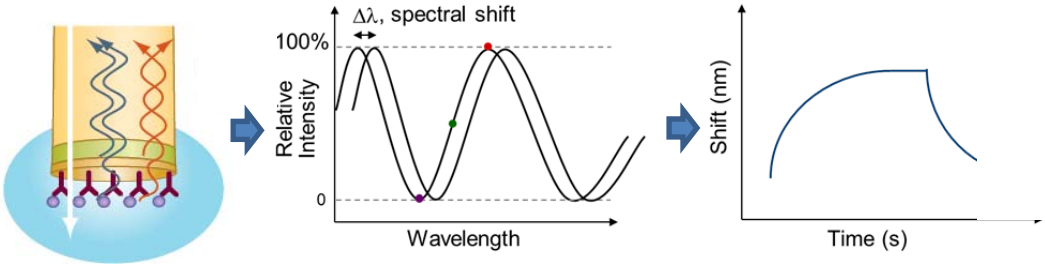

Figure 1: Global presentation of biolayer interferometry technique. Adapted from, https://www.fortebio.com/bli-technology.html, accessed 12.15.2017 and from (Dayne, 2012).

2. Materials and methods

2.1 Materials

Manual BioLayer Interferometry system (BLItz), High Precision Streptavidin (SAX) BLI biosensors and Drop holder from ForteBio/Pall Corp. (Menlo Park, USA) were used. Black 96-well non-binding microplate (Greiner Bio-One, France) and 0.5 ml black microcentrifuge tubes (Argos Technologies, USA) were also used.

2.2 Proteins and compounds

Different monoclonal and polyclonal Rubisco antibodies from different sources were tested (results not shown). Rabbit polyclonal antibody anti-RbcL / Rubisco large subunit, forms I and II, from Agrisera (Vännas, Sweden) was selected. EZ-Link NHS-PEG4-Biotin and Zeba Spin Desalting Columns were purchased from Thermo Scientific (Villebon sur Yvette, France). Hydrogen disodium phosphate (> 99%) and dihydrogen sodium phosphate monohydrate (> 99 %) were provided by Carl Roth (Karlsruhe, Germany). Kinetics Buffer 10x were purchased from ForteBio/Pall Corp. (Menlo Park, USA). Sucrose for microbiology (≥99 %) and Glycine (≥99 %) from Sigma-Aldrich/Merck (Darmstadt, Germany). All solutions, except 15% sucrose in Kinetics Buffer, were prepared with deionized Milli-Q water. Rubisco samples were extracted from alfalfa (Medicago Sativa) juices, purified by gel chromatography in a phosphate buffer at pH 7.4, and atomized by spray drying. Dry solid contains 63 % of Rubisco and 37 % of salts (Na_2HPO_4/NaH_2PO_4).

2.3 Biosensors preparation

Biosensor preparation is essential in order to ensure analysis precision and repeatability (Maragos, 2011). Batch preparation is recommended for industrial applications. Each batch must be characterized by a calibration curve. In this work, each biosensor has been prepared under controlled and monitored conditions in order to efficiently control all phases of the analytical protocol. Biosensor preparation consists in two steps (Delis, 2016): (1) biotinylation of the antibody and (2) biotin-antibody fixation onto SAX biosensors.
(1) The antibody anti-RbcL was reconstituted in 50 µL of deionized water to prepare a 1 µg/µL solution. Immediately before use, NHS-PEG4-Biotin (2 g) was diluted to 2 mM. 2 µL NHS-PEG4-Biotin solution were added to anti-RbcL solution and the reaction was incubated at room temperature for 30 min or on ice for 2 h.

NHS-PEG4-Biotin non-used was discarded. The non-reacted NHS-PEG4-Biotin was removed by gel filtration with Zeba Spin Desalting Columns, 2 mL. Biotinylated antibody anti-RbcL was stored at -20 °C.
(2) To load biotinylated antibody onto SAX biosensors, three steps are necessary (Table 1). Previously, they must be hydrated in Pall Kinetics Buffer for 10 min. To follow the preparation, experimental conditions for each step need to be indicated in Kinetics advanced mode of the BLITZ software, by creating the assay method.
After use, biosensors can be regenerated for reuse. For this, the sensor tips are incubated in glycine 10 mmol/L pH 1 for 10 s, followed by a washing step in kinetics buffer solution. This procedure is repeated 5 times. In most applications, biosensors can be usually regenerated 3 times.

2.4 Quantitation of Rubisco by bio-layer interferometry

Rubisco's powder at 63 % weight has been used to prepare a mother solution at 10 g Rubisco/L and 42.8 mM phosphate in deionized water. For the validation of the analytical method and in order to generate calibration curves, standard solutions of Rubisco were prepared from 0.5 to 2 g Rubisco/L by dilution of aliquots in four different PBS buffer solutions. Calibration curves have been determined by using Create Standard Curve module in Blitz Pro software. For each standard, 4 µL of Rubisco solution at the required concentration and buffer were placed into Blitz system using a drop-holder. A pre-hydrated biosensor was loaded into the equipment and the assay was run for 120 seconds with shaker enabled. As Blitz Pro software does not accept zero as a standard, measurements in buffer solutions have been done for each calibration curve considering Rubisco concentration as negligible (10^{-12} g/L).
All experimental data have been measured at least three times in order to establish the method's repeatability. First, experimental protocol was evaluated by using standard solutions of 1 g/L. The effect of biosensor regeneration has been validated by using: (a) new biosensors or biosensors regenerated until 3 times, or (b) biosensors regenerated at least four times. Then, effects inherent to proteins, such as protein dissolution or protein stability, have been analysed by considering the time between the preparation of the solutions and the measurements. The effect of buffer solution, especially pH, has been also considered. Tables 2 and 3 summarize all the experimental conditions investigated. Pall PBS and PBS1 buffer solutions have the same pH but different compositions, namely different ionic strength. In all the cases, prior to their use, biosensors were always hydrated in the corresponding buffer solution for at least 10 minutes. To determine Rubisco concentration in unknown samples, Quantitate Sample module in Blitz Pro software was used. In the same way as for standard solutions, 4 µL of sample were placed into Blitz System and the assay was run for 120 s.
All the tests have been carried out at controlled temperature (20°C).

*Table 1: SAX biosensor on-line immobilization protocol (*values determined after optimization of the method).*

Assay Method Experiment Creation				Additional Information	
Step	Step Type	Duration (s)	Position	Sample solution	View volume (µL)
1	Initial Baseline	60	Tube	Pall PBS	350
2	Loading	180*	Drop	Biotin-Antibody 25* µg/ml	4
3	Baseline	60	Tube	Pall PBS	350

Table 2: Experimental conditions for analytical method validation.

Standard solution (g/L)	Time from standards preparation to analysis	Replicates for each standard
0.5	2 h and 4 h	3
1	30 min, 2 h and 4 h	4 (+1 for reuse evaluation)
2	2 h and 4 h	3

Table 3: Buffer solutions used. For each buffer solution, a specific calibration curve has been established.

pH	PBS buffer solutions	Identified in this work as
7.40	Pall PBS buffer*	Pall PBS buffer
7.44	81 mM Na_2HPO_4/19 mM NaH_2PO_4	PBS 1
6.80	50 mM Na_2HPO_4/50 mM NaH_2PO_4	PBS 2
6.20	19 mM Na_2HPO_4/81 mM NaH_2PO_4	PBS 3

* Kinetics Buffer 10X concentration (ProClin®300, 0.1 mol/L phosphate, 1.5 mol/L NaCl, pH 7.4)

3. Results and discussion

During analysis, the experimental results show the evolution of interferometric-profile shifts (in nanometres) with time, as represented in the example of figure 2. Moreover, on streptavidin biosensors, the spectral shift

corresponds to the path increase, so a spectral shift of 1 nm corresponds to 1 nm of mass thickness (Wallner et al., 2013). These curves obtained from BLItz software are in on-line representations of the molecular binding that takes place at the tip of biosensors (protein/antibody association curves)(Pall Fortebio, 2014). From these results, two parameters can be determined, both characteristic for a Rubisco concentration in a buffer solution (Delis, 2016): the "maximal binding" (or maximal spectral shift) corresponding to the amount of Rubisco bounded at a definite time (usually 120 s), and the "binding rate", corresponding to the initial slope of the curves, which depends on the Rubisco/antibody association rate.

In figure 2, all the results obtained with the standard solution of 1 g Rubisco/L are represented. Table 4 summarizes experimental results of maximal binding (MB, in nm) and binding rate (BR, in nm/s) obtained for these standards measured 30 min after the solution preparation. For each buffer solution, left values are replicates measured with new biosensors (Rep 1, Rep 3 and Rep 4). Right values include the replicate measured with a four times regenerated biosensors (Rep 2*). All the experimental results obtained for this standard solution are clearly regrouped in four categories, depending on the buffer solution (figure 2). The results for Pall PBS and PBS1 buffer solutions match exactly, showing that the effect of pH is much important than the effect of ionic strength.

For each group, the repeatability of the measurements is quite good when maximal binding is considered, and very good when all the biosensors were new or regenerated 3 times maximum. When a biosensor regenerated more than 3 times is used, the risk to have less repeatable results increases. Moreover, maximal binding obtained with four-reused biosensors are often lower than those obtained with new biosensors, in accord with results obtained by Maragos (2011). An important effect of time between the solution preparation and the analysis has been observed especially in the values of binding rates determined (Table 5). Most of values obtained at 30 min seem to minimize the response. At pH 6.8 and 6.2, Rubisco (pI ~ 6) is less stable because the minimum of proteins solubility corresponds to a pH equal to their pI. In these conditions, the dispersion of the results increases strongly, which makes the analysis less repeatable. From this point of view, analysis performed more than 2 hours after the preparation of solutions, seems to be a good compromise for these conditions. In buffer solutions at pH 7.4, binding rate is well stabilized after 2 h, and values become much regular.

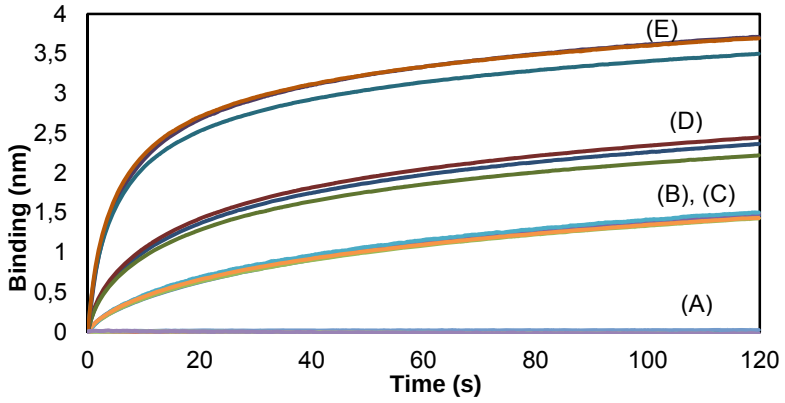

Figure 2: Signals for buffer solutions (4 curves)(A). Signals for standard solutions of 1 g/L in Pall PBS buffer pH 7.4 (B), in PBS 1 pH 7.44 (C), in PBS 2 pH 6.8 (D), and in PBS 3 pH 6.2 (E). All the results for standard of 1 g/L include 4 curves for 4 replicates.

Table 4: Maximal binding and binding rates obtained with standard solutions of Rubisco at 1g/L in different PBS buffer solutions. Asterisk (*) means a replicate obtained with a biosensor regenerated four times.

	Pall PBS		PBS1		PBS2		PBS 3	
	MB (nm)	BR (nm/s)	MB (nm)	BR (nm/s)	MB (nm)	BR (nm/s)	MB (nm)	BR (nm/s)
Rep 1	1.46	0.054	1.48	0.062	2.37	0.158	3.71	0.464
Rep 2*	1.49	/0.072	1.40	/0.062	2.09	/0.156	3.48	/0.526
Rep 3	1.49	0.056	1.50	0.059	2.45	0.148	3.50	0.492
Rep 4	1.43	0.056	1.44	0.067	2.22	0.163	3.69	0.438
Mean		0.056/0.060		0.063/0.062		0.156/0.156		0.465/0.480
SD		0.001/0.008		0.004/0.003		0.008/0.006		0.027/0.038
%RSD		1.797/14.049		5.933/4.901		4.986/4.073		5.888/7.931

In order to quantify Rubisco concentration in an unknown solution, calibration curves at the experimental conditions chosen must be established. Calibration curves give Rubisco concentration as a function of binding rate (Pall Fortebio, 2014). Standard solutions have been prepared and analysed 2 h later. Figure 3 shows experimental results for buffer solution PBS 1, as an example. For each curve, the baseline was determined with the buffer solution. Fitting of the experimental results was done by using a binomial relationship, which gives better regression coefficients. Calibration curves for all the buffer solutions used are indicated in Table 6. As for the effect of time, regression coefficients obtained at pH 6.8 and 6.2 were poor. Calibration curves were then used for determining Rubisco concentration in standard solutions of 1 g/L. Results are also included in Table 6. In accordance with the above results, Rubisco concentration was efficiently determined in buffer solutions at pH 7.4 but analysis conditions were not adequate for the analyses at pH 6.8 and 6.2.

Table 5: Binding rates in different PBS buffer solutions obtained with standard solutions of Rubisco at 1g/L at different times from the solution preparation. Each value is the mean of three replicates.

	Time between solution preparation and analysis		
PBS buffer solutions	30 min	2 h	4 h
Pall PBS	0.056	0.085	0.084
PBS 1	0.063	0.081	0.079
PBS 2	0.156	0.207	0.179
PBS 3	0.465	0.533	0.428

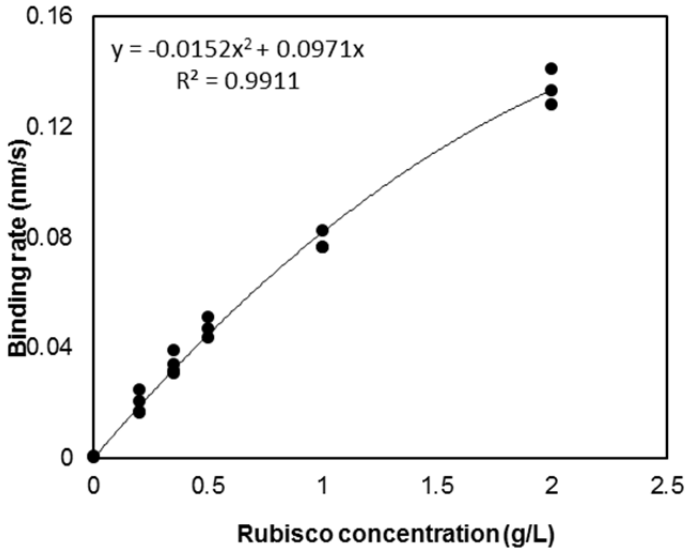

Figure 3: Calibration curve for buffer solution PBS 1. Each concentration has been triplicated.

Table 6: Calibration curves equations for each buffer solution. Rubisco concentration recalculated in standards solutions (theoretical concentration 1 g Rubisco/L).

PBS buffer solutions	Calibration curve equation	R^2	Rubisco concentration (g/L)
Pall PBS	BR = -0.0182C^2 + 0.1027C	0.9838	1.00
PBS 1	BR = -0.0152C^2 + 0.0971C	0.9911	0.96
PBS 2	BR = -0.0341C^2 + 0.2376C	0.9773	0.86
PBS 3	BR = -0.1399C^2 + 0.6819C	0.8613	0.74

Table 7: Rubisco quantitation in an unknown sample. All the measures were triplicated.

Unknown solution	BLItz analysis			Rubisco concentration (g/L)	
	Rubisco concentration (g/L)	SD	%RSD	HPLC SEC	Dry mass (SD)
Solution X8	8.0	0.38	7.54	10.3 ± 1.0	10.7 (0.05)
Solution X16	9.4	0.60	4.03		

BLI method was finally applied for determining Rubisco concentration in an unknown solution obtained from alfalfa juice and purified by ultrafiltration. Experimental results (Table 7) were then compared with analysis by HPLC SEC and dry mass determination. In order to keep concentrations in the interval of the calibration curve, and to guarantee the respect of buffer conditions, solutions were diluted in PBS 1 buffer solution at two dilution ratios. The results obtained were repeatable, with deviations between values under 8 %, and consistent for the three analytical methods applied in such a system.

4. Conclusions and perspectives

Biolayer Interferometry is a quickly selective technique for protein analysis and can be applied for Rubisco quantitation even if the results are strongly dependent on the experimental conditions, and must be well controlled. pH was the most important variable to be controlled, but time from sample preparation, biosensors preparation or reuse were also significant. In this work, the recommended conditions to measure Rubisco concentration by BLItz were established to be 0.1 mol/L PBS buffer at pH 7.4, 2 h between the sample preparation and its analysis and the use of new or 3 times-regenerated biosensors.

Acknowledgments

The authors gratefully acknowledge financial and material support from the Occitanie Region (France), Vivadour Cooperative and Ovalie Innovation.

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