Enzyme and Microbial Technology 97 (2017) 97–103

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Quantification of immobilized *Candida antarctica* lipase B (CALB) using ICP-AES combined with Bradford method

Paula Nicolás[a], Verónica L. Lassalle[b], María L. Ferreira[a,*]

[a] Planta Piloto de Ingeniería Química (PLAPIQUI-UNS-CONICET), Universidad Nacional del Sur, Camino La Carrindanga km 7, 8000, Bahía Blanca, Argentina
[b] Instituto de Química del Sur (INQUISUR-UNS-CONICET), Avda. Alem 1253, 8000, Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form
17 November 2016
Accepted 22 November 2016
Available online 25 November 2016

ABSTRACT

The aim of this manuscript was to study the application of a new method of protein quantification in *Candida antarctica* lipase B commercial solutions. Error sources associated to the traditional Bradford technique were demonstrated. Eight biocatalysts based on *C. antarctica* lipase B (CALB) immobilized onto magnetite nanoparticles were used. Magnetite nanoparticles were coated with chitosan (CHIT) and modified with glutaraldehyde (GLUT) and aminopropyltriethoxysilane (APTS). Later, CALB was adsorbed on the modified support.

The proposed novel protein quantification method included the determination of sulfur (from protein in CALB solution) by means of Atomic Emission by Inductive Coupling Plasma (AE-ICP). Four different protocols were applied combining AE-ICP and classical Bradford assays, besides Carbon, Hydrogen and Nitrogen (CHN) analysis. The calculated error in protein content using the "classic" Bradford method with bovine serum albumin as standard ranged from 400 to 1200% when protein in CALB solution was quantified. These errors were calculated considering as "true protein content values" the results of the amount of immobilized protein obtained with the improved method. The optimum quantification procedure involved the combination of Bradford method, ICP and CHN analysis.

1. Introduction

Comparison of enzymatic activity per milligram of protein between the free and immobilized enzyme is the best method to address immobilization efficiency. Protein loading onto the support is calculated indirectly, measuring initial and final protein concentration in supernatant of immobilization media. UV/visible spectroscopy-based methods are used to obtain protein content in immobilized proteins. Among them, the Bradford assay is the most employed technique for total protein quantification [1,2] using Coomassie Brillant Blue G-250 dye (CBB). The CBB-protein complex absorbs at 595 nm. Bovine serum albumin (BSA) is used as standard. Serious limitations have been found in quantification of proteins different than BSA when applying the Bradford method. Molecular weights, aminoacids sequence and exposed structure of BSA to aqueous solutions and those from the unknown protein are (or may be) very different [3]. Protein aggregation may change after stirring and/or after prolonged contact with buffers. Generally, pure target protein is not available. Other error source is the interaction among the dye and chemical species from the support [4].

Bradford and bicinchoninic acid methods are fraught with large deviations in the case of proteins with unusual amino acid composition (e.g. lysozyme) [5,6]. These results have been reported in several published manuscripts devoted to lipase immobilization and application of lipases in esterification of fatty acids [7–12], hydrolysis [13–15] acidolysis [16] and diglycerides synthesis [17].

The determination of enzymatic activity in a selected reaction using an aliquot of the supernatant of an immobilization solution is not a valid method for protein quantification. An improved method with the consideration of interferences to quantify protein when using *Candida antarctica* lipase B (CALB) in crosslinked-enzyme aggregates (CLEAs) was proposed in Guauque Torres et al. [13]. Aggregation of CALB was a very serious problem. Careful considerations about linear response of enzymatic activity versus protein concentration were needed. Even more, enzymatic activity is only valid for the selected test reaction and it can not be related to other reactions. Bradford method is still of widespread use in the field. Serious drawbacks have been not evident. The classical Bradford procedure does not include the checking of full UV/vis spectra and the analysis of interferences. Among such interferences are

* Corresponding author at: PLAPIQUI-UNS-CONICET, Camino La Carrindanga km 7, CC 717, 8000, Bahía Blanca, Argentina.
E-mail addresses: mlferreira@plapiqui.edu.ar, ferreiramaralujn@yahoo.com (M.L. Ferreira).

http://dx.doi.org/10.1016/j.enzmictec.2016.11.009
0141-0229/

dissolved compounds from supports, the ions of the buffers, the coordination of ions from buffers to proteins, the use of BSA as standard, the nature and extent of the protein aggregation and the role of colloids or nanoparticles in the solution. The checking of linear response Absorbance-Protein concentration at selected wavelength is the only criteria used to test accuracy but this is not a good test for suitability. Absorbance increase with the increase of concentration of BSA and the absorbance change with the increase in concentration of the unknown protein (in Bradford method) may be *both* linear but not similar in slope. Besides, abnormal results, such as higher absorbances than initial ones (not lower) in the supernatants solutions from immobilization media at the wavelengths used to protein quantification is an usual finding.

In the field of proteomics, inductively coupled plasma-mass spectrometry (ICP-MS) is more and more explored, due to great selectivity and sensitivity. The technique allows tackling the widespread problem of absolute protein quantification in specific applications. However, present conventional techniques of MS for protein quantification usually require isotope labeling for both 'relative' and 'absolute' quantifications. Sanz-Medel et al. [18] concluded that the natural presence of a heteroatom in a protein or a limited number of them opens new avenues for straightforward "ICP–MS-guided detection". Derivatization and labeling strategies are applied to visualize "protein" for ICP–MS detection [18]. A recent review of Martínez Sierra et al. [19] presents the approaches available for sulfur isotope ratio measurements by ICP–MS. Special emphasis is done in the quantification of peptides/proteins and the analysis of metallopeptides/metalloproteins via sulfur by LC–ICP–MS. Unfortunately, this approach is expensive and not widely available. However, the elemental analysis in biotechnology is full of methodological advantages and shows the potential in biotechnological applications [20]. Atomic emission spectroscopy with Inductively coupled plasma(AES ICP-), or inductively coupled plasma optical emission spectrometry (ICP-OES), is an analytical technique used for the detection of trace metals. It is usually selective enough not to be interfered by organic matrix constituents and it is very useful with non-contaminated matrixes [20]. See additional table in Supplementary material where drawbacks and benefits of each protein quantification method are presented.

The objective of the manuscript is to find an accurate method to quantify protein during immobilization procedures when using CALB. The selected support/lipase system was magnetite nanoparticles coated with oleic acid and chitosan and CALB as lipase. Amino-propyl trietoxisilane (APTS) and glutaraldehyde (GLUT) were employed as coupling and crosslinking agents.

A novel accurate procedure based on the measurement of sulfur (S) content in CALB using Atomic Emission Spectroscopy with Induced Coupling Plasma spectroscopy (AES-ICP or ICP) combined with CHN determination was developed to obtain ***quantitatively*** the amount of protein. Bradford method was applied to measure the ***relative*** decrease of the protein in solution (final versus initial protein content). This combined procedure has not been applied before to CALB to the best of the authorsí knowledge. The solvent-free ethyl oleate synthesis was the reaction test to evaluate the correlation between amount of immobilized protein with enzymatic activity. This method avoids the need of protein labeling and the use of MS.

2. Methods

The supports were magnetite nanoparticles functionalized with oleic acid and chitosan. The procedure to immobilize CALB on those supports was previously reported. [21] Impure CALB aqueous solution (raw broth) was donated by Novozymes (Brazil) batch LCN02103. SDS-PAGE of the commercial solution of CALB was performed and the main protein present was CALB (see Supplementary material). Bovine Serum Albumin (BSA, 30% w/v) was purchased from Wiener (Argentina), Bradford Reagent 5X (BR, 5X means five times the conventional concentration) was from BioRad, phosphate buffer pH 7 (which will be referred as buffer from here thereafter) was from Anedra (concentrations not provided by the manufacturer) and NaCl from Cicarelli (Argentina).

2.1. Immobilization procedure and esterification reaction

200 mg of solid support was dispersed in 50 mL of a CALB aqueous solution with different concentrations and stirred magnetically during 7 h at room temperature. The solid was allowed to decant completely and the supernatant was withdrawn and stored at −4 °C. Ten milliliter of water were added to the remaining catalyst to remove unbound enzyme. The dispersion was stirred five minutes and finally decanted using a Nd magnet. This washing step was repeated twice. This protocol was carried out with supports treated with APTS and/or GLUT and three different ratio concentrations of CALB/protein. Experimental design generated eight different biocatalysts. In Nicolás et al. [21] preliminary results were reported, using catalysts prepared by immobilization of CALB onto a magnetic support including chitosan, pretreated with GLUT.

The biocatalysts were used in the solvent-free synthesis of ethyloleate using experimental conditions previously reported: 30 mg of catalyst were added to 1 g of Oleic Acid (OA), 150 μL of absolute ethanol and 200 μL of distilled water. The reaction mixture was magnetically stirred at 900–1000 rpm at 24 °C for 3 h. Sampling was performed according to the protocol developed in the aforementioned contribution.

2.2. ***Bradfo****rd's assays A—calibration curves*

Calibration curves were built using BSA as standard in the range from 1.2 to 10 μg/mL (microassay) and 0.2 to 0.9 mg/mL (standard assay) measuring absorbance at 595 nm.

BSA stock solutions were prepared in NaCl 0.15 M. Distilled water was used to dilute the Bradford Reactive (BR) 5X (500 mg/L) to a fifth (Bradford Reactive 1X) when needed for the standard assay.

2.3. ***Bradf****ord's assays B—sample treatment*

The raw CALB broth was analyzed applying both, the micro and standard Bradford assays at 595 nm. Immobilization supernatants and washing solutions were analyzed only in the microassay range, diluting each sample appropriately to fit the absorbance in the linear zone at 595 nm.

2.4. Determination of protein/lipase content by AE-ICP

The technique was based on measuring sulfur (S) in the protein solutions before and after immobilization using ICP. One molecule of CALB (33 kDa) contains 5 units of methionine and 7 units of cysteine [22]. The protein content was calculated considering that 12 atoms of sulfur (from the 5 methionines and 7 cysteines) are present per each lipase molecule.

An UV–vis spectrophotometer PG Instruments, UK, model T60U with a silicon photodiode detector was used. Sulfur determinations were carried out in an atomic emission spectrometer by ICP (Shimadzu Sequential 1000 model III) with conventional nebulization and external calibration by certified patterns (Chem-Lab, Zedelgem B-8210, Belgium). Traces of S in powdered sample were qualitatively detected by an X-Ray Fluorescence (XRF) spectrometer PHILIPS PW 1400, with Rh anode and a Ge detector under He atmosphere. Selected samples were analyzed for CHN, using a

Series II 2400 CHNO/S Elemental Analyzer from PerkinElmer. SDS-PAGE measurements were done using the procedure reported in Llerena-Suster et al. [23].

3. Results and discussion

Several errors have been found using Bradford method. Important interferences arose from the reaction of ionic species (for instance phosphate ions) from buffer with the dye [24,25]. When adding a drop of BR to 3,3 mL of phosphate buffer with 7×10^{-2} M PO_4^{3-}, the solution turns blue. In the micro-macro arrays of Bradford method the phosphate buffer is diluted with BR (1:10 or 1:5) and the phosphate or other ions should be considered interferences.

Binding of the BR to basic amino acids is more efficient than to acidic amino acids. Differences in the relative content of acidic/basic aminoacids among proteins give different results using Bradford method. At pH near 1 the solution of BR is deep green. At pH $\geq$ 2 the solution is blue, with BR carrying one negative charge.

It is worth noting that the colours observed in CBB solution depend on the acidity of the solution and its interactions with amino acids from proteins. The different colours result from the differently charged states of the dye molecule, corresponding to the amount of positive charges at the three nitrogen atoms (please see Scheme 1 in Supplementary material), while the two sulfonic acid groups are normally always negatively charged.

Silvério et al. [5] found that several concentrations of salts and polymers have considerable effects in the BSA calibration curve obtained by the Bradford microassay. They assured that only traces of salts and polymers could be considered as non-interfering. The authors proposed further dilution of samples to avoid these errors, but this is not adequate to quantify very low protein concentrations.

3.1. Calibration curves: the media and the standard

Two calibration curves were built: one using distilled water and another employing phosphate buffer to dilute the BSA stock solutions (see Fig. 1).

Different slopes arose. In the case of the microassay, slopes differed about 12%, whereas the standard assay gave a slope 15% greater using water instead of PBS buffer.

These differences in the curves slopes are assigned to the presence of ionic moieties arising from the PBS. Such ions increase the aggregation of the proteins and also remain partially adsorbed on their surface. Similar results have been obtained in previous work using CALB and other proteins [3].

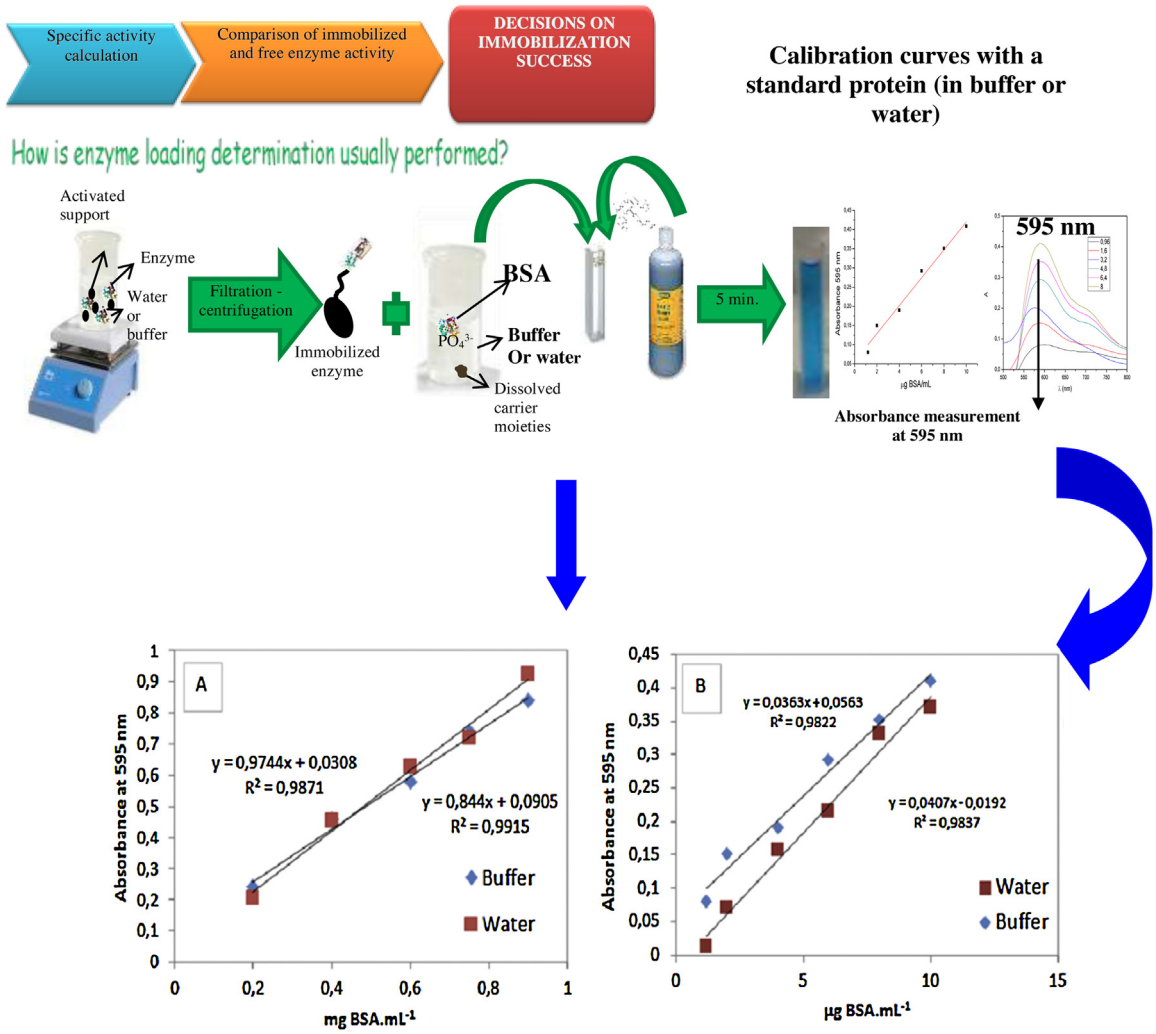

Fig. 1. Calibration curves for BSA with CBB using water (squares) or buffer (rhombus) to dilute the standard. A) Standard assay, B) Microassay The largest standard deviation for absorbance in each point was ±0,0081 in Absorbance units (n = 3).

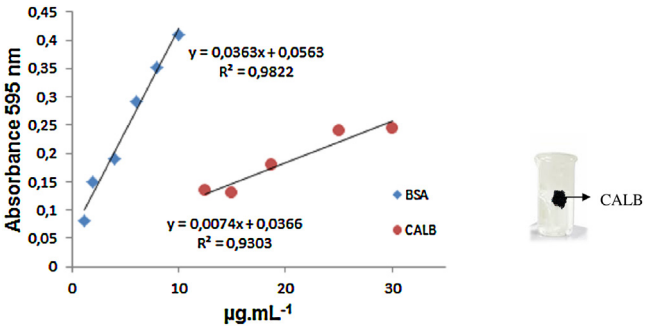

Fig. 2. Calibration curves of BSA (rhombus) and protein in CALB solutions (circles) with CBB. Protein concentrations were calculated using EA-ICP. CALB is used instead of BSA as calibration standard.

The slope of the calibration curve using impure CALB solution as standard was 77% lower than the value corresponding to the slope using BSA in Bradford microassay (see Fig. 2).

SDS-PAGE was performed on the raw broth CALB solution to confirm composition. Indeed, CALB was the main protein in the solution provided by Novo (see SDS-PAGE results in Supplementary material). Even if other molecules contain sulfur, the error would be almost depreciable since their molecular weight would be surely larger than 33 kDa. SDS-PAGE showed a minor signal near 66 kDa.

The protein content in the commercial "as received" CALB is 2,7 mg/mL by Bradfordís Standard assay, 3,5 mg/mL using the Bradford Microassay, 17,7 mg/mL using AE-ICP (this work) or 15,2 mg/mL following reference 23. Protein quantification in three diluted test commercial CALB solutions (see Fig. 2) gave 12,5, 18,8 and 25 µg protein (CALB)/mL by EA-ICP and 2,2, 3,4 and 5,0 µg protein/mL using Bradford.

The determination of free thiol (SH) groups has been done using the Ellman procedure. However, this method- that uses 5, 5′Dithiobis-(2 nitrobenzoic) acid or DTNB known as the Ellmanís Reagent- requires the S as the reduced form in the peptide (SH). The method recommends the reduction of sulfhydryl with a reducing agent. The application of Ellmanís method would result in erroneous CALB quantification because of the composition of the lipase in terms of the aminoacids sequence. In fact, CALB is formed by 357 aminoacids and 12 S atoms per molecule, many of them in S-S form [Protein Data Bank entry 4K6G, see sequence]. Then, the use a calibration solution containing cystein would interfere in the lipase quantification. This turns the Ellmanís method unviable to determine the amount of immobilized CALB [22].

3.2. Selection of an accurate method of quantification

Four procedures for protein quantification were selected:

I—To quantify the initial and final protein content in the supernatant and in the washing residues with BSA as standard and Bradford method.

II—To use AE-ICP method to quantify sulfur in the initial CALB solution and to use conventional, classical Bradford with BSA as standard to measure the final protein amount in supernatants after immoblization and in washing residues.

III—To use AE-ICP method to quantify sulfur in the initial CALB mixture but to apply Bradford method with CALB as standard protein to calculate the ***change relative to the initial protein concentration.*** The final protein in supernatants after immobilization and in washing residues is calculated with this relative change.

IV—To use AE-ICP method to quantify sulfur in the initial CALB mixture and the final sulfur content in supernatants after immobilization and in washing residues.

The data of mg of protein per 100 mg of support obtained with the methods presented above are shown in Table 1. Large discrepancies in Enzyme Loading (EL) were found when comparing data arising from method I and the average between results of methods III and IV.

In biocatalysts with high contents of nominal APTS and GLUT, the protein concentration obtained using Bradford in supernatant after immobilization time was higher than initial one (a result against conservation mass law). This behavior was attributed to residual, soluble moieties of APTS-GLUT from the support, leaching during immobilization procedure. These compounds would react with CBB [4], raising the absorbance signal.

In methods I, II and III the contribution of APTS/GLUT-dye complex was present. The interferences among APTS, GLUT and CBB were not previously reported in open literature at the best of the authorís knowledge. In many published articles the informed protein content may include this error (among others).

Catalyst 2 and 9 were the central replications. The best method for protein quantification gave 2.1 and 2.5%, w/w for the protein content of the biocatalysts, respectively. Conversions of oleic acid were 3.6 and 3.3% for both catalysts.

Experiments 2 and 9 are replications of the experimental design. In them, CALB was immobilized directly on APTS-coated support, i.e. without GLUT. As a consequence, the coupling mechanism is entirely different. In fact, their enzyme loadings (EL) were 2.1 and 2.5% but the conversions of oleic Acid to ethyl oleate were only 3–4%, meaning that lipase was bound in an inactive form. Therefore EL% did not correlate with conversion in the same fashion than the other 8 samples. This is a direct result of immobilization protocol and it is not at all associated to the applied protein quantification methods, so they were discarded from the discussion in this paper.

The error involved in protein quantification using an average in methods III and IV was much lower than the error involved when using methods I or II. The difference between catalysts 2 and 9 in the oleic acid conversion was lower than 10%. When two batches of Biocatalyst 7 were analyzed using CHN, carbon content was 8.47 and 8.05% whereas nitrogen content was 0.59 and 0.52% and hydrogen content was 0.88 and 0.81%, respectively for batch 1 and batch 2. Differences between batches were of 5,1% for C, 12,6% for N and 8,3% for H. The Biocatalyst 7 batch 1 showed 36% whereas the Biocatalyst batch 2 showed 38% conversion of oleic acid at the same conditions. Difference is less than 5,5% in oleic acid conversion between replicates.

Results from method I brought low protein loadings. These values were mainly affected by the choice of BSA as standard. The procedure II, which is based on the ICP application for the quantification of the initial amount of protein in solution, eliminated the mistake found in initial protein concentration. Finally, in method III the analyte protein and the standard protein were the same. Quite large "apparent" protein concentrations were found in the supernatants after immobilization. The source of this interference was iron leaching from the support, increasing absorbance at 595 nm. The support was stirred in distilled water under similar conditions than the immobilization but without protein available. The supernatants and washings solutions were analyzed with Bradford microassay (blank). A significant absorbance of 0.1 was found, assignable to dissolved iron (results not shown).

The partial dissolution of magnetite from the support was verified by ICP. Data arising from method IV suggested that the S content in the immobilization supernatant was higher than the total protein fed to the system. A contribution of S from the magnetite impurities (i.e $FeSO_4$) was considered as responsible for this erroneous value. An intense signal in XRF associated to S moieties was detected in magnetite (results not shown).

CHN analysis was performed for the supports without protein and the final biocatalysts 3, 7 and 10. For the Catalyst 3, the direct comparison between the support with Glutaraldehyde and with Glutaraldehyde plus the protein gave a total mass difference (or

Table 1
Protein loadings obtained with four methods for eight biocatalysts and percentage of associated errors.[a] The values considered more accurate are in bold.

Catalyst	Offered enzyme per 100 mg support (mg)[b]	Enzyme loading-EL (mg CALB per 100 mg support)					Error (%) of method I by defect	Error (%) of method II by excess
		I—Standard Bradford method	II—Bradford method (standard: BSA) combined with ICP	III—Bradford method (standard:CALB) combined with ICP	IV—ICP only	Average III and IV		
1	4,5	0,42	4,0	2,6	1,85	**2,2**	424	46
3	4,5	0,51	4,1	2,1	3,0	**2,6**	410	37
4	1,5	0,26	1,5	1,5	1,5	**1,5**	477	−2
5	1,5	[c]	1,0	0,8	0,62	**0,7**	∞	31
6	1,5	0,09	1,3	0,9	0,42	**0,6**	567	54
7	4,5	0,22	3,9	3,0	3,0	**3,0**	1264	22
8	1,5	0,09	1,3	1,3	0,55	**0,9**	900	31
10	4,5	0,29	3,9	3,1	3,75	**3,4**	1072	13

[a] Percentage error = [(number in bold/value obtained by the evaluated method) − 1] × 100.
[b] Determined by AE-ICP.
[c] The amount of protein ***detected after*** the immobilization time was higher ***than the initially measured*** in the initial solution following this method.

protein content) of 2.0% w/w, whereas for the catalyst 7 and 10 the results were 1.7–1.8% w/w. The CHN analysis of the support of biocatalyst 7, stirred 7 h, demonstrated partial dissolution of the APTS-GLUT–Magnetite. In summary, disaggregation/partial dissolution of the whole support has two main consequences. One is due to the coordination of the dissolved transition metal with CBB. The second was the overestimation of S content in final biocatalyst.

Correlation of oleic acid conversion with protein content obtained with method I or method III/IV is shown in Fig. 3. The profile of enzymatic esterification activity-protein content is clearly well correlated with the protein content in biocatalysts following the method proposed within this work. The Bradford method is not able to discriminate between different CALB loadings onto the particles.

Mistakes in the Bradford method have been reported before [5,6,26]. The research on quantification of proteins in enzymes or in food is continuous [6,27]. The main mistake found in this work with the Bradford method I was the low initial and final protein concentration detected. Method II decreased the errors found in method I. This method II gave very low results for the final protein concentration after the immobilization step. The use of Bradford method to measure only the *decrease* of protein concentration *relative to the initial one* is adequate, always when using the protein to quantify as standard.

It is important to highlight that the proposed procedure is applicable for only one enzyme and a limited number of variants of one basic type of nanoparticle. The authors are aware that the methods used for comparison [atomic emission, XRF, elemental C, H, N analysis] require specialized equipment & skills that will not be convenient for, or even available to, many investigators. However, they are available as services in many specialized accessible labs. Given the magnitude of the mistakes that were found, even when the authors agree in the difficulties involved in the application of the cited techniques, they encourage researchers in the field to take into account these considerations.

Considering a particular enzyme in a protein solution, for example a commercial one, it is very important, first, to filter the solution to avoid any insoluble macromolecule to interfere. Later, it is important to analyze the solution using SDS-PAGE to know the distribution of proteins in the commercial enzyme. If possible nucleic acids should be separated (but they do not contribute to sulfur content) Third, the structure of the protein should be checked in

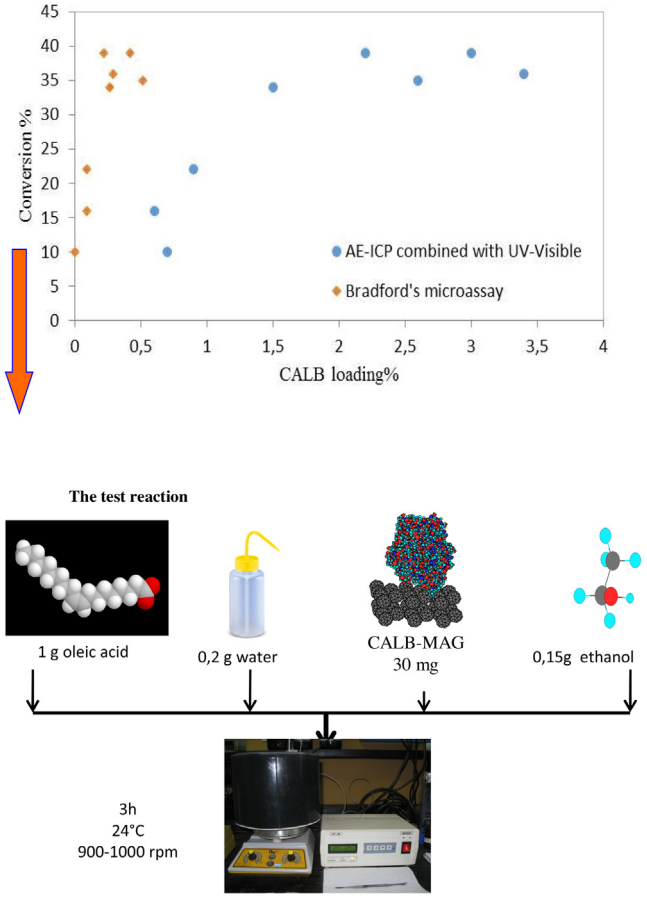

Fig. 3. Conversions of oleic acid (in percentage of initial amount) to ethyl oleate with eigth magnetic catalysts, correlated to protein content calculated with methods III and IV (average of both results, circles in blue) and calculated with the classical Bradford's microassay (method I in Table 1, rhombus in orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CALB structure from http://www.rcsb.org/pdb/explore.do?structureId=1tca.

the Protein Data Bank to know about the Sulfur content. With this information the adequate procedure can be carried out.

4. Conclusion

An innovative method was proposed to avoid (or at least minimize) mistakes in the application of Bradford method to quantify proteins using CALB. The magnitude of the mistakes regarding to protein immobilization were up to 1200% using "classic" Bradford method. The procedure proposed here is of easy application in the case of commercial CALB because:

- the sulfur content of the lipase (through the information about sequencing included in the Protein Data Bank for CALB) and the molecular weight of the lipase are known.
- the main protein present in the commercial solution or solid is CALB and this is checked through SDS-PAGE analysis of the commercial lipase solution.
- a simple step of dissolution and filtration to separate insoluble solids is performed (if the enzyme is received as solid)
- combined methods are applied, such as CHN analysis plus ICP-AES and Bradford.

The best result was obtained combining AE-ICP for the ***absolute protein quantification*** and Bradford method for the ***relative decrease*** in protein concentration, using CALB commercial solution as standard. The methodology may be applied other lipases, proteins and supports.

Classical Bradford Method using BSA as standard gives lower than "true" protein contents (up to ten times lower) in commercial CALB solutions, diluted with buffer or with distilled water, before and after immobilization steps in common procedures for CALB immobilization.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

Author authorizations

The work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint), it is not under consideration for publication elsewhere, its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

All authors materially participated in the research and/or article preparation.

Bachelor in Chemistry Paula Nicolas performed the experimental studies under the guidance of Drs Lassalle and Ferreira. This work is part of her PhD Thesis.

Drs Lassalle and Ferreira participated equally in the article preparation, all parts, including the design of the figures, discussion and conclusions. Bachelor Paula Nicolas also reviewed the article and all authors have approved the final article to be submitted.

Acknowledgements

The authors acknowledge the financial support from CONICET (Argentina, all the authors), the economic support from PICT0788-2010 (ANPCyT, Argentina, Dr M. L. Ferreira and Bachelor P. Nicolás) and the PGIN°24/ZQ09 (UNS, Argentina, Dr Verónica Lassalle). The authors acknowledge Dr Susana Morcelle (LIPROVE-UNLP-CONICET) the SDS-Page determinations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2016.11.009.

References

[1] H.K. Ku, H.M. Lim, K.H. Oh, H.J. Yang, J.S. Jeong, S.K. Kim, Interpretation of protein quantitation using the Bradford assay: comparison with two calculation models, Anal. Biochem. 434 (2013) 178–180.
[2] L.J. Daumann, J.A. Larrabee, D. Ollis, G. Schenk, L.R. Gahan, Immobilization of the enzyme GpdQ on magnetite nanoparticles for organophosphate pesticide bioremediation, J. Inorg. Biochem. 131 (2014) 1–7.
[3] V. Lassalle, S. Pirillo, E. Rueda, M.L. Ferreira, An accurate UV/visible method to quantify proteins and enzymes: impact of aggregation, buffer concentration and the nature of the standard, Curr. Top Anal. Chem. 8 (2011) 83–93.
[4] T. Ishii, A. Sorita, M. Sawamura, H. Kusunose, H. Ukeda, Determination of reaction product of glutaraldehyde and amine based on the binding ability of coomassie brilliant blue, Anal. Sci. 13 (1997) 5–9.
[5] S.C. Silvério, S. Moreira, A.M. Milagres, E.A. Macedo, J.A. Teixeira, S.I. Mussatto, Interference of some aqueous two-phase system phase-forming components in protein determination by the Bradford method, Anal. Biochem. 421 (2012) 719–724.
[6] E. Szöllösi, E. Házy, C. Szász, P. Tompa, Large systematic errors compromise quantitation of intrinsically unstructured proteins, Anal. Biochem. 360 (2007) 321–323.
[7] M.L. Foresti, M.L. Ferreira, Frequent analytical/experimental problems in lipase-mediated synthesis in Solvent Free Systems and how to avoid them, Anal. Bioanal. Chem. 381 (7) (2005) 1408–1425.
[8] M.L. Foresti, M.L. Ferreira, Solvent-free ethyl oleate synthesis mediated by lipase from *Candida antarctic*a B adsorbed on polypropylene powder, Catal. Today 107–108 (2005) 23–30.
[9] M.L. Foresti, M.L. Ferreira, Chitosan-immobilized lipases for the catalysis of fatty acid esterifications, Enzyme Microb. Technol. 40 (4) (2007) 769–777.
[10] M.L. Foresti, M.L. Ferreira, Analysis of the interaction of lipases with polypropylene of different structure and polypropylene-modified glass surface, Colloids Surf. A 294 (1–3) (2007) 147–155.
[11] K.V. Sandeep, K. Chandra, D Interference of *N*-hydroxysuccinimide with bicinchoninic acid protein assay, Biochem. Biophys. Res. Commun. 411 (2011) 455–457.
[12] K.V. Sandeep, Z. BinBin, Z. Dan, A. Khalid, J. Luong, F. Sheu, Sulfo-N-hydroxysuccinimide interferes with bicinchoninic acid protein assay, Anal. Biochem. 417 (2011) 156–158.
[13] Guauque Torres M. del Pilar, Foresti María Laura, Ferreira María Luján, Cross-linked enzyme aggregates (CLEAs) of lipases: a practical procedure for the calculation of their recovered activity, AMB Expr. 3 (1) (2013) 1–11.
[14] M. Guauque Torres, P. del, M.L. Foresti, M.L. Ferreira, Effect of different parameters on the hydrolytic activity of cross-linked enzyme aggregates (CLEAs) of lipase from Thermomyces lanuginosa, Biochem. Eng. J. 7 (2013) 218–223.
[15] M.P. Guauque Torres, M.L. Foresti, M.L. Ferreira, CLEAs of *Candida antarctica* lipase B (CALB) with a bovine serum albumin (BSA) cofeeder core. Study of their catalytic activity, Biochem. Eng. J. 90 (2014) 36–43.
[16] M.L. Foresti, M.L. Ferreira, Lipase-catalyzed acidolysis of tripalmitin with capric acid in organic solvent medium: analysis of the effect of experimental conditions through factorial design and analysis of multiple responses, Enzyme Microb. Technol. 46 (6) (2010) 419–429.
[17] D.A. Sánchez, G. Tonetto, M.L. Ferreira, Enzymatic synthesis of f 1,3-dicaproyglycerol by esterification of glycerol with capric acid in an organic solvent system, J. Mol. Catal. B: Enzyme 100 (2014) 7–18.
[18] Sanz-Medel Alfredo, Montes-Bayó Marían, Bettmer Jörg, Fernández-Sanchez M. Luisa, Encinar Ruiz, ICP–MS for absolute quantification of proteins for heteroatom-tagged, targeted proteomics, Trends Anal. Chem. 40 (2012) 52–64.
[19] J. Martínez-Sierra, O. Giner, Galilea San Blas, J.M. Marchante Gayón, J.I. García Alonso, Sulfur analysis by inductively coupled plasma-mass spectrometry: a review, Spectrochim. Acta Part B 108 (2015) 35–52.
[20] S. Hann, M. Dernovics, G. Koellensperger, Elemental analysis in biotechnology, Curr. Opin. Biotechnol. 31 (2015) 93–100.
[21] P. Nicolás, V. Lassalle, M.L. Ferreira, Development of a magnetic biocatalyst useful for the synthesis of ethyloleate, Bioprocess Biosyst. Eng. 37 (2014) 585–591.
[22] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, The sequence: crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*, Structure 2 (1994) 293–308.

[23] R. Llerena-Suster Carlos, E. Briand Laura, R. Morcelle Susana, Analytical characterization and purification of a commercial extract of enzymes: a case study, Colloids Surf. B 121 (2014) 11–20.
[24] J.K. Poppea, A.P. Costa, M.C. Brasil, R.C. Rodrigues, M.A. Ayuba, Multipoint covalent immobilization of lipases on aldehyde-activated support: characterization and application in transesterification reaction, J. Mol. Catal. B: Enzyme 94 (2013) 57–62.
[25] G.R. Grimsley, C.N. Pace, Unit 3.1 spectrophotometric determination of protein concentration, in: J.E. Coligan, M. Dunn, D.W. Speicher, P.T. Wingfield (Eds.), Current Protocols in Protein Science, John Wiley & Sons Inc., Hoboken, NJ, 2004, 3.1.1–3.1.9.
[26] S.J. Compton, C.G. Jones, Mechanism of dye response and interference in the Bradford protein assay, Anal. Biochem. 151 (1985) 369–374.
[27] H. Goetz, M. Kuschel, T. Wulff, C. Sauber, C. Miller, S. Fisher, C. Woodward, Comparison of selected analytical techniques for protein sizing: quantitation and molecular weight determination, J. Biochem. Biophys. Methods 60 (2004) 281–293.