Colloids and Surfaces B: Biointerfaces 136 (2015) 1202–1206

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

PEG-induced molecular crowding leads to a relaxed conformation, higher thermal stability and lower catalytic efficiency of *Escherichia coli* β-galactosidase

Verónica Nolan, Julieta M. Sánchez, María A. Perillo*

Instituto de Investigaciones Biológicas y Tecnológicas (IIBYT), (CONICET-Universidad Nacional de Córdoba), ICTA & Cátedra de Química Biológica, Depto. de Química, FCEFyN, UNC, Av. Velez Sarsfield 1611, X 5016GCA Córdoba, Argentina

ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form 30 October 2015
Accepted 2 November 2015
Available online 11 November 2015

Keywords:
PEG-induced molecular crowding
Enzymatic activity
Protein structure
Thermal stability
Water activity

ABSTRACT

Enzymatic activities were historically assayed in dilute solutions where molecular crowding, molecular confinement and their consequences were not taken into account. Here we report how macromolecular crowding tunes catalytic parameters for the tetrameric β-Galactosidase from *Escherichia coli*, β-Gal. We detected increases in K_M (weaker substrate binding) and a nonlinear variation in V_{max}, with a minimum at 25% W/P of the crowding agent (polyethyleneglycol molecular mass 6000, PEG^{6000}) resulting in a linear decrease in the catalytic efficiency (k_{cat}/K_M) within the whole [PEG^{6000}] range tested). Presence of crowding agent affected β-Gal structural content and increased its thermal resistance. Steady state fluorescence and Fourier transformed infrared spectroscopic observations are compatible with crowding-induced disordering and restricted internal dynamics as a result of excluded volume and solvent structuring effects. This leads to a non-optimal substrate-binding site and a less conformationally strained protein.

1. Introduction

β-D-galactosidase [EC 3.2.1.23] (β-Gal) is a soluble enzyme capable of catalyzing lactose hydrolysis into its constitutive monosaccharides: glucose and galactose [1]. β-Gal has been extensively studied because of its nutritional, biotechnological and therapeutic impact [2,3]. Usually, β-Gal exerts its activity in macromolecular crowded environments and heterogeneous media (e.g. intracellular milieu and conditions of technological interest like during encapsulation [4]). Molecular crowded systems involve non-ideal solutions, with a significant proportion of structured water – thus, a low water thermodynamic activity – and steric and diffusional restrictions imposed to solutes [5]. Under these conditions reactions are kinetically and thermodynamically affected. Enzyme catalyzed reactions are further affected due to the effect of crowding on the protein structure and function.

In this paper we investigate the effect that molecular crowding induces on the catalytic activity of β-Gal from *Escherichia coli* (β-Gal) with focus on the effects on the protein structure. PEG^{6000}, a non-charged highly water-soluble polymer with well known effects on water dynamics [6] was used to produce the crowded environment. The main results suggest that PEG-induced crowding favors a more relaxed protein conformation associated with lower catalytic efficiency and higher protein thermal stability. Opposed correlations were found in a heterogeneous system, upon analyzing the fraction of β-Gal bound to the lipid–water interface [7]. This suggests that on the one hand both systems seem to conduct the protein through similar energy landscapes but, the potentiation or depotentiation of the catalytic activity seem to reflect the differential accessibility of water molecules, one of the substrates, in both systems.

2. Materials and methods

2.1. Materials

The enzyme β-Gal from *E. coli* [EC3.2.1.23] Grade VII (specific activity 600–1200 UI/mg protein), and the substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma Chem. Co. (St. Louis, MO). PEG^{6000} was from Anedra. Other reagents and solvents were of analytical grade.

* Corresponding author. Fax: +54 351 4334139.
E-mail addresses: mperillo@efn.uncor.edu, mariekator@yahoo.com (M.A. Perillo).

http://dx.doi.org/10.1016/j.colsurfb.2015.11.003
0927-7765/

2.2. Enzyme activity assays

2.2.1. Incubation system

β-Gal activity was measured as described in Sánchez et al. [7]. The incubation system contained: 0.1 M, pH 6.8 sodium phosphate buffer with or without PEG^{6000} within the range from 0 to 35% W/V, 0–3 mM ONPG final concentration and 0.1 ml of β-Gal (specific activity 650 UI/mg protein; 1 UI = 1 mmol/min of ONP formed at 37 °C) at a final concentration of 0.033 mg/L. The hydrolysis reaction was stopped by the addition of 0.2 M NaOH. The absorbance of the *ortho*-nitrophenoxide (ONPx) formed was measured at 420 nm. Elsewhere, we described the occurrence of a non-enzymatic ONPG hydrolysis in the presence of PEG^{6000}. Thus, this was taken into account to avoid introducing a sistematic error in β-Gal catalyzed ONP production.

2.2.2. Protein quantitation

Protein concentration was measured by the Lowry's method [8].

2.2.3. Determination of β-Gal kinetic parameters

The initial reaction rate (V_0), defined as the concentration of product formed per sec per mg of protein, was plotted vs. the substrate concentration (S). The values of K_M (Michaelis constant) and V_{max} (maximal reaction rate) were determined by fitting the Michaelis–Menten equation (Eq. (1)) to the experimental data by a computer aided nonlinear regression analysis by the least squares method.

$$V = \frac{V_{max} \times S}{K_M + S} \quad (1)$$

2.3. Effect of PEG on β-Gal structure and thermal stability

2.3.1. Fluorescence spectroscopy

Fluorescence spectra were recorded in a Fluoromax Spex-3 Jovin Yvon (Horiba, New Jersey, USA) spectrofluorometer. A quartz cell with 10 mm path length and a thermostated holder was used. The slits were set at 2 nm. λ_{exc} was set at 290 nm. Emission spectra were acquired within the 300–400 nm range. Protein concentration used was 0.2 mg/ml. Raman contribution from water and the probable PEG^{6000} contribution were subtracted in all spectra.

To facilitate comparisons, the center of spectral mass (CSM) was calculated for each of the fluorescence emission spectra [9] according to Eq. (2), where I_i is the absorbance or the fluorescence intensity measure at the wavelength λ_i.

$$\lambda = \frac{\sum \lambda_i \times I_i}{\sum I_i} \quad (2)$$

2.3.2. FTIR Spectroscopy

Spectra were recorded in a Nicolet-Nexus spectrometer using a cell for liquid samples with CaF_2 windows and 75-μm Teflon spacers. The spectrometer was flushed with dry air to reduce water vapor distortions in the spectra. Five hundred scans were collected both for the background and the sample at a nominal resolution of 2 cm^{-1}. For sample preparation, 0.1 mg of lyophilized protein was suspended with 30 μL of D_2O and incubated for 24 h at room temperature to allow deuterium exchange of the amide protons. All measurements were done at 25 °C. Blank samples contained PEG^{6000} at the same concentration present in the protein sample.

2.4. Statistical calculations

The least squares method was applied to fit functions through a regression analysis. The propagation error method was used to calculate the error associated to variables calculated from other several experimentally determined ones [10]. The effects on kinetic parameters exerted by the different PEG^{6000} concentrations were analyzed with a post-hoc test of LSD.

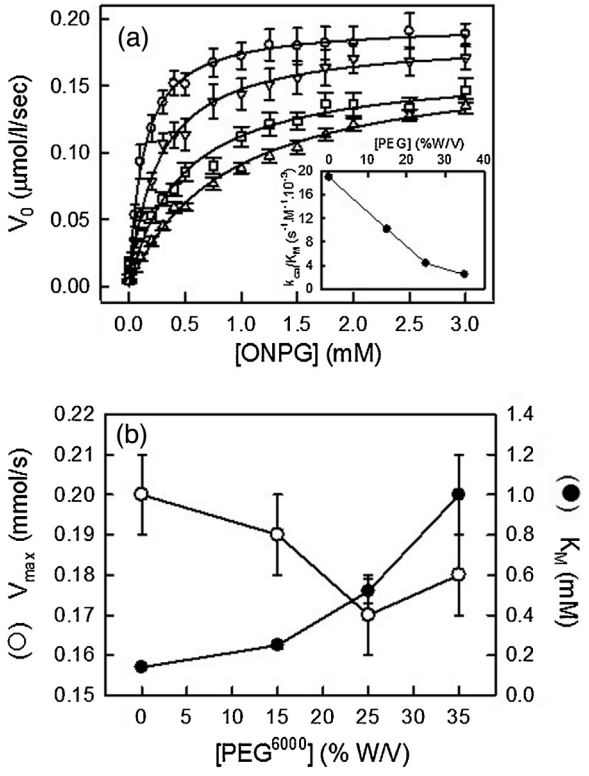

Fig. 1. Effect of PEG-induced molecular crowding on β-Gal enzymatic activity. (a) Reaction rate vs. substrate concentration at different PEG^{6000} concentrations, from bottom to top, 0 (○), 15 (▽), 25 (□) and 35 (△) W/V. Inset: catalytic efficiency (calculated from data shown in (b) vs. PEG^{6000} concentration. (b) Kinetic parameters, (○) K_M and (●) V_{max}, derived from curves shown in (a) through a least squares analysis. Error bars represent the mean ± s.e.m. of triplicates.

3. Results and discussion

3.1. Effect of PEG-induced molecular crowding on β-Gal enzymatic activity

β-Gal enzymatic activity was assayed in the absence and in the presence of increasing concentrations of the crowding agent PEG^{6000}. Optimal incubation conditions were determined in previous assays and were found to be 7 min and 0.033×10^{-3} mg/ml for the optimal incubation time and enzyme concentration, respectively.

The kinetics of *E.coli* catalyzed ONPG hydrolysis exhibited a Michaelian behavior at all PEG^{6000} concentrations tested (from 0 to 35% W/V) (Fig. 1a) with an almost linear reduction in the catalytic efficiency (Fig. 1a, insert). The kinetic parameters, K_M and V_{max}, (Fig. 1b), were calculated from curves in Fig. 1a. Upon PEG^{6000} concentration increased from 0% W/V to 35% W/V, K_M grew up from 0.14 mM to 1 mM. V_{max} tended to a minimum at 25% W/V PEG^{6000} and showed a recovery at a higher concentration, although values were not statistically different.

3.2. Effect of PEG-induced molecular crowding on β-Gal structure and thermal stability

3.2.1. Fluorescence spectroscopy

The fluorescence emission of Trp is sensitive to the polarity of the medium and can be used as an effective detector of conformational changes in proteins. When excited selectively at 290 nm,

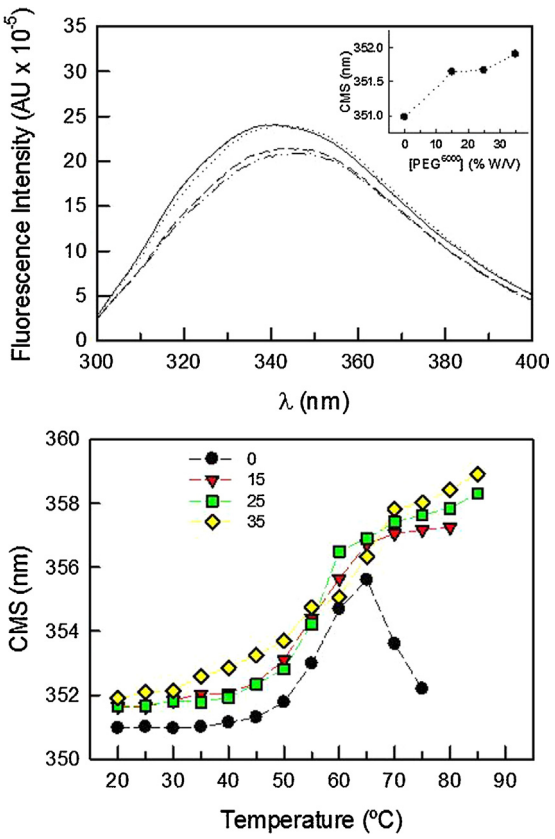

Fig. 2. Effect of PEG-induced molecular crowding on β-Gal fluorescence spectrum. (a) Spectra of 0.2 mg/ml (4.54 nM) solution of *E.coli* β-Gal with or without crowding agent are shown. Numbers in the graph refer to PEG^{6000} concentrations in %W/V. The inset shows the variation in the CMS at the different PEG^{6000} concentrations tested. (b) Effect of temperature on the CMS of the fluorescence emission spectrum at each PEG^{6000} concentration. Note that CSM values are based on the description of whole spectra; its reproducibility level is within ±0.01 nm.

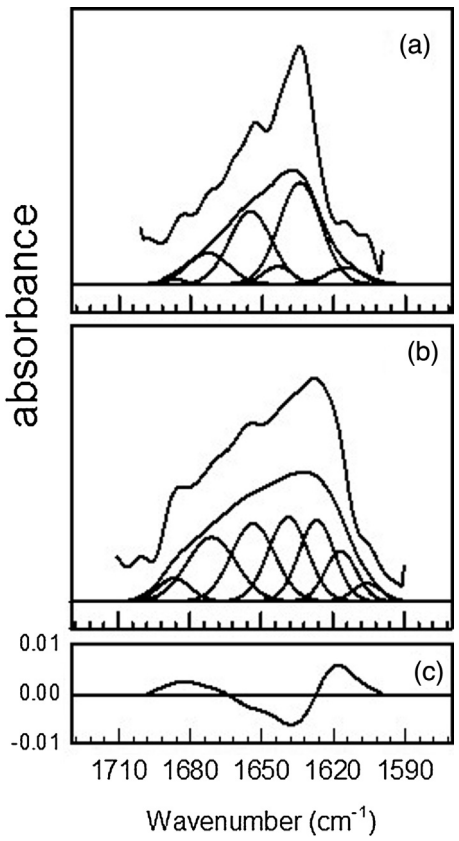

Fig. 3. Effect of PEG-induced molecular crowding on FT-IR spectrum of *E.coli* β-Gal. (a) 0% W/V PEG^{6000}; (b) 35% PEG^{6000}; (c) difference spectrum obtained by subtracting a normalized spectrum (a) from a normalized spectrum (b). Upper trace in panels (a) and (b) are deconvolved spectra using $K = 2$ and FWHH = 18 cm^{-1}; middle trace in each panel is non-deconvolved spectra. Gaussian curves below the middle traces are the spectral components obtained by curve fitting. The positions of the components are displayed in Table 1S.

it is the main contributor to the protein fluorescence [9]. β-Gal is a tetrameric protein with 39 Trps residues per protomer. In the absence of PEG^{6000}, the β-Gal fluorescence spectrum showed a CMS at around 350 nm (Fig. 2) which was compatible with Trp residues localized in an environment quite polar if compared to what was reported for Trp localized in a buried or non-polar medium ($\lambda_{CMS} \sim 330$ nm). In the presence of PEG^{6000}, at 24 °C, spectra exhibited a bathocromic shift and decrease in fluorescence emission intensity in a molecular crowding concentration dependent manner (Fig. 2a). A detailed analysis (insert of Fig. 2a), showed that CMS moved from 351 nm at 0 to 352 nm at 35% W/V PEG^{6000}. These results indicated that Trp residues became, on average, more accessible to the solvent suggesting that PEG-induced molecular crowding leads to at least a partial unfolding of β-Gal.

Results from thermally induced denaturation of β-Gal at different PEG^{6000} concentrations, studied from fluorescence emission spectra, are shown in Fig. 2b. In the absence of PEG^{6000} protein denaturation, evidenced a highly cooperative increase in CSM up to 65 °C. At higher temperatures a sudden decrease (blue shift) in CSM is observed. This could be due to self-aggregation of the thermally unfolded protein leading Trp residues towards a more hydrophobic environment provided by protein aggregates. The change in CSM became less cooperative as a function of PEG^{6000} concentration and tended to a plateau above 65. This suggests that PEG-induced molecular crowding inhibits the self-aggregation of thermally unfolded protein.

3.2.2. *Infrared spectroscopy*

β-Gal in D_2O was studied in the absence and in the presence of 35% W/V PEG^{6000} using FT-IR spectroscopy (Fig. 3). The amide *I′* band, between 1600 and 1700 cm^{-1}, mainly due to the C=O stretching of the peptide bonds, is shown. This spectrum region provides information on the secondary structure of the protein [11]. In the absence of crowding agent, the spectral shape resembles that of a protein with high content of β-sheet structure.

Fourier deconvolution of the spectra, shown in Fig. 3a and b, and the second derivative (not shown) allow the identification of the different band components. Fitting of the components to the original (not deconvolved) spectrum was essentially performed according to the procedure described in Nolan et al. [12].

In the absence of PEG^{6000}, six components were resolved (Fig. 3a). Their positions and contributions to the area of the amide *I′* band are shown in Table 1S. Bands have been assigned according to Byler and Susi [11]. The band centred at 1634 cm^{-1} corresponds to parallel β-chains. Unordered structures generate the band appearing at 1643 cm^{-1}. The band at 1655 cm^{-1} can be assigned to the α-helical structure. Turns and bends, including β-turns, are responsible for the bands at 1673, and 1683 cm^{-1} which may also be assigned to extended β-structures. These results are consistent with previous data from FT-IR reported by Arrondo et al. [13], as well as with the crystalline structure of β-Gal [14]. The β-Gal monomer is composed of five compact domains [14]. The first domain corresponds to a "jelli-roll" β-barrel, the second and forth domains are a fibronectin-type III β-sandwich fold, a topology that

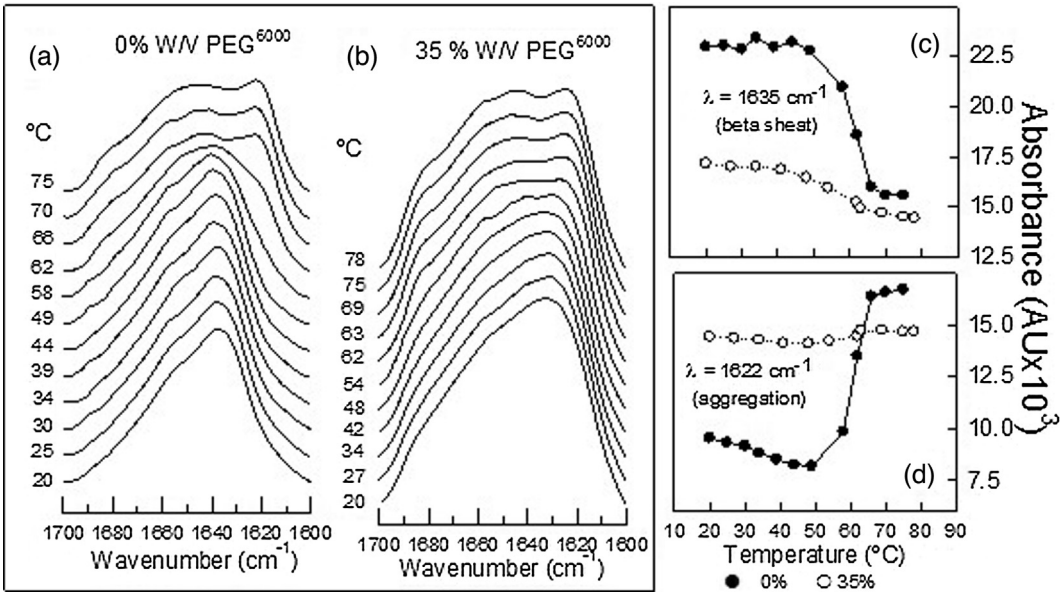

Fig. 4. Effect of PEG^{6000} on β-Gal thermal stability. FT-IR spectra in the absence (a) and in the presence (b) of 35% W/V PEG^{6000} at temperatures varying from 20 to 78 °C. Effect of PEG^{6000} on the absorbance at 1635 cm^{-1} (c) and 1620 cm^{-1} (d).

is similar to immunoglobulin constant domains. The third domain forms a distorted "TIM barrel" (a α/β barrel with 7 α helices and a distorted sixth parallel strand) and contains the catalytic site. The fifth domain consists of a 18-stranded antiparallel sandwich. The joint presence of β-parallel and α-helix features in the FT-IR spectrum reflect the α/β barrel domain.

In the presence of PEG^{6000} the FT-IR spectrum of β-Gal exhibits 7 bands (Fig. 3b), the absence of a band at 1634 cm^{-1} and a significant decrease in the proportion of α-helix content (the signal at 1655 cm^{-1} is shifted to 1658 cm^{-1} with a lowered intensity if compared with the spectrum in the absence of PEG^{6000}). Taken together this features suggests the unfolding of the TIM barrel leading to negative effects on the active site and thus in the catalytic activity. A significant increase in the contribution from 2 to 23% of the band at 1642 cm^{-1} (associated to unordered structures) also indicates a more relaxed structure of the protein in crowded media.

To provide a generalized picture of changes induced by the presence of PEG^{6000}, the area of the non-deconvolved spectra was normalized to 1 cm^{-1} between 1700 and 1600 cm^{-1} and the spectrum of the protein in absence of PEG^{6000} was subtracted from the spectrum in the presence of PEG^{6000}. A negative band was obtained centered at 1636 cm^{-1} revealing a strong decrease in the content of parallel β-chains and α-helix while the increase in the bands at 1682 and 1618 cm^{-1} indicates an increase in turns and bends as well as in low frequency β-components (Fig. 3c).

3.2.3. *β-Gal thermal stability studied by infrared spectroscopy*

FT-IR spectra of β-Gal were recorded as a function of temperature, in the absence (Fig. 4a) and in the presence of PEG^{6000} (Fig. 4b). In the absence PEG^{6000} the main β-structure band suffered an important and cooperative decrease at 1635 cm^{-1} (Fig. 4c) and an increase in the band at 1620 cm^{-1} (Fig. 4d). The later band has been assigned to intermolecular contacts and generally appears when unfolded chains are self-aggregated [15].

In the presence of 35% W/V PEG^{6000} changes in absorbance intensity at 1635 and 1620 cm^{-1} were less marked reflecting the polymer effect on preventing thermal-induced protein folding and aggregation.

4. Conclusions

The amount of free water in crowded solutions bears little resemblance to typical dilute solution experiments, and a reduction in free water volume may increase effective macromolecular concentrations or activities by several orders of magnitude. Increasing concentration, or decreasing water content, has a marked positive effect on concentrations and negative effect on diffusion, both of reactants and products. So, due to a combination of these opposite effects it is generally observed a resultant nonlinear change in V_{max}. In our system, whilst the V_{max} is just mildly affected by the presence of crowding agent, the ratio k_{cat}/K_M (where $k_{cat} = V_{max}/[E]_{tot}$), a parameter that is typically used to compare catalytic efficiency of enzymes, was strongly affected. Hence, the impairment in the diffusion prevailed over increasing the effective concentration of system components and a negative effect on the enzymatic activity was observed.

The affinity diminution we observed can also be a consequence of changes of water in our system as the crowding agent concentration is increased. As it was stated, molecular crowded systems are characterized by having large surfaces in contact with the solvent promoting an ordered water structure. Water structure enhancement produces a diminution in the hydrophobic effect; through this mechanism, interactions between the enzyme and the substrate can be affected. A direct enzyme-PEG binding phenomenon, already mentioned previously for other protein-PEG systems [16], might not be excluded. As a resultant both fluorescence as well as FT-IR spectroscopies revealed a protein structure more opened in the presence of PEG^{6000} compared with the conformation in its absence being the substrate binding domain the most affected. The latter would support the deleterious effect of PEG on K_M.

On the other hand, the effect of crowded systems on β-Gal thermal stability was noticeable being denaturation less cooperative and aggregation prevented.

Taken together our results showed that in crowded conditions β-Gal is thermodynamically trapped in a more open, flexible and stable conformation than in dilute solutions.

Acknowledgements

This work was partially supported by grants from SeCyT-UNC, ANPCyT and CONICET from Argentina. JMS, MVN and MAP are Career members of CONICET.

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.colsurfb.2015.11.003.

References

[1] D.L. Nelson, A.L. Lehninger, M.M. Cox, Lehninger Principles of Biochemistry, Worth, New York, 2000.
[2] E.W.F.W. Alton, U. Griesenbach, D.M. Geddes, Nat. Med. 4 (1998) 1121–1122.
[3] A. Hoskova, J. Sabacky, A. Mrskos, R. Pospisil, Arch. Dis. Child. 55 (1980) 304–305.
[4] M.C. Crescimbeni, V. Nolan, P.D. Clop, G.N. Marín, M.A. Perillo, Colloids Surf. B Biointerfaces 76 (2010) 387–396.
[5] R.J. Ellis, Trends Biochem. Sci. 26 (2001) 597–604.
[6] E.M. Clop, A.K. Chattah, M.A. Perillo, J. Phys. Chem. B 118 (2014) 6150–6158.
[7] J.M. Sanchez, V. Nolan, M.A. Perillo, Colloids Surf. B Biointerfaces 108 (2013) 1–7.
[8] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[9] J.R. Lakowicz, J. Kusba, W. Wiczk, I. Gryczynski, H. Szmacinski, M.L. Johnson, Biophys. Chem. 39 (1991) 79–84.
[10] J.R. Green, D. Margerison, Statistical Treatment of Experimental Data, 2nd revised ed., Elsevier Scientific, Amsterdam, Oxford, 1978.
[11] D.M. Byler, H. Susi, Biopolymers 25 (1986) 469–487.
[12] V. Nolan, M. Perduca, H.L. Monaco, B. Maggio, G.G. Montich, Biochimica Biophysa Acta 1611 (2003) 98–106.
[13] J.L.R. Arrondo, A. Muga, J. Castresana, C. Bernabeu, F.M. Goñi, FEBS Lett. 252 (1989) 118–120.
[14] R.H. Jacobson, X.J. Zhang, R.F. DuBose, B.W. Matthews, Nature 369 (1994) 761–766.
[15] J.L.R. Arrondo, J. Castresana, J.M. Valpuesta, F.M. Goni, Biochemistry 33 (1994) 11650–11655.
[16] H.X. Zhou, G. Rivas, A.P. Minton, Annu. Rev. Biophys. 37 (2008) 375–397.