Assessment of the Effect of Triton X-114 on the Physicochemical Properties of an Antibody Fragment

Luciana P. Malpiedi
Biochemical and Pharmaceutical Technology Dept./FCF, University of São Paulo. Av. Prof. Lineu Prestes, 580—Bloco 16, CEP 05508-000, São Paulo, Brazil

Physical Chemistry Dept., Faculty of Biochemistry and Pharmaceutical Sciences, National University of Rosario, Suipacha 570, S2002LRK, Rosario, Argentina

Bibiana B. Nerli
Physical Chemistry Dept., Faculty of Biochemistry and Pharmaceutical Sciences, National University of Rosario, Suipacha 570, S2002LRK, Rosario, Argentina

Dulcineia S. P. Abdalla
Dept. of Clinical Analysis and Toxicology, University of São Paulo, São Paulo, Brazil

Adalberto Pessoa Jr
Biochemical and Pharmaceutical Technology Dept./FCF, University of São Paulo. Av. Prof. Lineu Prestes, 580—Bloco 16, CEP 05508-000, São Paulo, Brazil

DOI 10.1002/btpr.1882
Published online in Wiley Online Library (wileyonlinelibrary.com)

The effect of Triton X-114 on the physicochemical properties of a single-chain antibody fragment (scFv) has been studied. According to the far UV circular dichroism spectroscopy, the secondary structure of the recombinant antibody was not significantly affected by the presence of Triton. From the antibody tertiary structure analysis, it was found that the surfactant could be located around the tryptophan molecules accessible to the solvent, diminishing the polarity of its environment but maintaining most of the protein structure integrity. However, in certain conditions of high temperature and high concentration of denaturant molecules, the presence of TX could compromise the antibody fragment stability. These results represent a previous step in designing scFv purification protocols and should be considered prior to developing scFv liquid–liquid extraction procedures. © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2014
Keywords: nonionic surfactant, single-chain antibody fragment, spectroscopic study

Introduction

Triton X-114 is a non ionic surfactant that belongs to the family of polyoxyethylene-based detergents. Its structure, usually abbreviated to C_8E_7, is presented in Figure 1.[1]

This detergent presents a high water solubility and above certain concentration, known as critical micelle concentration (cmc), the surfactant forms aggregates in which the hydrophobic (non polar) regions of the molecule flock to the interior in order to minimize their contact with water, and the hydrophilic groups remain on the outer surface in order to maximize their contact with the solvent.[2] Upon temperature increment, micellar solutions of Triton X-114 become turbid and the micelles become immiscible with water, thus, forming a surfactant-rich phase that separates from a surfactant-poor phase.[3]

In 1981 Bordier[4] demonstrated that proteins selectively partitioned between the different Triton X-114 micellar phases. Since then, numerous compounds have been recovered by using a similar methodology. For example, lipase was successfully purified ($R > 85\%$) from *Burkholderia sp* by Ooi et al.[5] while Kumar et al.[6] used an affinity based reverse micellar extraction technique to separate bromelain from pineapple (*Ananas comosus* L. Merryl) waste. Mathias et al.[3] were also successful at purifying liver integral membrane proteins by applying a Triton X-114 based micelles methodology and Patchornik et al.[7] achieved the purification of bacterial membrane proteins using engineered micelles.

Particularly, our research group presents a wide experience in using aqueous micellar two phase systems (AMTPS) to extract and purify biological molecules. For example, clavulanic acid was successfully purified ($R > 85\%$) from *Streptomyces clavuligerus* by Carvalho et al.[8] and Haga et al.[9] Besides, Lopes et al.[10] used this type of system to remove lipopolysaccharide endotoxins from *E. coli* and Jozala et al.[11] achieved the purification of nisin from *Lactobacillus lactis*. The AMTPS is a simple methodology which consists

Additional Supporting Information may be found in the online version of this article.

Correspondence concerning this article should be addressed to L. P. Malpiedi at lucianapelleg@yahoo.com.ar

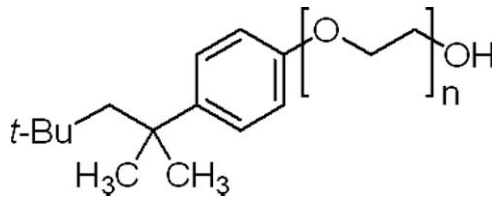

Figure 1. Schematic presentation of Triton X-114 structure.

in mixing the complex sample which contains the target molecule with a surfactant solution. After heating the system above certain temperature, the phase separation takes place and the target protein partitions into the phases according to its physic-chemical properties.

At present, we are interested in applying this technique to purify an anti-electronegative LDL single-chain antibody fragment (scFv) from *Pichia pastoris* supernatant.[12] ScFv molecules present several advantages respect to full-sized antibodies, such as, easy manipulation, high permeability and the fact that their immune-complexes can be cleared quickly from the human body.[13] Nevertheless, some drawbacks of scFv application are their low pharmacokinetic and poor stability.[13,14]

Pioneering stability studies, carried out by Wörn and Plücktum,[15,16] have demonstrated that scFv stability is highly dependent on its sequence as well as on the medium conditions. Other analyses, accomplished by Ewert et al.,[17] have showed that scFv hydrophobic patches accessible to solvent were responsible for protein aggregation and loss of structural stability.

Because the protein integrity maintenance comprises one of the most important points to be considered in any downstream process,[18,19] the main objective of this work was to obtain information about the effect of the non-ionic surfactant Triton X-114, above its cmc (0.009% w/w in water at 23°C), on the physicochemical properties of a single-chain antibody fragment (scFv) by means of spectroscopic measurements. Triton X-114 was selected since this surfactant presents the following advantages:—the low temperature required to separate the phases, close to ambient temperature,[20] and —the absence of a strong electrostatic interaction, which is reported as the main cause for surfactant-based protein denaturation[21] when using ionic surfactants.

Additionally, this work also aims to evaluate the impact of Triton X-114 on the scFv structure as a first step prior to developing recombinant antibody fragment purification protocols.

Materials and Methods

Chemicals

Triton X-114 (TX), 1-anilino-8-naphthalene sulfonate (ANS) and acrylamide were purchased from Sigma–Aldrich and used without further purification.

Biologicals

The recombinant *Pichia pastoris* SMD 1168 (Δpep4::URA3 Δkex::SUC2 his4 ura3, phenotype His− Mut+), an anti-LDL electronegative his-tagged single-chain antibody fragment (scFv) producing yeast (Invitrogem), was kindly provided by Professor Dulcineia Saes Parra Abdalla from the São Paulo University (Brazil) and stored at −70°C with glycerol 20 wt%.[12]

ScFv purification

The anti-LDL His-tagged scFv was purified from *Pichia pastoris* culture supernatant by using Ni-Sepharose 6 Fast Flow resin following the protocol provided by GE Health-Care®. The eluted sample, containing 80% of the initial scFv content with a purity superior to 90% according to electrophoreses analysis (data not shown), was extensively dialyzed against phosphate buffered saline (PBS) solution pH 7.2 before been used. Besides, the specificity and affinity of the purified antibody fragment against LDL(-) was confirmed by ELISA.[12]

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of scFv in solution (9.6 μM), without and with TX (0.5% w/w), were performed in a Jasco J-810 spectropolarimeter using a thermostatized cuvette (20°C) of 1 mm of pathlength. The scan rate was of 50 nm min^{-1} and the data acquisition were performed with a bandwidth of 1 nm. In all cases, 10 scans were made. The signal corresponding to the medium components was subtracted in all the assayed conditions.

Fluorescence spectroscopy

Fluorescence measurements were performed on an Aminco Bowman S2 spectrofluorometer with a thermostated circulating water bath attached. Maximum error of fluorescence measurements was of ±5%.

Effect of Triton X-114 on the Native Protein Fluorescence. Fluorescence measurements were performed using a thermostated (20°C) cuvette of 1 cm of optical pathway and a bandwidth of 2 nm. The scan rate was of 100 nm min^{-1}. The protein (1.6 μM) was excited at 294 nm and the emission was recorded from 300 to 420 nm. Emission fluorescence spectra were corrected using the software supplied by the manufacturer and the signal corresponding to the medium components was subtracted in all the assayed conditions. The fluorescence emission maximum was determined by fitting the fluorescence emission spectra to a Gaussian function. The measurements were made in triplicate.

Native Protein Fluorescence Quenching by Acrylamide: Effect of Triton X-114 Presence. The quenching of the native protein fluorescence (scFv 1.6 μM; $\lambda_{excitation}$ 294 nm, $\lambda_{emission}$ 320 nm) was carried out by titration with acrylamide (10 μL aliquots of a 4M solution) in absence and presence of Triton X-114 (1.0 % w/w) at 20°C. The signal corresponding to the medium components was subtracted in all the assayed conditions and the measurements were made by triplicate.

The data were analyzed using the "sphere of action" mathematical model according to Lakowicz[22]:

$$\frac{F_0}{F} = (1 + Ksv[Q])e^{([Q]V)} \quad (1)$$

where F_0 and F are the protein fluorescence in absence and presence of the quencher, respectively; *K*sv, the Stern Volmer constant; *[Q]*, the quencher concentration; and *V*, a constant. The Ksv, considered as the dynamic component of the quenching, is related to both the fluorophore lifetime and

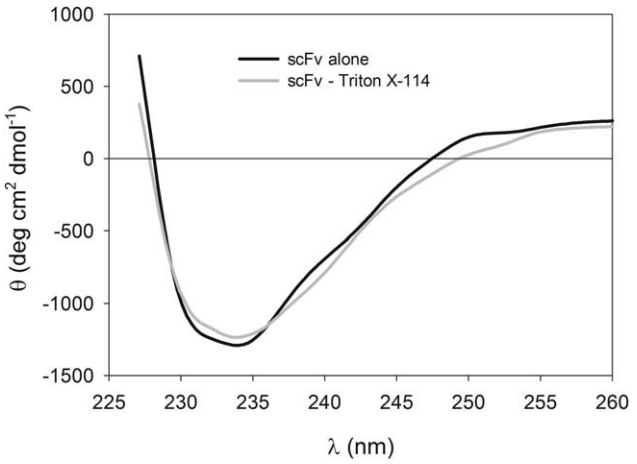

Figure 2. Effect of Triton X-114 (0.5% w/w) on scFv far UV spectrum. Protein concentration: 9.6 μM. Medium: phosphate buffered saline solution 10 mM, pH 7.2. Temperature: 20°C. Repetitive scanning of ten cycles was used.

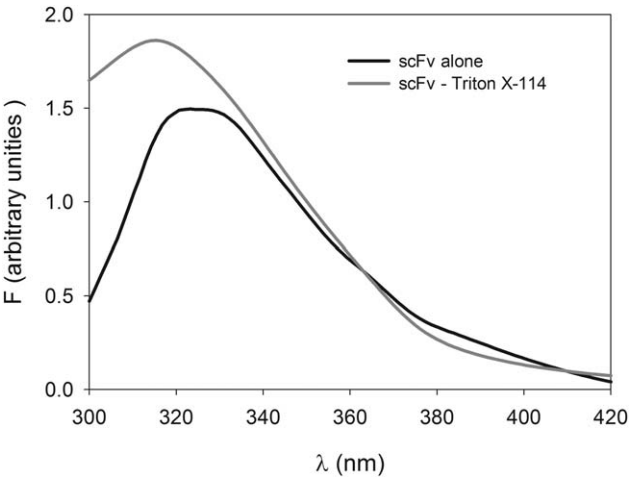

Figure 3. Effect of Triton X-114 (1.0 % w/w) on scFv native fluorescence. Protein concentration: 1.6 μM. Medium: phosphate buffered saline solution 10 mM, pH 7.2. Temperature: 20°C. $\lambda_{excitation}$: 294 nm.

the bimolecular quenching constant. The constant V is considered the static component of the quenching[23] and is related to v, the volume of the "sphere of action" through the following equation:

$$V = \frac{N_A V}{1000} \quad (2)$$

where N_A is the Avogadro number and v is the volume of the sphere that surrounds the fluorophore within which it can be considered in contact with the quencher making the probability of immediate quenching be unity. With the aid of the software Sigma Plot 10 Copyright © 2006 Systat Software, Inc; the V and Ksv constants were obtained by non linear fitting of experimental data ([Q], F) to the Eq. 1.

Measurements of the Protein Relative Surface Hydrophobicity (S^0). The relative surface hydrophobicity of the protein was determined by using the fluorescent probe ANS, as it was previously reported.[24] Aliquots (from 0 to 200 μL) of scFv solution (final concentration from 0 to 3.6 μg mL^{-1}) were added to a 3-mL initial sample containing ANS 40 μM in sodium phosphate buffer 50 mM pH 7.40.

The fluorescence emission intensity at 470 nm at 20°C was measured when excited at 382 nm. The relative fluorescence (F_{rel}) at each protein concentration was calculated by subtracting the initial signal (without protein) which contains response from the medium components. Therefore, the slope of the F_{rel} vs. protein concentration plot was determined and considered to be correlated to the protein relative surface hydrophobicity (S^0).[25] The same procedure (protein concentration from 0 to 3.6 μg/mL, $\lambda_{excitation}$ 382 nm, $\lambda_{emission}$ 470 nm) was repeated for scFv in presence of surfactant (by including TX 1.0% at the initial sample) and for two model proteins: bovine serum albumin (BSA) and trypsin (TRY) in absence of TX. All the measurements were made in triplicate.

ScFv thermal and structural stability

Thermal stability of the protein was assayed by incubating scFv in PBS solutions (1.6 μM), without and with TX (1.0% w/w), at 4, 20, and 40°C. In each case the fluorescence spectra (see Effect of Triton X-114 on the Native Protein Fluorescence section) were determined at different incubation times (2 and 4 h).

The structural stability of the antibody was evaluated by measuring the protein fluorescence spectra (see Effect of Triton X-114 on the Native Protein Fluorescence section) in media of increasing urea concentration (0–10 M), in absence and presence of TX (1.0% w/w) at 20°C. The percentage progress of the unfolding process was estimated through the ratio, in which $\Delta\lambda_{obs}$ is the increase in the wavelength of the emission peak caused by denaturant at a given concentration and $\Delta\lambda_{max}$ is the limit $\Delta\lambda_{obs}$ value when the protein is completely denatured. The maximum fluorescence emission was determined by fitting the fluorescence emission spectra to a Gaussian function.

The signal corresponding to the medium components was subtracted in all the assayed conditions and the measurements were made in triplicate.

Effect of TX on medium viscosity

The viscosity experiments were carried out in a Brookfield DV-II viscosimeter, the temperature was maintained constant at 25°C (±0.1°C). Measurements were carried out at a shear rate of 10 s^{-1} (Newtonian behavior) and repeated at least three times for each solution. The accuracy of the viscosity determination is within 1%.

RESULTS

Circular dichroism spectroscopy

Circular dichroism spectra of proteins are known to be sensitive to protein structure.[26] Figure 2 shows the effect of TX (0.5 % w/w) on the secondary structure of scFv in solution. Unfortunately, remaining imidazole molecules from the elution buffer used in scFv affinity purification (see ScFv Purification section), caused a high background around 200 nm, hindering the spectral analysis at wavelength lower than 220 nm.[27] Nevertheless, the data collected in the range of 225–260 nm show a negative band with a minimum at 235 nm. This has been previously observed for other scFv

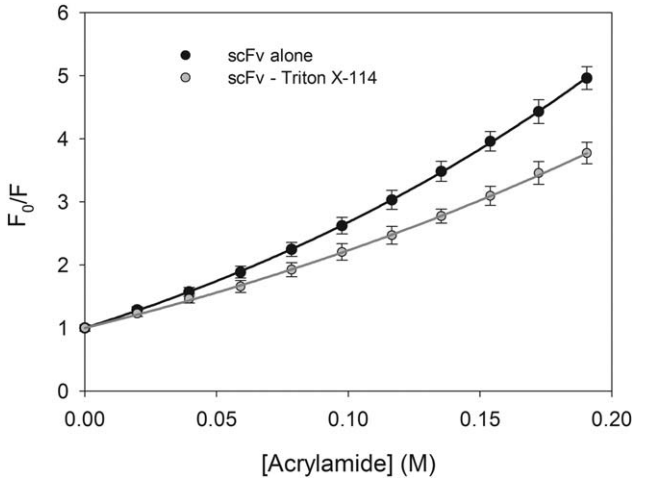

Figure 4. Stern–Volmer plots for the quenching of the native fluorescence emission of the scFv by acrylamide. Protein concentration: 1.6 μM. Medium: phosphate buffered saline solution 10 mM, pH 7.2. Temperature: 20°C. $\lambda_{excitation}$: 294 nm, $\lambda_{emission}$: 320 nm.

molecules[28–30] and assigned to the contributions from the aromatic and/or cystinyl side-chains within the far-UV. For example, Baden et al. reported a similar minimum at 235 nm for V_L domain,[29] which was attributed to the interaction of the aromatic residues with the conserved Trp35. This pattern has been only observed in β-proteins since in $\alpha/\alpha+\beta-$proteins the 230–235 nm band would be hidden by the alpha-helix negative peak (228 nm). This finding agrees with the β-rich characteristic of other antibody fragments.[31] The presence of TX did not modify the CD signal significantly, which suggest that the conformation was not altered (Figure 2).

Fluorescence spectroscopy

Effect of Triton X-114 on the Native Protein Fluorescence. Fluorescence measurements were used to monitor eventual changes on the tertiary structure produced by the protein interaction with surfactant. It is a well-known fact that the characteristics of fluorescence signal are highly dependent on the environment of the fluorophore molecule. Particularly, the native protein fluorescence, which is mainly dominated by the tryptophan emission, is sensitive to changes in the polarity of this residue environment.[32,33] Modifications in either the position or the orientation of the tryptophan residues, caused by protein interaction with surfactant, could alter their exposure to solvent and therefore, modify the quantum yield and/or the position of the emission peak. Figure 3 shows the native fluorescence spectra obtained for scFv when excited at 294 nm. At this condition, tryptophan residues dominate the protein fluorescence because both their absorbance at the wavelength of excitation and their quantum yield of emission are considerably higher than the respective values of the rest of amino acids able to have fluorescence emission such as tyrosine and phenylalanine.[22] A main peak at 324 nm, observed in buffer medium (without TX), indicates that the tryptophan residues, responsible for fluorescence emission, possess low exposure to solvent. The surfactant presence induces both an enhancement of fluorescent intensity and a significant blue shift on the position of emission peak (315 nm).This behavior could be attributed to an approximation of surfactant molecules to the tryptophan

Table 1. Dynamic and Static Quenching Constants Calculated According to Eq. (1), for the Acrylamide-based Extinction Process of scFv Fluorescence Without and with TX 0.5 % w/w

	scFv Alone	scFv—TX
*K*sv (M^{-1})	10.64 ± 0.15	8.24 ± 0.25
V (M^{-1})	2.60 ± 0.06	2.00 ± 0.10
R (Å)	10.1	9.3

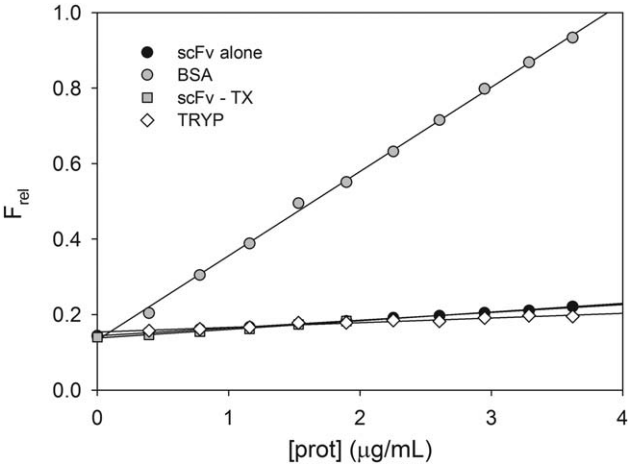

Figure 5. Variation in fluorescence intensity of ANS (40 μM) with protein concentration in the range 0–3.6 μg mL^{-1}. Medium: sodium phosphate buffer 50 mM, pH 7.4. Temperature: 20°C. $\lambda_{excitation}$: 382 nm, $\lambda_{emission}$: 470 nm.

neighbors which produces an increment in the hydrophobic character of the environment. Besides, the presence of TX (1% w/w) produced an increase of the medium viscosity from 1.02 to 1.52 mPa s^{-1}. This fact should be responsible of the fluorescence enhancement by decreasing the probability of nonradiant de-excitation processes.[22]

Effect of Triton X-114 on the Native Protein Fluorescence Quenching by Acrylamide. Acrylamide quenching has been used to investigate the microenvironments of tryptophan residues in different proteins.[34] This molecule is able to diffuse into the interior of the protein, thus testing both the degree of tryptophan accessibility to the solvent and any modification at the tryptophan microenvironment induced by a cosolute presence.

In this work, scFv was titrated with acrylamide in absence and presence of TX at a concentration of 1.0% w/w. Figure 4 shows the obtained quenching data expressed as Stern–Volmer plot. Positive deviations from the linear behavior were observed for both conditions, suggesting the coexistence of dynamic and static quenching.[23] Generally, this pattern is observed when the extent of quenching is large. It is interpreted as an instantaneous tryptophan quenching by the immediately adjacent quencher molecules. Therefore, the analysis of the quenching data was made by using a modified Stern Volmer equation, based on the sphere of action model (see Eq. (1)). Table 1 summarizes the values of quenching constants calculated from the mentioned equation. As it can be noticed, the presence of surfactant produced a decrease of the Stern–Volmer constant (K_{sv}). This parameter is directly related to the acrylamide diffusion coefficient and depends on its friction coefficient. The observed decrease in K_{sv} could be then assigned to an increase in viscosity caused

Table 2. Comparative Values of Relative Surface Hydrophobicity of scFv and Model Proteins

Protein	S^0 (10^{-3} μg^{-1} mL^{-1})
scFv alone	20.0 ± 3.0
scFv—TX	22.0 ± 1.3
TRYP	12.0 ± 1.2
BSA	223.0 ± 2.7

Table 3. Changes on Fluorescence Emission Spectra of scFv in Absence and Presence of TX (1.0 % w/w) at Different Temperatures

	Fluorescence Emission			
	scFv Alone		scFv—TX	
Incubation Condition	Peak Position	Fluorescence Intensity	Peak Position	Fluorescence Intensity
2 h—4°C	323.1	1.690	315.1	2.096
4 h—4°C	322.8	1.774	315.2	2.280
2 h—20°C	322.1	1.495	315.5	1.920
4 h—20°C	323.5	1.556	315.5	1.928
2 h—40°C	319.2	1.739	315.7	1.465
4 h—40°C	316.1	1.822	320.3	1.052

by the surfactant presence, which slows the diffusive process of the quencher towards the protein fluorophores.

On the other hand, from V static constant, the radius of the "sphere of action" was calculated through the Eq. (2). Both values (10.1 and 9.3 Å) are slightly higher than the sum of van der Waals radii of indol and acrylamide (6–7 Å),[22] supporting the existence of a contact interaction at the instant of tryptophan excitation, thus validating the selected quenching model. The slight decrease in V, observed in TX presence, indicates that the proximity between TX molecules and tryptophan decreases the probability the quencher being within the volume V at the time of excitation.[22]

Measurements of the Relative Surface Hydrophobicity (S^0). Figure 5 shows the ANS fluorescence intensity (F_{rel}) vs. protein concentration for all the assayed conditions. Besides, the S^0 values estimated according to Measurements of the Protein Relative Surface Hydrophobicity (S^0) section are presented in Table 2. Taking into account that this methodology allows to obtain only relative values, the same assay was carried out with two well-known model proteins (BSA and TRYP) in order to compare them each other. The S^0 values observed for these proteins show that BSA exhibits a significantly higher hydrophobic character than TRYP does. These results agree with reports on hydrophobic scales of proteins determined by alternative methods such as partitioning in aqueous two phase systems, fluorescence determination of energy transfer efficiency and use of cis-parinaric acid as probe.[35] Moreover, scFv presented a S^0 value similar to that of TRYP, suggesting a superficial character predominantly hydrophilic for this antibody fragment. On the other hand, the presence of surfactant did not modify significantly the scFv relative surface hydrophobicity (S^0) suggesting that the interaction between the recombinant antibody and TX does not generate a significant change in the protein surface properties.

Thermal stability

The effect of TX on scFv thermal stability was analyzed by the native fluorescence emission (See Supplementary Figures 1 and 2). Table 3 shows the spectrum parameters obtained at different incubation conditions.

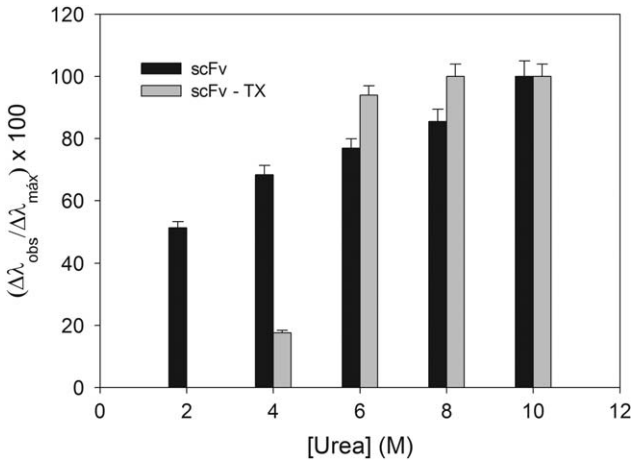

Figure 6. ScFv structural stability curves in absence and presence of Triton X-114 at a concentration of 1.0% w/w. Protein concentration: 1.6 μM. Medium: phosphate buffered saline solution 10 mM, pH 7.2. Temperature: 20°C. $\lambda_{excitation}$: 294 nm.

In absence of TX, practically no changes in the position of the emission peak are observed when incubating scFv at 4 and 20°C for at least 4 h. This indicates that no significant modifications on tertiary protein structure affecting the tryptophan microenvironment are taking place. However, a decay of fluorescence signal is observed when increasing temperature. It can be assigned to the increase in the temperature-dependent collisional de-excitation phenomena. The mentioned trend is not maintained when incubating at 40°C. A blue shift of the peak and a slight enhancement of fluorescence intensity are observed after 2 and 4 h of incubation, thus suggesting a decrease in the environmental polarity of the tryptophan residues accessible to solvent. This behavior could be attributed to the interaction between hydrophobic patches of different scFv molecules which would be more exposed after heating at 40°C, thus forming scFv aggregates (See Supplementary Figure 3).[17]

The presence of TX shifts the emission peak to lower wavelengths and increases the fluorescence, as it was discussed in Effect of Triton X-114 on the Native Protein Fluorescence section. However, after this initial effect no additional changes are observed after incubating at 4, 20°C for 2 and 4 h. This suggests that the tertiary structure of scFv is not being affected by the surfactant presence at the assayed conditions. When the temperature rises to 40°C and the incubation time extends to 4 h, a red shift of the emission peak and a decrease of fluorescence are observed, thus suggesting a higher exposure of tryptophans with the consequent increase in its environment polarity. This behavior at 40°C does not represent any significant disadvantages in designing purification protocols since the temperature of phase separation in AMTPSs formed by TX-114 is around 24–27°C.[20]

Structural stability

Addition of small molecules on protein aqueous solutions can affect the protein stability, structure, and function.[36] Particularly, the chemical denaturation with an agent such as urea is one of most widespread ways to assess protein stability. At present, the molecular mechanism for urea's ability to denature proteins is not completely known. However, different reports based on molecular dynamics simulations[37] have suggested urea to denature directly by binding to the protein, or indirectly, by altering the solvent environment.

Figure 6 shows the effect of TX presence on the scFv unfolding by urea. The first step in unfolding is the expansion of the hydrophobic core, and then, its solvation by water. The red shift ($\Delta\lambda_{obs}$) of the fluorescence peak at any denaturant concentration presents a direct relationship with the degree of exposition of fluorescent amino acids and therefore, with the progress of protein denaturation.[32] In this context, this parameter ($\Delta\lambda_{obs}$) related to its maximum value when the denaturation is completed ($\Delta\lambda_{max}$) was used to follow the process.

It can be appreciated that denaturation patterns in absence and presence of TX show cooperative behavior, characteristic of this type of structural transition. In absence of surfactant, the major enhancement of $\Delta\lambda_{obs}/\Delta\lambda_{max}$ is observed in the range of urea 0–2*M*, which is followed by a constant increment of the study parameter, suggesting a non two-state unfolding mechanism.[17]

In presence of TX a different behavior was observed. At urea 4–6*M*, a drastic enhancement of $\Delta\lambda_{obs}/\Delta\lambda_{max}$ is obtained, but without showing further significant shifts. These results evidenced that in presence of surfactant, scFv unfolding is delayed, but the maximum denatured state is reached at lower urea concentration.

Discussion

Most of purification protocols imply the use of several separative steps which could lead to protein denaturation. In this context, new extractive methodologies that present a reduced number of purification stages and therefore shorter time consumptions are being evaluated. One of them is the liquid–liquid extraction with aqueous micellar two-phase systems (AMTPS). Non-ionic surfactants, such as TX, are the most widely used due to its low protein denaturation index.[21] However, due to the fact that several works have reported loss of activity and/or structure after the protein being in contact with non-ionic surfactant,[38,39] the effect of the detergent on the protein stability should be studied as a first important step prior to developing the extraction process.

In this work, the effect of Triton X-114 (TX) on structural features of a single-chain antibody fragment (scFv) was evaluated as a first attempt to search for scFv purification conditions by using liquid–liquid extraction with AMTPS.

First, the scFv secondary structure was study by far UV CD spectroscopy. This analysis showed a pattern observed for other proteins containing predominantly β-sheet secondary structure, agreeing with other author works which confirm that these antibody formats are β-rich proteins.[16,30] Alternatively, Umetsu et al.[18] have demonstrated that the formation of β-sheet could represent an early step in scFv aggregation, suggesting that the antibody fragment solution should be rigorously controlled in order to avoid protein nucleation.

In presence of TX, practically no changes in scFv CD spectra was found (Figure 2), suggesting that this protein retained much of its native secondary structure. Other non-ionic surfactants, such as Tween's serie and Triton X-100, have demonstrated to have low impact in the native secondary structure of different proteins.[39,40]

The antibody tertiary structure was analyzed by different fluorescence measurements. According to scFv native fluorescence spectra, TX could be located around the tryptophan molecules accessible to the solvent, diminishing the polarity of its environment.[22] These results agreed with those obtained from quenching experiments, which showed that surfactant molecules would act as barriers to the attack of the acrylamide molecules.[34,39,40] The affinity of non ionic surfactants for hydrophobic patches accessible to solvent has already been demonstrated by other authors. For example, Tween 40 presents a high affinity for the molten globule state of hGh because of the numerous exposed hydrophobic regions.[39] Similar results were reported by Doñate et al.[40] for the interaction between aspartate aminotrasferase and Triton X-100. In both examples, the binding between the protein hydrophobic patches and surfactant did not resulted in protein destabilization. Indeed, it was suggested that the surfactants could act as a chaperonines.[11] These findings also agree with the results presented in Table 1 of this manuscript, which suggest that the surfactant did not increase the accessibility of the TRP and with the results exposed in Table 2, which shows that the relative surface hydrophobicity of scFv resulted to be practically unaffected by TX.

Thermal and structural stability of the scFv have also been accessed. According to Table 3, scFv fluorescence spectrum was significantly affected by the presence of TX at 40°C, shifting the emission maximum toward longer wavelength. That behavior could be attributable to a stronger interaction between the surfactant and the thermally unfolded recombinant antibody as a consequence of a major exposure of hydrophobic regions in comparison to the native scFv, thus favoring the denatured state. A similar behavior has been reported for the interaction between Triton X-100 and different isozymes.[40]

As far as the structural stability is concerned, it was observed that the chemical unfolding of scFv resulted to be a cooperative process and TX significantly affected the denaturation mechanism (Figure 6). The study of scFv chemical denaturation has already been approached by other authors[17,41,42] and as it was reported, scFv stability depends on the intrinsic stability of their variable domains (V_H and V_L) and on the interaction between them.[42] Indeed, it was demonstrated that slight changes in the structure of one of the scFv domains, can modify the unfolding profile of the whole antibody fragment.[17] In this context, it could be suggested that the interaction between hydrophobic scFv regions and TX may modify the complete antibody structural stability, favoring the total protein denaturation at low urea concentration.

Conclusion

We conclude that the surfactant Triton X-114 does not modify extensively the secondary structure of the single-chain antibody fragment. Besides, it was found that the surfactant could be located around the tryptophan molecules accessible to the solvent, diminishing the polarity of its environment but maintaining most of the protein structure integrity. However, in certain conditions of high temperature and high concentration of denaturant molecules, the presence of TX could compromise the antibody fragment stability. In this context, these work conditions should be avoided in order to maintain the antibody integrity.

These results represent a previous step in designing scFv purification protocols and will be considered prior to developing scFv liquid–liquid extraction procedures. Besides, it is worth of consideration the possible extension of the present study to others non ionic surfactants and scFv molecules.

Acknowledgments

This work has been supported by the following grants: Process N° 2011/20521-0 (FAPESP) and the International Cooperation Program FAPESP-CONICET 2012/2014.

Literature Cited

1. Sánchez-Ferrer A, Pérez-Gilabert M, Núñez E, Bru R, García-Carmona F. Triton X-114 phase partitioning in plant protein purification. *J Chromatogr A*. 1994;668:75–83.
2. Rangel-yagui CO, Pessoa A, Blankschtein D. Two-phase aqueous micellar systems—an alternative method for protein purification. *Braz J Chem Eng*. 2004;21:531–544.
3. Mathias RA, Chen Y, Kapp EA, Greening DW, Mathivanan S, Simpson RJ. Triton X-114 phase separation in the isolation and purification of mouse liver microsomal membrane proteins. *Methods*. 2011;54:396–406.
4. Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem*. 1981;256:1604–1607.
5. Ooi CW, Hii SL, Kamal SMM, Ariff A, Ling TC. Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from *Burkholderia pseudomallei*. *Process Biochem*. 2011;46:68–73.
6. Kumar S, Hemavathi AB, Hebbar HU. Affinity based reverse micellar extraction and purification of bromelain from pineapple (*Ananas comosus L. Merryl*) waste. *Process Biochem*. 2011;46: 1216–1220.
7. Patchornik G, Danino D, Kesselman E, Wachtel E, Friedman N, Sheves M. Purification of a Membrane protein with conjugated engineered micelles. *Bioconjug Chem*. 2013;24:1270–1275.
8. Carvalho V, Andréia F, Converti A, Pessoa A. Liquid–liquid extraction by mixed micellar systems: a new approach for clavulanic acid recovery from fermented broth. *Biochem Eng J*. 2011; 56:75–83.
9. Haga RB, Santos-Ebinuma VC, de Siqueira Cardoso Silva M, Pessoa A, Rangel-Yagui CO. Clavulanic acid partitioning in charged aqueous two-phase micellar systems. *Sep Purif Technol*. 2013;103:273–278.
10. Lopes AM, Santos-Ebinuma VC, Celia L, Novaes DL, Dutra JV. LPS–protein aggregation influences protein partitioning in aqueous two-phase micellar systems. *Appl Microbiol Biotechnol*. 2013;97:6201–6209.
11. Jozala AF, Lopes AM, Mazzola PG, Magalhaes PO, Vessoni Penna TC, Pessoa Jr A. Liquid–liquid extraction of commercial and biosynthesized nisin by aqueous two-phase micellar systems. *Enzyme Microb Technol*. 2008;42:107–112.
12. Kasuma SM, Cavalcante MF, Telles AER, Maranhao AQ, Abdalla DSP. Cloning and expresion of an anti-LDL(-) single-chain variable fragment, and its inhibitory effect on experimental atherosclerosis. *Landes Biosci mAbs*. 2013;5:563–575.
13. Weisser NE, Hall JC. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. *Biotechnol Adv*. 2009;27:502–520.
14. Malpiedi LP, Díaz CA, Nerli BB, Pessoa A. Single-chain antibody fragments: purification methodologies. *Process Biochem*. 2013;48:1242–1251.
15. Wörn A, Plückthun A. Different equilibrium stability behavior of ScFv fragments: identification, classification, and improvement by protein engineering. *Biochemistry*. 1999;38:8739–8750.
16. Wörn A, Plückthun A. Stability engineering of antibody single-chain Fv fragments. *J Mol Biol*. 2001;35:989–1010.
17. Ewert S, Huber T, Honegger A, Plu A. Biophysical properties of human antibody variable domains. *J Mol Biol*. 2003;2836: 531–553.
18. Umetsu M, Ashish K, Tsumoto K, Kumagai I. Pathway to insoluble aggregates on the refolding of a single-chain Fv antibody: morphological changes of aggregated protein on refolding. *Chem Lett*. 2004;33:1600–1601.
19. Valetti NW, Boeris V, Picó G. Characterization of chymotrypsin-carrageenan complex in aqueous solution: a solubility and thermodynamical stability study. *Int J Biol Macromol*. 2013;52:45–51.
20. Fischer I, Franzreb M. Direct determination of the composition of aqueous micellar two-phase systems (AMTPS) using potentiometric titration—a rapid tool for detergent-based bioseparation. *Colloids Surf A*. 2011;377:97–102.
21. Otzen D. Protein—surfactant interactions: a tale of many states. *BBA Proteins Proteom*. 2011;1814:562–591.
22. Lakowicz JR, Jhonson ML, Joshi N, Gryczynski I, Laczko G. Transient effects in quenching detected by harmonic-content frequency-domain fluorometry. *Chem Phys Lett*. 1986;131:343–348.
23. Geddes CD. Optical halide sensing using fluorescence quenching: theory, simulations and applications—a review. *Meas Sci Technol*. 2001;12:R53–R88.
24. Malpiedi LP, Picó G, Nerli BB. Features of partitioning pattern of two pancreatic enzymatic precursors: trypsinogen and chymotrypsinogen in polyethyleneglycol–sodium citrate aqueous biphasic systems. *J Chromatogr B*. 2008;870:1–7.
25. Haskard CA, Li-chan ECY. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS^-) fluorescent probes. *J Agric Food Chem*. 1998;46,2671–2677.
26. Corrêa DHA, Ramos CHI. The use of circular dichroism spectroscopy to study protein folding, form and function. *J Biochem*. 2009;3:164–173.
27. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. *Biochim Biophys Acta*. 2005;1751:119–139.
28. Marín-Argany M, Rivera-Hernández G, Martí J, Villegas S. An anti-Aβ (amyloid β) single-chain variable fragment prevents amyloid fibril formation and cytotoxicity by withdrawing Aβ oligomers from the amyloid pathway. *Biochem J*. 2011;437:25–34.
29. Baden EM, Owen BL, Peterson FC, Volkman BF, Ramirez-Alvarado M, Thompson JR. Altered dimer interface decreases stability in an amyloidogenic protein. *J Biol Chem*. 2008;283: 15853–15860.
30. Sreerama N, Manning MC, Powers ME, Zhang JX, Goldenberg DP, Woody RW. Tyrosine, phenylalanine, and disulfide contributions to the circular dichroism of proteins: circular dichroism spectra of wild-type and mutant bovine pancreatic trypsin inhibitor. *Biochemistry*. 1999;38:10814–10822.
31. Ramm K, Gehrig P, Plückthun A. Removal of the conserved disulfide bridges from the scFv fragment of an antibody: effects on folding kinetics and aggregation. *J Mol Biol*. 1999;290:535–546.
32. Burstein EA, Vedenkina NS, Ivkova MN. Fluorescence and the location of tryptophan resudues in protein molecules. *Photochem Photobiol*. 1973;18:263–279.
33. Boeris V, Farruggia B, Nerli B, Romanini D, Picó G. Protein-flexible chain polymer interactions to explain protein partition in aqueous two-phase systems and the protein–polyelectrolyte complex formation. *Int J Biol Macromol*. 2007; 41:286–294.
34. Boeris V, Romanini D, Farruggia B, Picó G. Interaction and complex formation between catalase and cationic polyelectrolytes: chitosan and Eudragit E100. *Int J Biol Macromol*. 2009; 45:103–108.
35. Tubio G, Nerli B, Pico G. Relationship between the protein surface hydrophobicity and its partitioning behaviour in aqueous two-phase systems of polyethyleneglycol–dextran. *J Chromatogr B*. 2004;799:293–301.
36. Timasheff SN. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annu Rev Biophys Biomol Struct*. 1993;22: 67–97.
37. Canchi DR, Paschek D, García AE. Equilibrium study of protein denaturation by urea. *J Am Chem Soc*. 2010;132:2338–2344.
38. Prakash V, Nandi PK. Studies on the conformation of α-globulin from *Sesamum indicum L.* in cationic and nonionic detergents. *Bioscience*. 1982;4:331–345.
39. Bam NB, Cleland JL, Randolph TW. Molten globule intermediate of recombinant human growth hormone: stabilization with

surfactants. *Pace Pacing Clin Electrophysiol*. 1996;7938:801–809.
40. Doñate F, Artigues A, Iriarte ANA. Opposite behavior of two isozymes when refolding in the presence of non-ionic detergents. *Protein Sci*. 1998;7:1811–1820.
41. Jäger M, Plückthun A. Domain interactions in antibody Fv and scFv fragments: effects on unfolding kinetics and equilibria. *FEBS Lett*. 1999;462:307–312.
42. Zhao J-X, Yang L, Gu Z-N, Chen H-Q, Tian F-W, Chen Y-Q, Zhang H, Chen W. Stabilization of the single-chain fragment variable by an interdomain disulfide bond and its effect on antibody affinity. *Int J Mol Sci*. 2010;12:1–11.

Manuscript received Oct. 26, 2013, and revision received Jan. 29, 2014.