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Triton™ X-100 Behaves Similarly to Tyrosine-Containing and Tryptophan-Free Proteins in UV-Vis Spectroscopy

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As a known non-ionic, mild non-denaturing detergent and emulsifier, Triton™ X-100 is often used in various biochemical studies such as the isolation of membrane-protein complexes for solubilizing membrane proteins, the process of periplasmic protein extraction as a component of the lysis buffer, and as a permeabilization reagent in indirect immunofluorescence staining and flow cytometry. It has been shown that the diluted solution of Triton™ X-100 with the optimal pH range of 6.0-8.0 has a significant absorption of UV light. In the present project, we show that the absorption spectrum of Triton™ X-100, when dissolved in 1X phosphate-buffered saline, is similar to that of α-synuclein, as a representative of those proteins lack tryptophan but contain tyrosine as their main UV absorber. These results show that whenever the use of Triton™ X-100 for extracting membrane and periplasmic proteins is inevitable, scavenging it before the characterization of the proteins by UV-Vis spectroscopy, especially the determination of their concentration using Beer-Lambert Law, would be necessary.

Keywords: Triton™ X-100, Detergent, Membrane proteins, Periplasmic proteins, α-Synuclein, UV-Vis spectroscopy, Beer-Lambert law

INTRODUCTION

As a non-ionic, mild non-denaturing surfactant, Triton™ X-100 (polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether) is a colorless viscous (about 270 centipoise at 25 °C) liquid with a melting point of 6 °C. It is composed of an aromatic ring with two substituents present at the *para* position relative to each other. One substituent is a hydrophilic polyethylene oxide chain, and another one, a hydrophobic hydrocarbon chain (Fig. 1). On average, each Triton™ X-100 molecule has 9.5 ethylene oxide units. As its hydrophilic part can form hydrogen bonds with the surrounding protons, Triton™ X-100 is soluble in water and some other solvents such as toluene, trichloroethylene, ethylene glycol, ethyl ether, ethanol, isopropyl alcohol and 1,2-dichloroethane at 25 °C [1].

Triton™ X-100 has various biochemical applications. It is often used, for example, as an inactivating reagent of some lipid-enveloped viruses (*e.g.*, HIV, HBV and HCV),

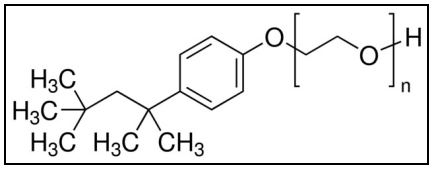

Fig. 1. Chemical Structure of Triton™ X-100.

as an ingredient in influenza vaccines (*e.g.*, Fluzone), as the cell permeabilization and blocking agent before immunostaining, immunofluorescence staining, flow cytometry, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) technique [2-14]. Also, Triton™ X-100 has the potential of extracting cellular organelles and DNA as a part (about 0.1%) of lysis buffer and membrane proteins in their native states in conjunction with zwitterion detergents (*e.g.*, CHAPS), of lowering membrane contaminants of protein extracted, of insolubilizing membrane lipids to probe their structures, and in a mixture with phospholipids, of producing effective

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substrates for studying enzymes involved in phospholipid metabolism [15-22]. Another important application of Triton™ X-100 is to extract native and recombinant periplasmic proteins [23-27]. Since Triton™ X-100 significantly absorbs UV light [28], its presence in the final protein solutions could strongly affect their quantitation by UV-Vis spectroscopy. In the present study, how Triton™ X-100 covers protein absorption spectra, especially those having tyrosine as their main UV light absorber.

EXPERIMENTAL

Materials

Triton™ X-100 (CAS Number 9002-93-1), hen egg-white lysozyme (HEWL, CAS Number 12650-88-3), isopropyl β-D-1-thiogalactopyranoside (IPTG, CAS Number 367-93-1), ethylenediaminetetraacetic acid (EDTA, CAS Number 60-00-4), phenylmethanesulfonyl fluoride (PMSF, CAS Number 329-98-6), ammonium persulfate (APS, CAS Number 7727-54-0), N,N,N',N'-tetramethyl ethylenediamine (TEMED, CAS Number 110-18-9), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ready to use 1X phosphate-buffered saline (PBS) powder was purchased from DNA biotech Inc. (Tehran, Iran) and dissolved in double-distilled water (ddH2O). pT7-7 asyn WT plasmid was a gift from Hilal Lashuel (Addgene plasmid # 36046; http://n2t.net/addgene:36046 ; RRID:Addgene_36046).

Methods

Preparation of α-synuclein monomeric solution. pT7-7 asyn WT plasmid was transformed into a calcium competent E. coli BL21 (DE3) strain. A single colony of the E. coli transformed, grown overnight at 37 °C on the ampicillin-containing lysogeny broth (LB) agar plate, was transferred to a 10 ml LB medium containing ampicillin (100 μg ml^{-1}) followed by overnight incubation at 37 °C. The resulting pre-culture was poured into a 1.0 liter LB medium containing the same ampicillin concentration and incubated at 37 °C until its absorbance reached 0.5 at 600 nm. Then, 0.5 mM IPTG was added to induce recombinant α-synuclein production. After 3-5 h, bacteria were harvested by centrifugation at 12000 × g for 5 min and then lysed in the lysis buffer (20 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 1.0 mM PMSF, and 0.5% protamine sulfate) by boiling at 95 °C for 20 min followed by 35W sonication for 2 min. Bacterial debris was discarded by centrifugation at 12000 × g for 30 min. Following precipitation of protein using 0.36 g ml^{-1} ammonium sulfate, the precipitant was re-suspended in the running buffer (20 mM Tris-HCl (pH 8.0)). The solution was filtered sequentially by 30 and 10 kDa Amicon® Ultra-15 Centrifugal Filters (Merckmillipore Inc.) (two-filter-assisted filtration). The fraction that did not pass through the 10 kDa filter was loaded to a 5-ml HiTrap Q FF column anion exchange column (GE Healthcare) balanced with the running buffer. Following washing the column with the washing buffer (20 mM Tris-HCl (pH 8.0), 100.0 mM NaCl), α-synuclein was eluted with the elution buffer (20 mM Tris-HCl (pH 8.0), 300.0 mM NaCl), analyzed by SDS-PAGE, and dialyzed against 1X PBS buffer (pH 7.4). Finally, the concentration of α-synuclein was obtained spectrophotometrically using ε_{280} = 5,960 M^{-1} cm^{-1} [29].

Preparation of Triton™ X-100 working solutions. To prepare a 1% v/v solution, 10 μl of Triton™ X-100 was mixed with 990 μl of the 1X PBS buffer in a 1.5 ml microtube and then vortexed to be dissolved completely.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

A mini 12% SDS-PAGE gel was prepared according to the following recipe (Table 1) [30]. HEWL was used here as an efficient marker because the molecular weights of both lysozyme and α-synuclein are about 14 kDa.

UV-Vis Spectroscopy

UV absorption spectra of 70 μM α-synuclein and Triton™ X-100 solutions (1.0, 0.5, 0.25, 0.125, 0.06 and 0.03%) dissolved in the 1X PBS buffer were recorded on a Carry 100 Bio UV-Vis Spectrophotometer at 200-300 nm. The baseline was corrected beforehand in the presence of the same buffer.

RESULTS

Using two-filter-assisted filtration accompanied by the low-pressure ion-exchange chromatography, recombinant

Table 1. 12% SDS-PAGE Recipe[30]

Separating gel (10 ml)					
ddH_2O	30% Acrylamide mix	1.5 M Tris-HCl (pH 8.8)	10% SDS	10% APS	TEMED
3.3 ml	4.0 ml	2.5 ml	100 µl	100 µl	4 µl
Stacking gel (5 ml)					
ddH_2O	30% Acrylamide mix	1.0 M Tris-HCl (pH 6.8)	10% SDS	10% APS	TEMED
3.4 ml	830 µl	630 µl	50 µl	50 µl	5 µl

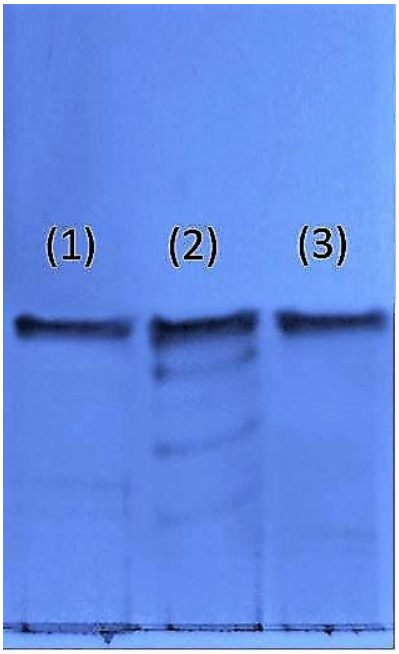

Fig. 2. 12% SDS-PAGE to analyze recombinant α-synuclein purification. (1) Lysozyme as a marker, (2) α-synuclein purified only by ion-exchange chromatography and (3) α-synuclein purified using two-filter-assisted filtration accompanied by the low-pressure ion-exchange chromatography.

α-synuclein was purified as appropriate as possible (Fig. 2). As shown by the widely-used 12% SDS-PAGE technique, purifying the protein only by the chromatography method could be associated with contaminations in the final solution, which may interfere with the protein absorbance in UV-Vis spectroscopy assay. However, this problem was addressed using two Amicon filters by which those contaminants with molecular weights above 30 kDa and below 10 kDa were removed.

After its dialysis against 1X PBS buffer (pH 7.4), α-synuclein, as a representative of tryptophan-free, tyrosine containing proteins, showed a significant UV absorption at 200-300 nm (Fig. 3). As shown, the protein's absorbance has two peaks, a sharp one at 274-276 nm with a shoulder to

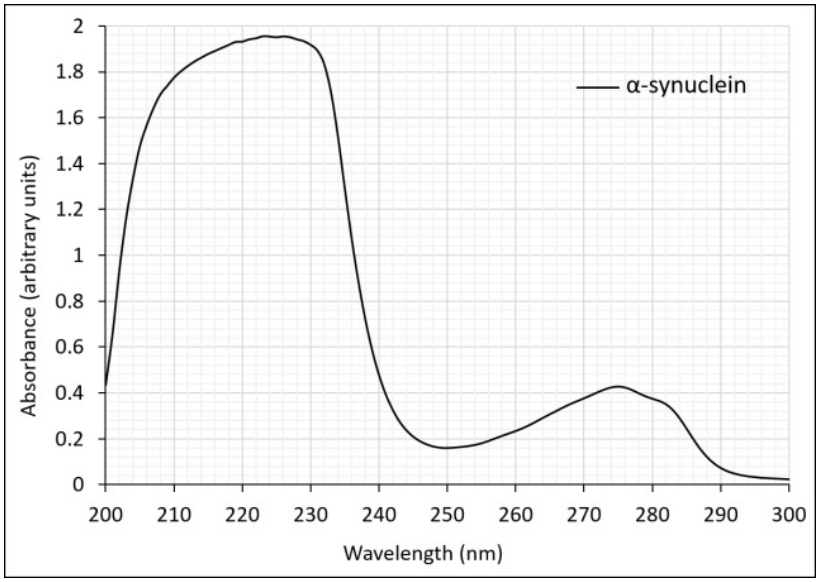

Fig. 4. The UV absorption spectrum of 70 μM α-synuclein dissolved in the 1X PBS buffer. As shown, α-synuclein represents a sharp peak at 274-276 nm with a shoulder to 280 nm and a broader and stronger peak at 210-230 nm. Its 260/280 and 230/375 ratios were calculated to be 0.62 and 3.36, respectively.

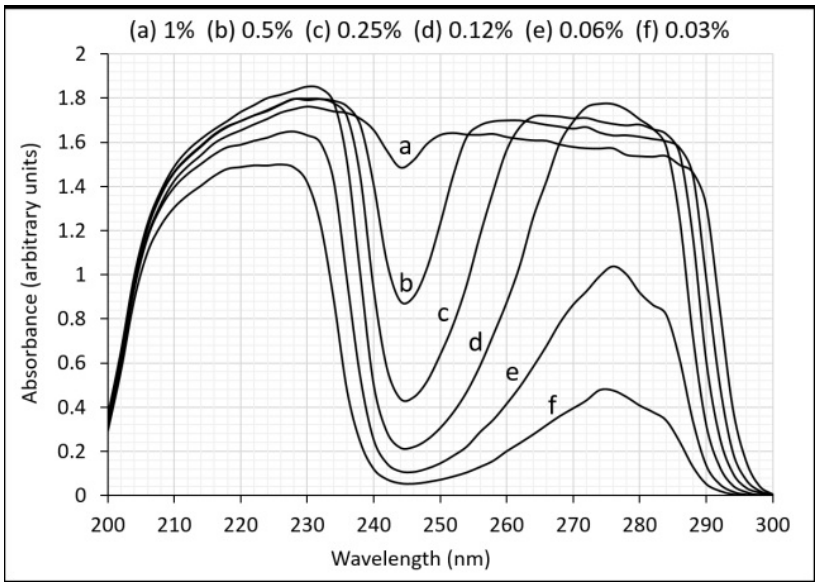

Fig. 4. UV spectra of the Triton™ X-100 solutions (1.0, 0.5, 0.25, 0.12, 0.06 and 0.03%) prepared in the 1X PBS buffer; 1% of Triton™ X-100 shows (a) a broad absorbance with a valley at 244 nm. Reducing its percentage gradually deepened the valley and sharpened the spectra at both sides, so that 0.03% of Triton™ X-100 shows (f) a sharp peak at 274-276 nm with a shoulder to 280 nm and a broad peak at 210-230 nm. Its 260/280 and 230/275 ratios were calculated to be 0.49 and 2.97, respectively.

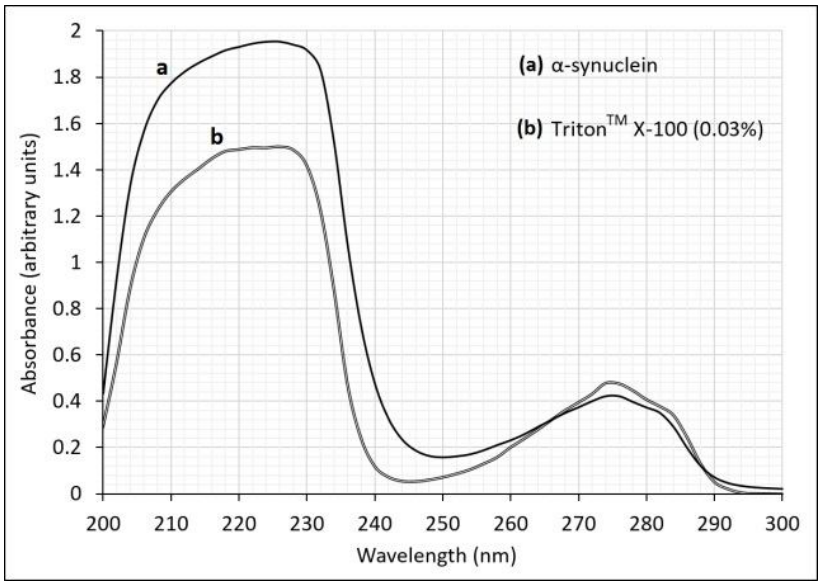

Fig. 5. Overlap of ultraviolet absorption spectra of 70 μM α-synuclein and 0.03% of Triton™ X-100. As shown, these two spectra are too similar to be distinguished from one another if displayed separately.

280 nm and a broad one of greater magnitude at 210-230 nm. Knowing absorbance values of 70 μM α-synuclein at 230 nm (1.92), 260 nm (0.23), 275 nm (0.42), and 280 nm (0.37), 260/280 ratio and 230/275 ratio were calculated to be 0.62 and 3.36, respectively.

The absorbance of Triton™ X-100 dissolved in the 1X PBS buffer was recorded at 200-300 nm. As shown in Fig. 4, 1% of Triton™ X-100 showed a broad absorbance in this region with a valley at 244 nm. By diluting Triton™ X-100 percentage to half in the same buffer, the valley deepened, but the broadness of the spectrum on both sides of it remained unchanged. Further dilution of Triton™ X-100 to 0.25, 0.12, 0.06 and finally 0.03% caused the valley to be deepened further and simultaneously the absorption spectrum at its right wing be sharpened so that at 0.03% an obvious maximum peak at 274-276 nm with a shoulder at 280 nm appeared. However, the shape of the left wing was largely remained unchanged. Knowing absorbance values of 0.03% of Triton™ X-100 at 230 nm (1.42), 260 nm (0.20), 275 nm (0.48) and at 280 nm (0.41), the 260/280 and 230/275 ratios were calculated to be 0.49 and 2.97, respectively.

DISCUSSION

Comparing Figs. 3 and 4 shows that the absorption spectra of α-synuclein and low percentage (< 0.06%) Triton™ X-100 are similar to a great extent in shape. Both have a maximum peak at 274-276 nm with a shoulder to 280 nm and a much stronger broad peak in 210-230 nm. As seen in Fig. 4, Triton™ X-100 also has an obvious absorbance at 280 nm, the maximum absorption wavelength of a large number of proteins. Overlapping the absorption spectra of 70 μM α-synuclein and 0.03% of Triton™ X-100 (Fig. 5) showed many similarities between them with a little difference in their absorbance at 210-230 nm. Thus, displaying these two spectra separately makes it difficult to distinguish them from each other.

There are two ways to find out some differences between these two spectra, especially when the peaks at 274-276 nm have the same absorption values. The first way is to compare their 260/280 ratios, the feature needed to be calculated for any protein purified to estimate its contamination by nucleic acids. According to our calculations, the ratio values for 70% μM α-synuclein and 0.03% of Triton™ X-100 were 0.62 and 0.49, respectively.

Since the minimum ratio of the highest purity protein has already been calculated to be 0.57 [31], obtaining a lower value (0.49 for Triton™ X-100) indicates the existence of a non-protein substance. The second way could be comparing their 230/275 ratios. As calculated, the ratio values for 70 μM α-synuclein and Triton™ X-100 are 3.36 and 2.97, respectively. It means that, for a specific concentration of α-synuclein, the maximum UV absorption of peptide bonds at 230 nm could be three folds more that absorption of total tyrosine at 275 nm.

CONCLUSIONS

As a non-ionic detergent, Triton™ X-100 is usually used in biochemical studies for different purposes, especially in the isolation and solubilization of the periplasmic and membrane proteins. Furthermore, it has a significant absorption in the UV region of light with a significant peak at 274-280 nm just as proteins. Therefore, it is concluded that the presence of even a little amount of Triton™ X-100 in a protein solution can greatly affect the calculation of its concentration by Beer-Lambert law, which depends on the absorbance value at 280 nm, whether the protein contains tryptophan or not. If the protein extracted is tryptophan-free, it will contain tyrosine instead, since the absorption spectrum of Triton™ X-100 is similar to that of such proteins, its presence could mislead the researcher even when the final solution lacks any protein. Therefore, using other methods of protein characterization is suggested.

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