Protocol for spectrophotometric determination of native and desialylated apo-transferrin molar absorption coefficients

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Abstract

Absorbance of a protein around 280 nm depends on its tryptophan (Trp), tyrosine (Tyr) and cystine (i.e. the disulfide bond) content. Molar absorption coefficient of a folded (native) protein can be determined by measuring absorbance of two protein solutions with identical concentrations, one containing only buffer and the other containing the same buffer and 6.0 M guanidine·HCl. Since molar absorption coefficients of Trp, Tyr and cystine in 6.0 M guanidine·HCl are known, molar absorption coefficient of an unfolded (denatured) protein at given wavelength can be calculated if the protein amino acid sequence and structure (i.e. the protein Trp, Tyr and cystine content) is known and the absorbances of the folded and unfolded protein are measured. The molar absorption coefficient of a folded protein at given wavelength is then equal to the product of a molar absorption coefficient of an unfolded protein and absorbance ratio of folded and unfolded protein at given wavelength. The required amount of the protein is 1 mg but approximately 60 % of folded protein can be recovered and used for further studies.

Keywords

transferrin, absorbance, molar absorption coefficient, guanidine

1.1 Materials and instrumentation

1) 1% Tergazyme solution (Alconox, cat.no. 1104)
2) 25 mM sodium phosphate (Kemika, cat.no. 14067 07) buffer, pH = 7.4
3) Protein (Sigma-Aldrich cat.no. G9885) stock solution: approximately 1 mg protein in 0.25 mL of 25 mM sodium phosphate buffer, pH = 7.4
4) 6.6 M guanidine·HCl (PanReac AppliChem, A14990500), 25 mM sodium phosphate buffer, pH = 7.4 adjusted with NaOH
5) Cary 50 UV-Vis spectrophotometer, Varian UV Scan application Software Version: 3.00(303)
6) Ultrasonic bath (Sper Scientific Direct, 42000 Hz, 100004)
7) Quartz cuvette, 1 cm optical path (Hellma, cat.no. 105.253-QS)
8) Water, double distilled in an all-glass apparatus

1.2 Method

The molar absorption coefficient of an unfolded protein in 6.0 M guanidine·HCl, ε_u, is calculated using Eq. 1, where #Trp is number of tryptophan residues, #Tyr is number of tyrosine residues and #cystine is number of cystine bridges in the protein structure, [1] and $\varepsilon_{\lambda Trp}$, $\varepsilon_{\lambda Trp}$ and $\varepsilon_{\lambda cystine}$ are their respective molar absorption coefficients in 6.0 M guanidine·HCl at a certain wavelength (Table 3.1.):

$$\varepsilon_{\lambda u} / M^{-1}\,cm^{-1} = (\#Trp)(\varepsilon_{\lambda Trp})+(\#Tyr)(\varepsilon_{\lambda Trp})+(\#cystine)(\varepsilon_{\lambda cystine}) \qquad \text{(Eq 1)}$$

If the concentrations of the folded and unfolded protein solutions are equal, the molar absorption coefficient of a folded protein, ε_f, the values ε_f and ε_u are related by (Eq. 2):

$$\frac{A_f}{A_u} = \frac{\varepsilon_f}{\varepsilon_u}, \qquad \text{(Eq. 2)}$$

and ε_f can be calculated by using Eq. 3:

$$\varepsilon_f = \varepsilon_u \cdot \left(\frac{A_f}{A_u}\right). \qquad \text{(Eq. 3)}$$

Amino acid sequence and structure of the native apo-transferrin has been previously reported, so numbers of Trp, Tyr and cystines are known (Table 3.1.). [2,3] Importantly, **the method does not require knowing the exact mass of the dissolved protein** and its concentration can be calculated from the measured value of A_u (Eq. 4):

$$c_u = c_f = \frac{A_u}{\varepsilon_u \cdot l}, \qquad \text{(Eq. 4)}$$

where l = 1 cm is the cuvette pathlength.

If there is a significant absorbance of the protein above 320 nm, which should be zero, the corrected absorbance for the light scattering should be calculated with either (Eq. 5) or (Eq. 6), depending on the wavelength of interest:

$$A_{280\,corr} = A_{280} - (A_{330} \cdot 1.929) \qquad \text{(Eq.5)}$$

$$A_{278\,corr} = A_{278} - (A_{330} \cdot 1.986) \qquad \text{(Eq.6)}$$

where $A_{280\ corr}$ and $A_{278\ corr}$ are scatter-corrected absorbance values at 280 nm and 278 nm, respectively, whereas A_{280}, A_{278}, and A_{330} are absorbance values at 280 nm, 278 nm, and 330 nm, respectively. Correction for light scattering should be made for both folded and unfolded protein. This approach assumes that the scattering contribution varies as the inverse fourth power of the wavelength as in Rayleigh scattering.[3]

Table 1. Wavelength-dependent molar absorption coefficients, ε_λ, of Trp, Tyr and cystine in 6 M guanidine·HCl. Values in parentheses denote the number of respective amino acid residues in the sequence of native transferrin. [3,4]

	ε_λ / M^{-1} cm^{-1}			
λ / nm	Trp (8)	Tyr (26)	cystine (19)	Transferrin*
282	5600	1200	100	77900
280	5690	1280	120	81080
279	5660	1345	120	82530
278	5600	1400	127	83613
276	5400	1450	145	83655

* Values for unfolded apo-transferrin in 6.0 M guanidine·HCl calculated using Eq. 1.

Molar absorption coefficients for a range of wavelengths can be calculated using (Eq. 7) after concentration of the native (folded) protein is determined using (Eq. 4.):

$$\varepsilon_\lambda = \frac{A_\lambda}{c_f}, \qquad \text{(Eq. 7)}$$

where ε_λ is molar absorption coefficient at given wavelength, A_λ is absorbance of a native (folded) protein at given wavelength and c_f is concentration of a native (folded) protein.

1.3 Measurement

1.3.1 Measure absorbance spectrum of the folded protein

The quartz cuvette is soaked in 1% Tergazyme solution and placed in the ultrasonic cleaner for 360 seconds, rinsed with redistilled water and dried.

For absorbance baseline measurement, a volume of 1000 µL of 25mM sodium phosphate buffer, pH = 7.4, is added to the cuvette. Baseline is recorded by scanning absorbance between 250 and 300 nm (resolution 1 nm, medium scanning speed in the Varian UV Scan application). The cuvette is then rinsed with redistilled water and dried.

For the protein absorbance measurement, a volume of 910 µL of the same buffer used for baseline and 90 µL of protein stock solution is added to the

cuvette. Absorbance of the protein is then measured in the same range as baseline. The solution in the cuvette should be mixed well before measurement. The Varian UV Scan application automatically applies the baseline correction.

The solution in the cuvette and the stock protein solution remaining after measuring the absorbance spectrum of the unfolded protein (section 1.3.2.) can be recovered and used for further studies (approximately 60% of the total protein in the stock solution).

1.3.2 Measure absorbance spectrum of the unfolded protein

Repeat the cuvette cleaning procedure described in section 1.3.1.

For absorbance baseline measurement, a volume of 910 µL of 6.6 M guanidine·HCl, 25 mM sodium phosphate buffer, pH = 7.4 is added to the cuvette. A volume of 90 µL of 25 mM sodium phosphate buffer, pH = 7.4, is then added to the cuvette. The solution in the cuvette should be mixed well before measurement. Baseline is then recorded as described in section 1.3.1. Cuvette is then rinsed with redistilled water and dried.

For the protein absorbance measurement, a volume of 910 µL of 6.6 M guanidine·HCl, 25 mM sodium phosphate buffer, pH = 7.4, and 90 µL of protein stock solution is added to the sample cuvette. Absorbance of the protein is then measured as described in section 1.3.1.ten minutes after mixing for denaturation to take place. The solution in the cuvette should be mixed well before measurement.

The solution in the cuvette contains denatured protein and cannot be recovered and used for further studies.

Typical results of absorbance measurements are given in Fig. 1. Molar absorption coefficient of folded native human serum transferrin as a function of wavelength (calculated using Eq. 7) is shown in Fig 2.

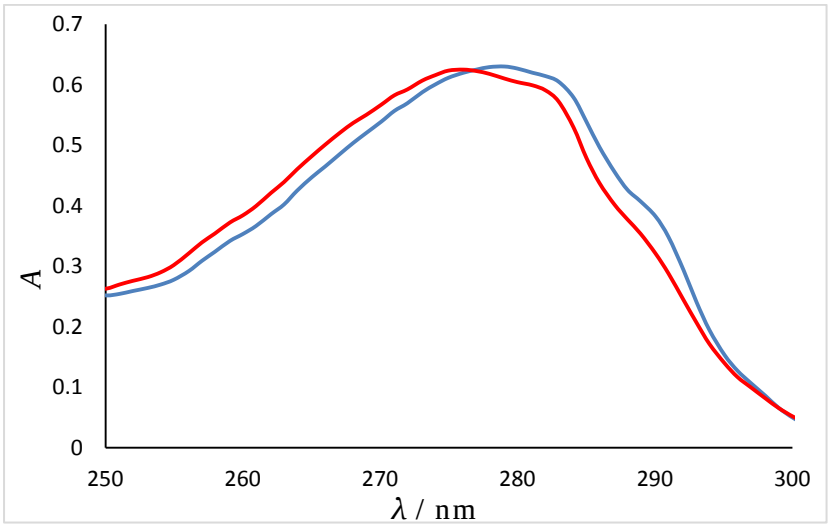

Figure 1. Absorbance spectrum of folded (A_f, blue) and unfolded (A_u, red) native human serum transferrin.

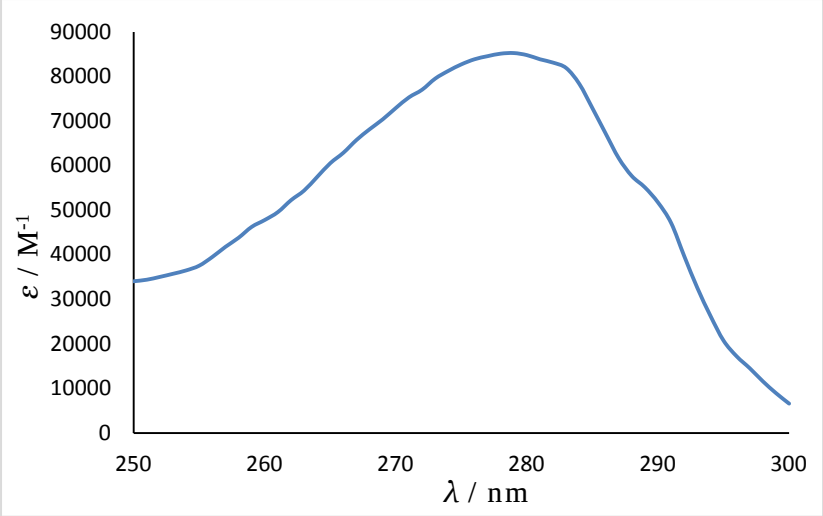

Figure 2. Molar absorption coefficient of folded native human serum transferrin as a function of wavelength (calculated using Eq. 7).

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