APPLICATION OF THE GLOBAL FIT IN PRESSURE SHIFT ASSAY METHOD

REPORT

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1 Itroduction

Pressure unfolds proteins and this feature is used to determine volumetric properties of proteins and their binding to other molecules. Monitoring folded protein fraction dependence on pressure applied allows to determine protein unfolding pressure (pressure when ratio of folded and unfolded protein fractions is equal 1), Gibbs free energy and volume changes. Analyzing intrinsic tryptophan emission spectra is one of methods used to follow protein structure changes. However, choosing spectral parameters, that represent unfolding of the protein, is not trivial. In this work we present the research on determination of lysozyme protein unfolding parameters by monitoring various tryptophan spectra parameters including the application of global fit algorithm.

2 Methods

The procedure of determination of unfolding parameters was as follows. Unfolding profile was constructed by taking a parameter of fluorescence spectra (taken directly from experimental data or from model fitted to experimental data) as function of pressure. Protein unfolding volume change($\Delta_u V$) and Gibbs free energy change($\Delta_u G$) were obtained by fitting the sigmoidal function parameters to unfolding profile.

2.1 Experimental data

The intrinsic tryptophan fluorescence spectra of lysozyme solution under pressure were analyzed in this work. The solution contained 10 μM lysozyme, 3.5 M guanidine hydrochloride, 10mM Bis-Tris buffer, pH 7.0. The fluorescence was measured by exciting at 285 nm and the emission spectra were recorded in the wavelength range of 320-380 nm. Pressure was increased in steps of 20 MPa up to 380 MPa. Several selected spectra are shown in Fig. 1

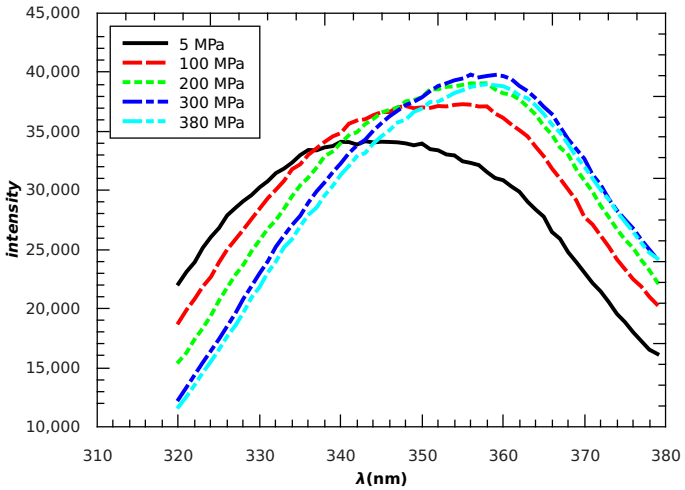


Fig. 1. The intrinsic tryptophan fluorescence spectra of lysozyme solution at selected pressures.

2.2 Single and global fit

The Levenberg–Marquardt algorithm was selected to fit models to data. If one assumed that several curves should be described by the same value of model parameter then model was fitted to the several curves simultaneously setting corresponding parameter as shared (global

fit) [1]. Software was developed for single and global fits. We used PYTHON [2] programming language for scripting. LMFIT [3] package for PYTHON was used as a core to fit a model to data. The global fit algorithm was implemented as described in [4]. Scripts was written as extension of QTIPLOT [5] computer program, used for managing graphical information and data analysis.

2.3 Direct fluorescence spectrum parameters for unfolding profiles

2.3.1 Intensity at selected wavelength

Fluorescence intensities at wavelength in range of 329 nm-334 nm were used to construct unfolding profiles.

2.3.2 Average emission wavelength

The average emission wavelength was also used to construct unfolding profiles. The following formula was used to calculate the average emission wavelength [6]:

$$< \lambda >= \frac{\sum_i F_i \lambda_i}{\sum_i F_i}, \tag{1}$$

where:

- F_i – Intensity at ith point of recorded spectrum;
- λ_i – wavelength at ith point of recorded spectrum.

All variables of Eqn. 1 are shown in Fig.2.

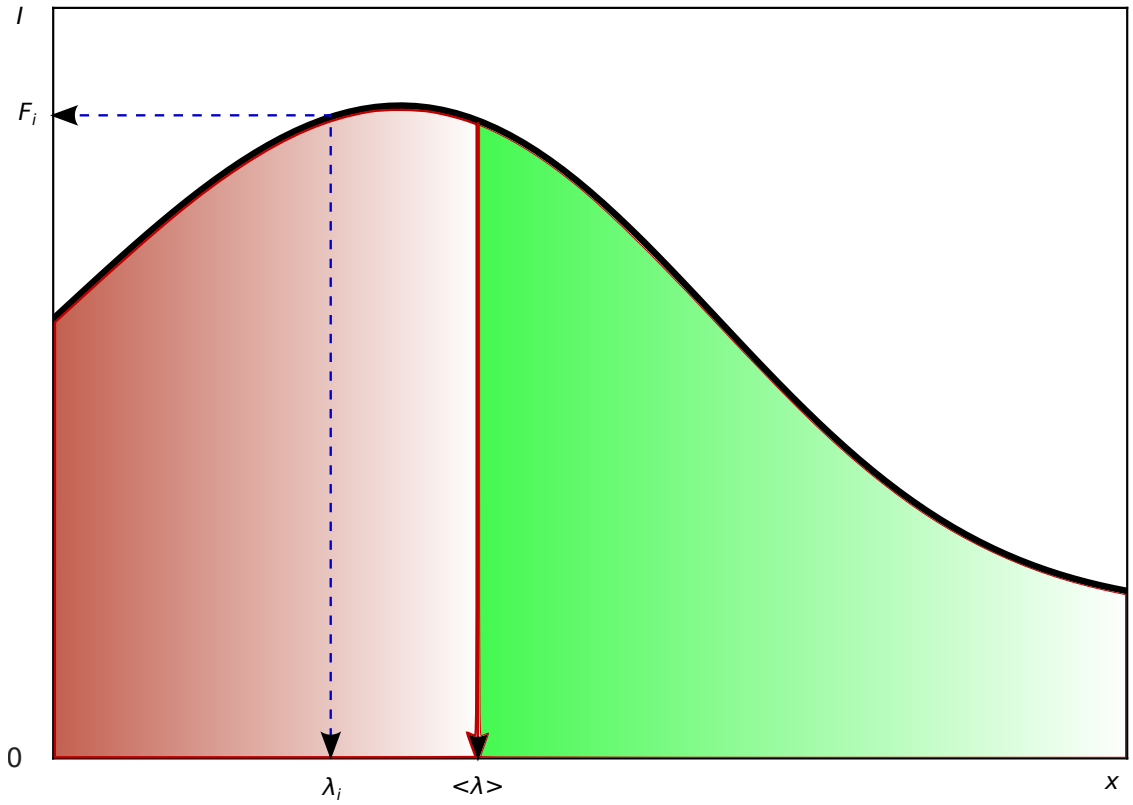


Fig. 2. Graphical explanation of the average emission wavelength: **Green** and **red** areas are equal.

2.4 Models for fluorescence spectra

2.4.1 Symmetrical Gaussian model

The following symmetrical Gaussian function was used to fit experimental spectra:

$$I(x) = a\frac{\sqrt{\frac{2}{\pi}}}{w}exp\Big(-2\Big(\frac{x-xc}{w}\Big)^2\Big) + y_0, \tag{2}$$

where:

- I – Intensity of the fluorescence;
- y_{max} – Intensity of the peak;
- x – Wavelength;
- x_c – Wavelength of the peak;
- w – Width of the peak at half of y_{max};
- y_0 – Intensity shift.

All variables of Eqn. 2 are shown in Fig. 3.

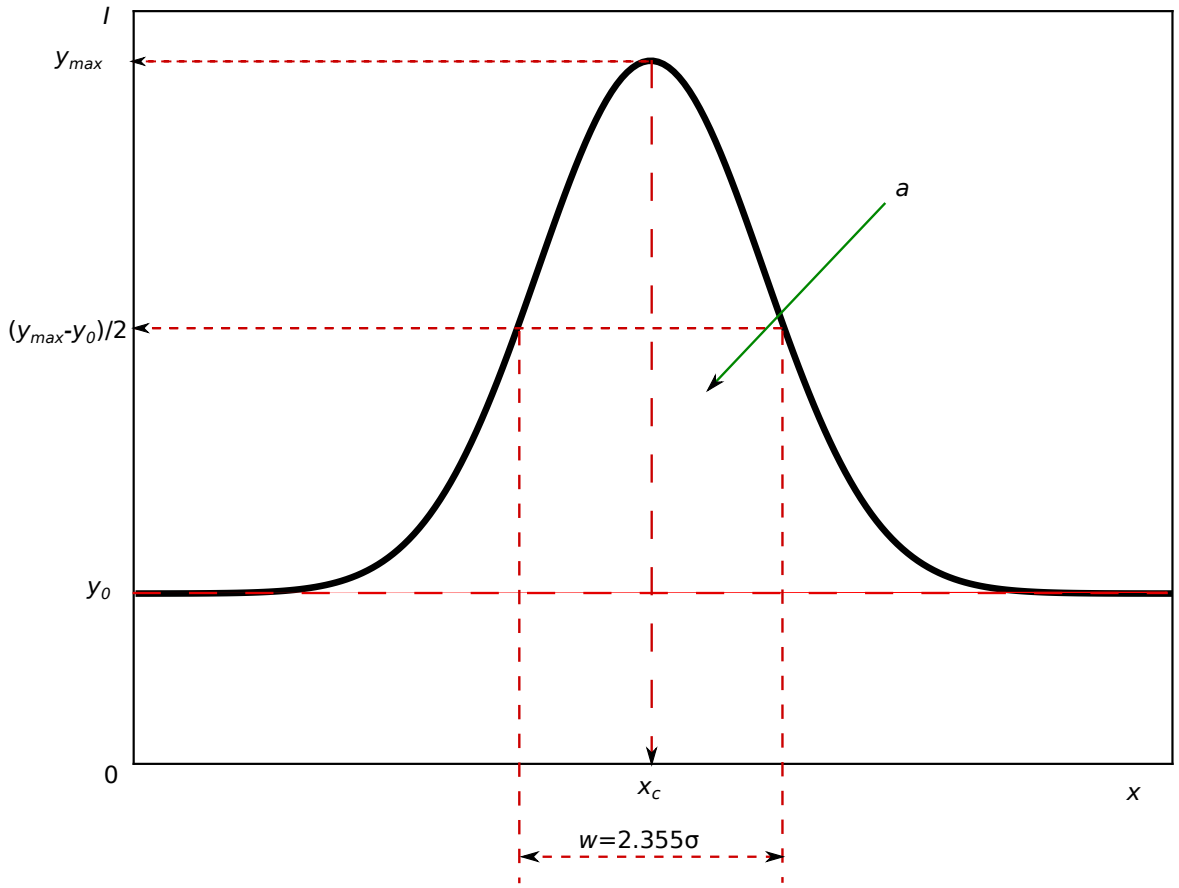


Fig. 3. Symmetrical Gaussian parameters.

2.4.2 Asymmetrical Gaussian model

The following asymmetrical Gaussian function was used to fit experimental spectra [6]:

$$I(x) = I_{max} \times exp\left\{-\frac{ln(2)}{ln(\rho^2)} \times \left[ln\left(\frac{(x_{max} + \frac{\Gamma\times\rho}{\rho^2-1}) - x}{(x_{max} + \frac{\Gamma\times\rho}{\rho^2-1}) - x_{max}}\right)\right]^2\right\}, \quad (3)$$

where:

- I – Intensity of the fluorescence;
- I_{max} – Intensity of the peak;
- x – Wavelength;
- x_{max} – Wavelength of the peak;
- ρ – Asymmetry parameter;
- Γ – Width of the peak at half of I_{max}.

All variables of Eqn. 3 are shown in Fig.4.

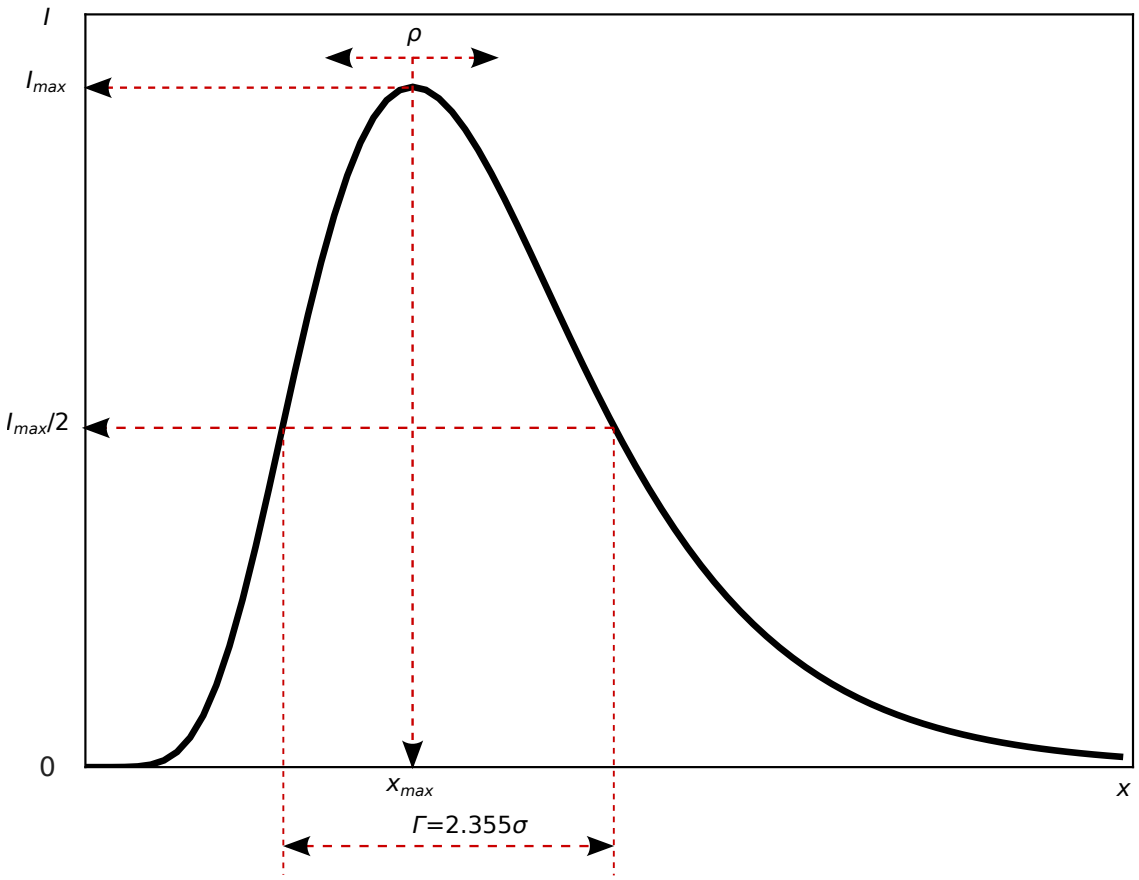


Fig. 4. Asymmetrical Gaussian parameters.

2.4.3 Two Gaussian model

Two Gaussian model consists of two symmetrical Gaussian functions:

$$I(x) = a1\frac{\sqrt{\frac{2}{\pi}}}{w1}exp\Big(-2\Big(\frac{x-xc1}{w1}\Big)^2\Big) + a2\frac{\sqrt{\frac{2}{\pi}}}{w2}exp\Big(-2\Big(\frac{x-xc2}{w2}\Big)^2\Big) + y_0, \tag{4}$$

where:

- I – Intensity of the fluorescence;
- i_{max1} – Intensity of the first peak - first Gaussian maximum;
- i_{max2} – Intensity of the second peak – second Gaussian maximum;
- $a1$ – First peak area - area between first Gaussian(red line) and $y_0/2$(blue line);
- $a2$ – Second peak area - area between second Gaussian(black line) and $y_0/2$(blue line);
- x – Wavelength
- x_{c1} – Wavelength of the first peak – center of the first Gaussian;
- x_{c2} – Wavelength of the second peak – center of the second Gaussian;
- w_1 – Width of the first peak at half of i_{max1}. σ_1- first Gaussian standard deviation;
- w_2 – Width of the second peak at half of i_{max2}. σ_2- second Gaussian standard deviation;
- y_0 – Intesity shift.

All variables of Eqn. 4 are shown in Fig. 5.

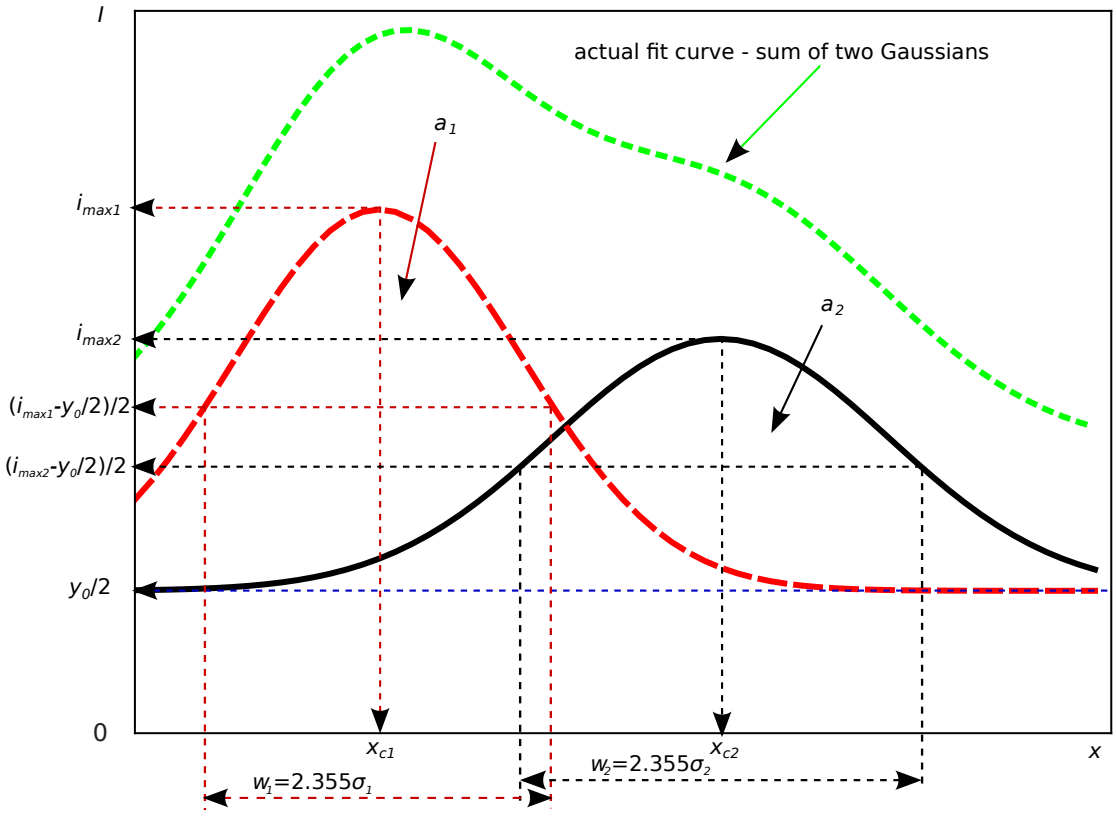


Fig. 5. Two Gaussian model parameters. **Green** line is a fit curve, **red** line – first Gaussian curve, **black** line – second Gaussian curve.

2.5 Model for unfolding profile

Unfolding profiles were fitted by sigmoidal function [7]:

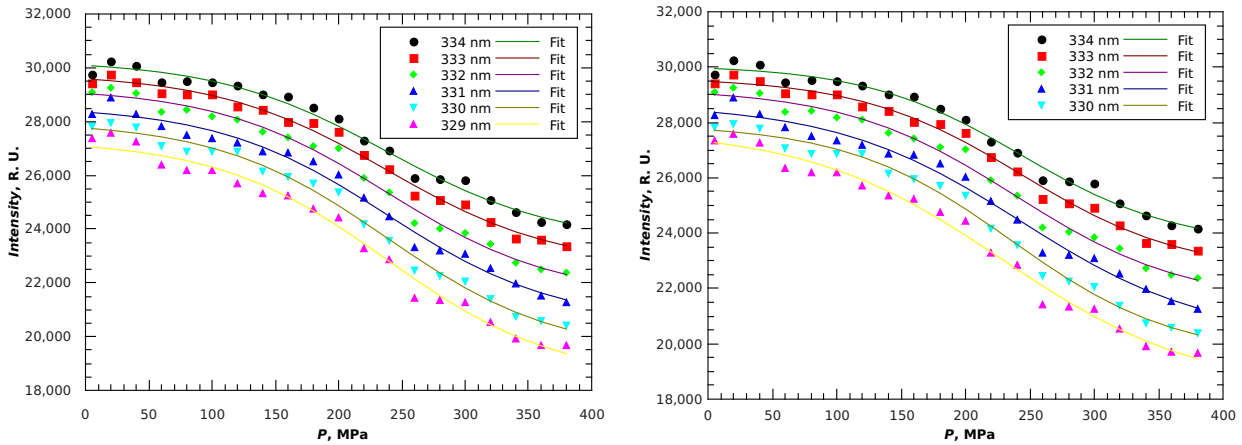
$$f = f_n + \frac{f_u - f_n}{1 + exp\left[\frac{\Delta_u G + \Delta_u V(P - P_0)}{RT}\right]}, \tag{5}$$

where:

- f – Fluorescence parameter;
- f_n – Fluorescence parameter before unfolding;
- f_u – Fluorescence parameter after unfolding;
- $\Delta_u G$ – Gibbs free energy of unfolding;
- $\Delta_u V$ – Volume of unfolding;
- P – Pressure applied on the protein;
- P_0 – Reference (atmospheric) pressure;
- R – Ideal gas constant;
- T – Experiment temperature.

3 Unfolding parameters of lysozyme

3.1 Case of intensities at fixed wavelength



(a) (b)

Fig. 6. Lysozyme unfolding profiles constructed from fluorescence intensities at selected wavelengths: a) global fit with shared parameters $\Delta_u G$ and $\Delta_u V$, b) single fits.

According to the litrature [6] it is possible to follow the transition of the protein unfolding by measuring the fluorescence intensity at constant wavelength for each pressure. Six unfolding profiles were constructed from experimental fluorescence intensities at wavelengths of the range 329-334 nm (points in Fig. 6). The sigmoidal function was fitted to each unfolding curve. The $\Delta_u V$ values determined from single fits (Fig. 6b) were scattered in range of 32-40 cm^3 mol^{-1}. Global fit to unfolding curves (Fig. 6a) found $\Delta_u V = -36.4$ cm^3 mol^{-1}.

3.2 The average emission wavelength case

The average emission wavelengths were calculated from experimental fluorescence data using Eqn. 1. The constructed unfolding profile is shown in Fig. 7. Values of unfolding parameters obtained by fit of sigmoidal function to the unfolding profile of full range of pressures were abnormal small. Therefore, The points of the average emission wavelenghts up to 50 MPa were excluded from fit (line in Fig. 7). Obtained unfolding parameters were: $\Delta_u V = -41$ cm^3 mol^{-1}, $\Delta_u G = 7.2$ kJ mol^{-1}.

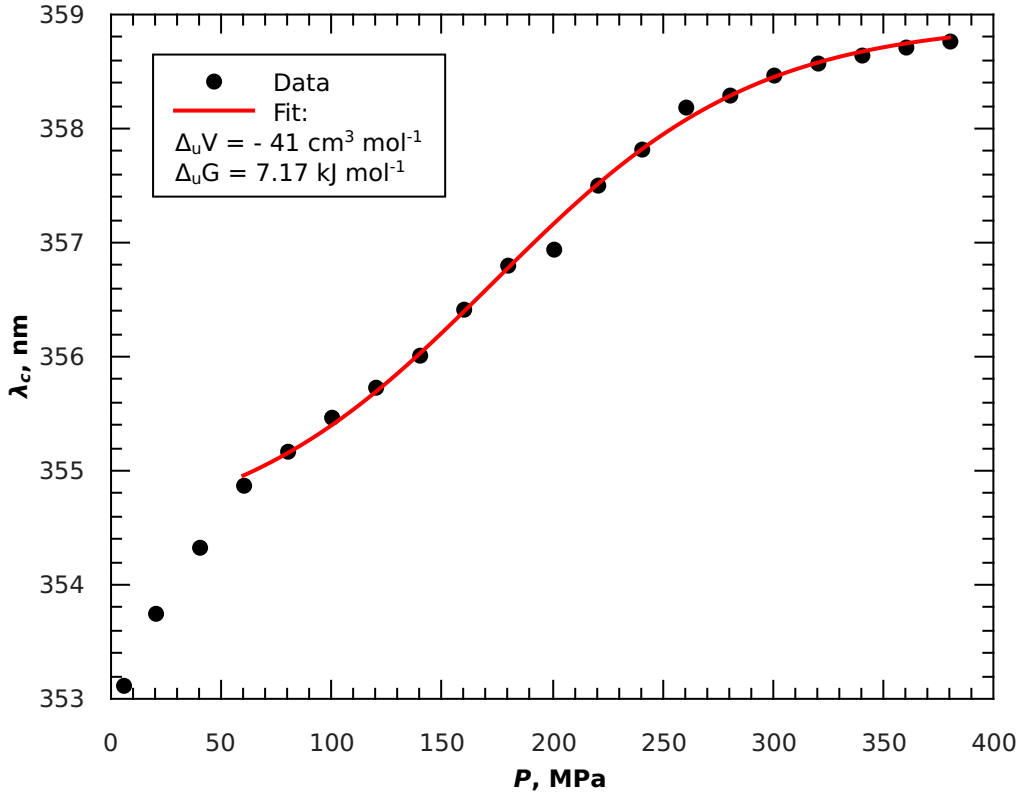
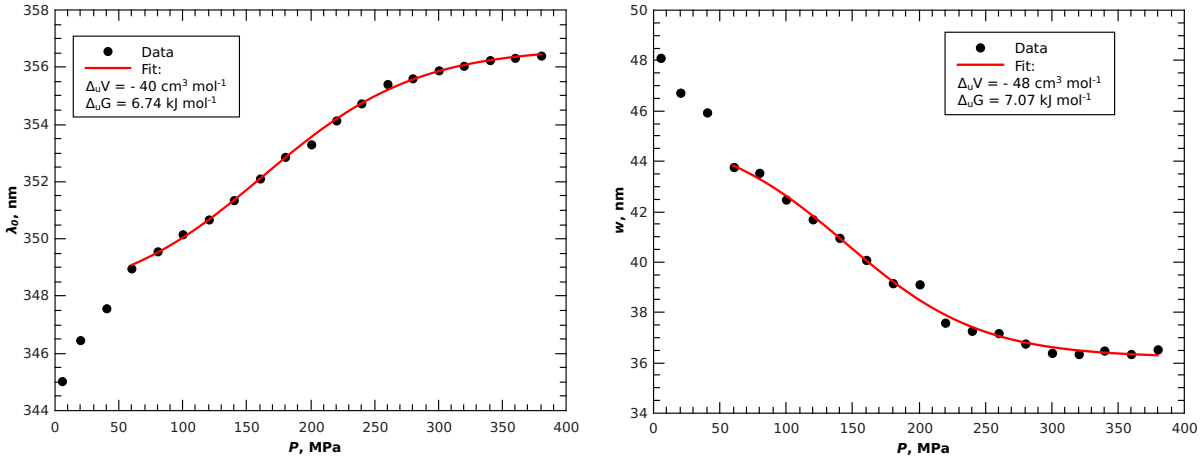


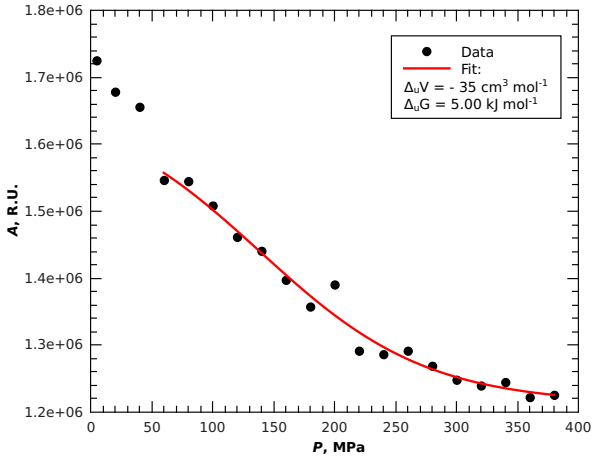
Fig. 7. The average emission wavelength dependence on pressure.

3.3 Symmetrical Gaussian model parameters approach

Symmetrical Gaussian model (Eqn. 2) was fitted to experimental fluorescence spectrum at each pressure. Fit was very sensitive to initial values of model parameters. Unfolding profiles were constructed from fitted model parameters λ_0, w, A and are shown in Fig. 8. The exclusion of points at pressures up to 50 MPa from analysis gave better fit results.



(a) (b)



(c)

Fig. 8. Unfolding profiles constructed from fitted symmetrical Gaussian model parameters λ_0(a), w(b), A(c).

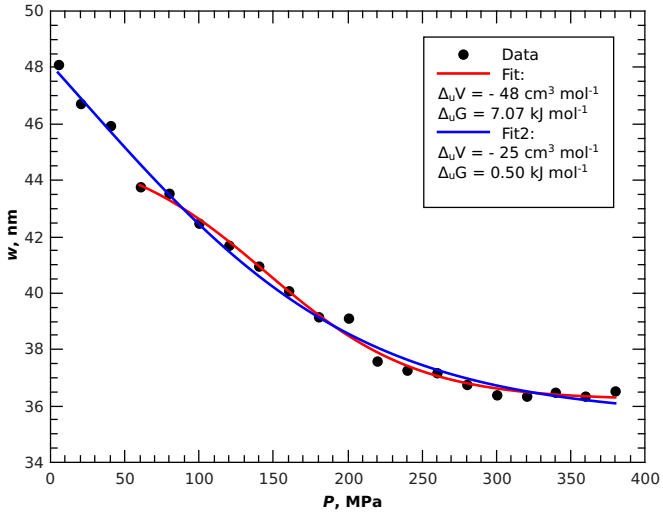


Fig. 9. Two fits of unfolding profile: when points at pressures between 5 MPa and 380 MPa are fitted (Fit2) and when points at pressures of 5-50 MPa are excluded (Fit).

3.4 Asymetrical Gaussian model parameters approach

Asymmetrical Gaussian model (Eqn. 3) was fitted to experimental fluorescence spectrum at each pressure. Unfolding profiles were constructed from fitted model parameters I_{max}, λ_c and are shown in Fig. 10 and Fig. 11 . The exclusion of points at pressures up to 50 MPa from analysis gave better fit results in both cases.

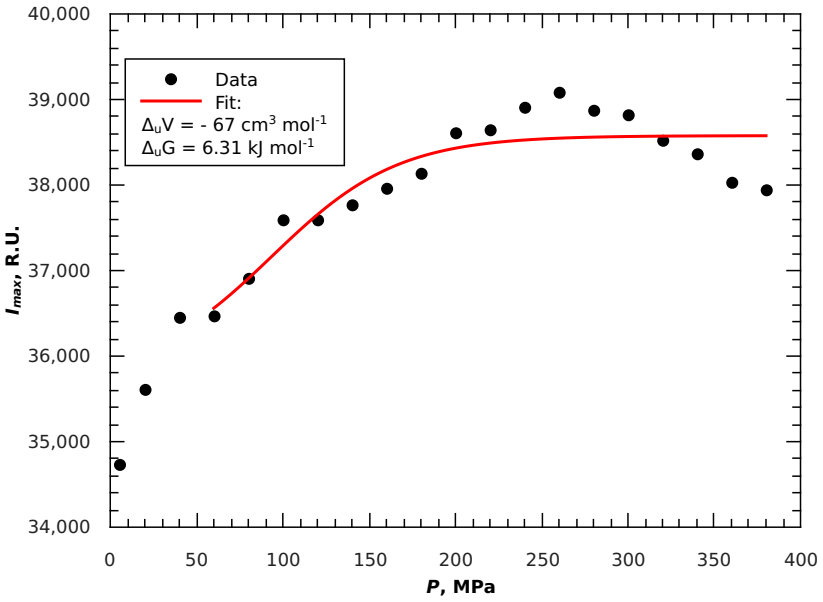


Fig. 10. Unfolding profile constructed from fitted asymmetrical Gaussian model parameter I_{max}

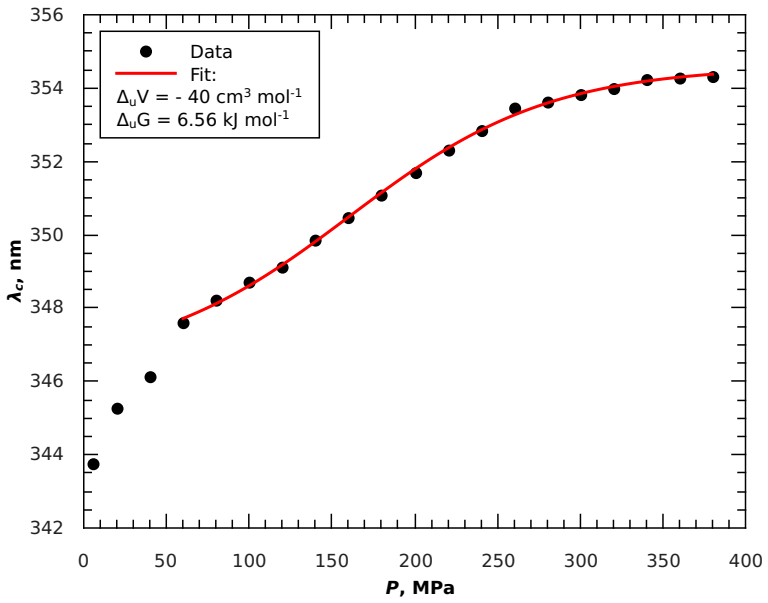


Fig. 11. Unfolding profile constructed from fitted asymmetrical Gaussian model parameter λ_c

3.5 Two Gaussian model parameters approach

Two Gaussian model (Eqn. 4) was globally fitted to experimental fluorescence spectra. Model parameters λ_{c1} and λ_{c2} were set as global for all spectra. Unfolding profiles were constructed from combinations of fitted model parameters: a_2/a_1, w_2/w_1, $a_2 - a_1$, $w_2 - w_1$ and are shown in Fig. 12. The exclusion of points at pressures up to 50 MPa from analysis gave better fit results in all cases.

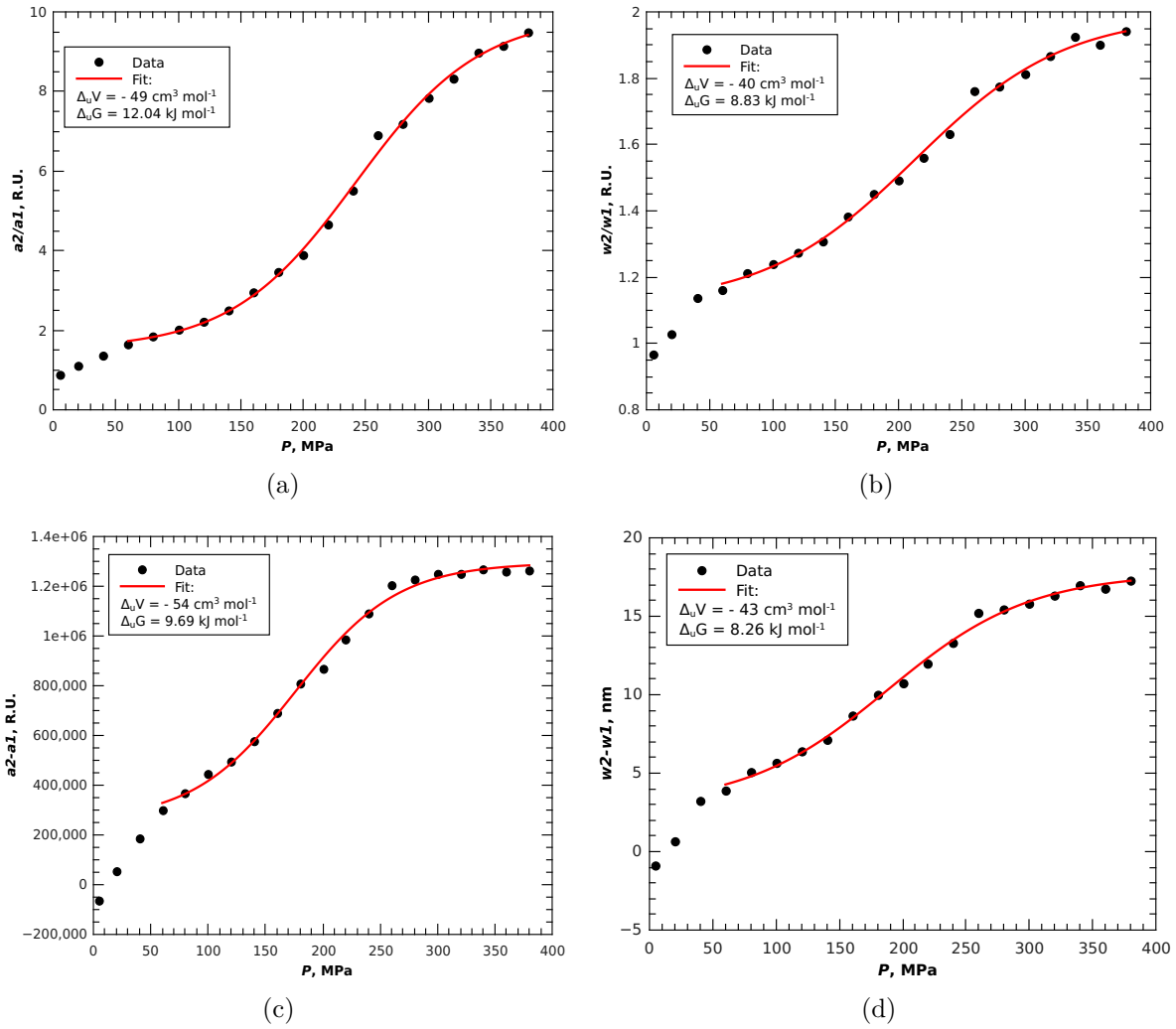


Fig. 12. Unfolding profile constructed from globally fitted Two Gaussian model parameters: a_2/a_1, w_2/w_1, $a_2 - a_1$, $w_2 - w_1$.

3.6 Summarized unfolding parameters

Lysozyme unfolding parameters obtained by variuos methods are summarized in Table 1.

Table 1. Values of protein unfolding parameters using different models.

Model	Parameter	Parameter description	$\Delta_u V$, $cm^3\ mol^{-1}$	$\Delta_u G$, $kJ\ mol^{-1}$	P_m, Mpa
Asymmetrical Gaussian:	I_{max}	max. intensity	-67	6.31	93
	λ_c	wavelength	-40	6.56	162
Symmetrical Gaussian:					
	A	area	-35	5.00	141
	λ_0	wavelength	-40	6.74	167
	w	width	-48	7.07	146
Two symmetrical Gaussians, two global wavelengths:					
	a2/a1	areas ratio	-49	12.04	243
	w2/w1	widths ratio	-40	8.83	218
	a2-a1	areas difference	-54	9.69	177
	w2-w1	widths difference	-43	8.26	190
Spectral Centroid:					
	λ_c	wavelength	-41	7.17	173
Intensities at different wavelenghts:	I_0	intensity	-36	8.78	239

4 Conclusions

Lysozyme fluorescence spectra up to 50MPa pressure should be excluded from determination of unfolding parameters. Future investigation on this effect should be performed. For determination of unfolding parameters from fluorescence intensity at selected wavelength the several unfolding curves (pressure dependency of intensity at several wavelengths) should be used. The global fit with shared parameters $\Delta_u V$ and $\Delta_u G$ is appropriate in this case.

5 Added value of work

The global fit software created during this work could be used in:

- Pressure shift method:
  - for construction of unfolding curves from fluorescence spectra.
  - for determination of unfolding parameters from unfolding curves.
- Other research areas.

6 Aknowledgements

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Reference

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Projektą „Mokslo pieva“ organizuoja mokslininkų ir dėstytojų komanda iš Baltijos pažangių technologijų instituto, Kauno technologijos universiteto, Socialinių inovacijų instituto, Vilniaus universiteto ir Vytauto Didžiojo universiteto.
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