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Method and Protocol

Deuteration Aiming for Neutron Scattering

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The distinguished feature of neutron as a scattering probe is an isotope effect, especially the large difference in neutron scattering length between hydrogen and deuterium. The difference renders the different visibility between hydrogenated and deuterated proteins. Therefore, the combination of deuterated protein and neutron scattering enables the selective visualization of a target domain in the complex or a target protein in the multi-component system. Despite of this fascinating character, there exist several problems for the general use of this method: difficulty and high cost for protein deuteration, and control and determination of deuteration ratio of the sample. To resolve them, the protocol of protein deuteration techniques is presented in this report. It is strongly expected that this protocol will offer more opportunity for conducting the neutron scattering studies with deuterated proteins.

Key words: neutron scattering, protein deuteration, contrast matching method

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Introduction

The solution scattering techniques are powerful tools for structural and dynamical studies on a large biological complex or system under physiological environments. Especially, neutron solution scattering is quite advantageous for the structural and dynamical studies on a large biological complex or system. One of unique features of neutron as a scattering probe is an isotope effect on the scattering length [1], which is particularly evident on hydrogen. To be more specific, the neutron scattering length of hydrogen (H) (–3.739 fm) is markedly different from that of deuterium (D) (6.671 fm), rendering the

◀ *Significance* ▶

Selective studies on the target protein in the complex or the multi-component system are possible with neutron scattering assisted by deuterated protein. However, following three problems impeded the neutron scattering studies by normal users. The first one is the requirement of deuterated protein of which the deuteration level is controlled. The second one is the difficulty for efficient determination of the degree of deuteration of protein. The third one is the high cost for sample preparation. Then, we provide the protocol of protein deuteration technique, offering more opportunity for neutron scattering coupled with deuterated protein by normal users.

different visibility to a molecule by replacing the hydrogen with the deuterium. We shall explain the utilization of the isotope effect for the structural study of a large biological complex or system in detail afterwards.

Let's consider a large biological complex or system comprised of several macromolecular components. These components are protein, nucleic acid, and carbohydrate with different scattering length densities (SLDs) from one another. Here, scattering intensity from one component is proportional to the square of $\Delta\rho_{component}$ [1], where $\Delta\rho_{component}$ is called contrast defined as the absolute difference between SLDs of the component ($\rho_{component}$) and of solvent ($\rho_{solvent}$), $\Delta\rho_{component}=|\rho_{component}-\rho_{solvent}|$. By setting the condition of $\Delta\rho_{component}=0$, namely "matching condition", the scattering from the component almost becomes disappeared, implying that the component becomes "scatteringly invisible". This is the most notable technique to selectively observe the target component, as a visible component, in the multi-components system and/or the complex with the solution neutron scattering.

There is one inevitable prerequisite to allow the matching technique: The components in the system should have different SLD values. One of the suitable research targets with this technique is a solution structure of nucleosome, which consists of histones (protein) and DNA. Because the SLD values of histone and DNA are $\sim2.2\times10^{-6}$ Å^{-2} and $\sim3.9\times10^{-6}$ Å^{-2}, respectively, the matching condition for histone is achieved with 40% (v/v) D$_2$O solvent ($\rho_{solvent}=2.2\times10^{-6}$ Å^{-2}) and that for DNA is also with 65% (v/v) D$_2$O solvent ($\rho_{solvent}=3.9\times10^{-6}$ Å^{-2}), as shown in Figure 1. Then, we can selectively observe the DNA structure of nucleosome by making the histones scatteringly invisible in 40% (v/v) D$_2$O solvent, and can selectively observe the histones structure by making the DNA scatteringly invisible in 65% (v/v) D$_2$O solvent as well. This has been extensively applied for the several structural studies of nucleosomes with small-angle neutron scattering (SANS) [2–4].

Here, there arises one issue, "*How can we selectively observe the target protein when the all components in the complex or system are proteins?*" The answer is "protein deuteration". As shown in Figure 2, the SLD value of the protein is varied with its deuteration ratio. To selectively observe the target subunit in the complex or the target protein in the multi-component solution, the standard combination for the proteins (subunits) is 100% deuterated (100d-) proteins (subunits) and non-deuterated (hydrogenated (h-)) one(s). By using 40% (v/v) D$_2$O as the solvent, the h-proteins become invisible (refer to Fig. 1) and the 100d-proteins are selectively visible with the high contrast. This method (CM-SANS) using small-angle neutron scattering (SANS) assisted by contrast matching (CM) has solved the structure of the target component in the complex or system [5–7].
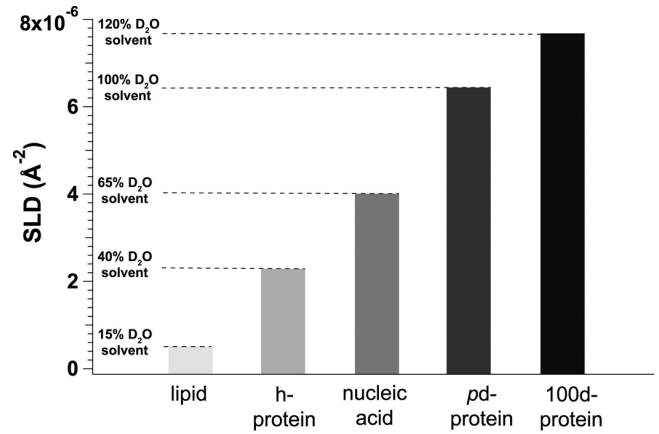

Figure 1   Neutron scattering length densities (SLD) of lipid, hydrogenated protein (h-protein), nucleic acid, partially-deuterated protein (*p*d-protein) and 100% deuterated protein (100d-protein).
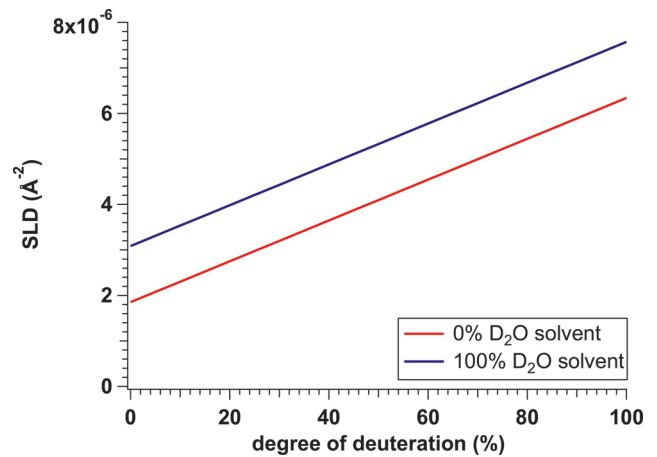

Figure 2   SLD of protein in 0% (red line) and 100% (blue line) D$_2$O solvent as a function of the degree of deuteration.

However, there is a drawback in CM-SANS method, which is attributed to a large number of hydrogens in 40% (v/v) D$_2$O solvent. The hydrogen atom causes the intensive incoherent scattering, which becomes the background scattering and deteriorates the signal-to-noise (S/N) ratio, especially in the higher scattering vector (Q) range of SANS intensity profile. To overcome the drawback, inverse contrast matching SANS (iCM-SANS) method has been proposed [8]. In the iCM-SANS, partially deuterated (c.a. 75%) and non-deuterated protein components are 'invisible' and 'visible', in terms of scattering, in 100% D$_2$O solvent, respectively. In the iCM-SANS, it should be noted that partially deuterated (*p*d-) proteins become invisible (refer to Fig. 1) and the h-proteins are selectively observed under the low incoherent background scattering thanks to 100% D$_2$O as the solvent. Recently, iCM-SANS is utilized for the investigation of partial structures in the complicated complexes [8–10].

As mentioned above, the protein deuteration and related techniques are indispensable for the neutron scattering study with taking the advantage of the neutron characteristics. However, there are obstacles for the normal users to perform the neutron scattering studies with deuterated protein as a conventional technique. In this report, we introduce the full protocol package for CM- and iCM-SANS, and the other neutron solution scattering. This protocol is comprised of following three sections.

In the first section, the procedure for the preparation of deuterated protein of which degree of deuteration is controlled is introduced. In the second section, the efficient procedure for the determination of the degree of deuteration of protein is presented. In the third section, the procedure for recycling of D_2O, which serves to the cost reduction of sample preparation, is summarized. It is strongly expected that this protocol will offer more opportunity for structural study of a large biological complex or system with neutron scattering for the structural biologists from various research fields.

Materials and Methods

Chemical Reagents

Hydrogenated and deuterated D-glucose-1,2,3,4,5,6,6-d7 (97% atom D) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Isotec Inc. (Miamisburg, OH, USA) respectively. D_2O (99.8% atom D) was purchased from Taiyo Nippon Sanso Corporation (Tokyo, Japan). LC/MS grade 0.1% aqueous trifluoroacetic acid and acetonitrile were purchased from GL Science Inc. (Tokyo, Japan). Sinapinic acid (Matrix substance for MALDI-MS) and protein standard II were purchased from Bruker Daltonics (Billerica, MA, USA). All other chemical reagents were purchased from Sigma Aldrich (St Louis, MO, USA) or Wako Pure Chemical Industries.

MALDI-TOF Mass Spectrometry (MALDI-TOF MS)

The protein sample solution and a saturated solution of sinapinic acid in TA30 (30% acetonitrile in 70% of 0.1% TFA in water) were mixed with the volume ratio of 1 to 9 and dropped on the ground steel MALDI plate: 1 µl protein for sample solution and 9 µl for saturated sinapinic acid one in the standard case. After drying of MALDI-TOF MS sample and crystallization of droplets, the measurement was conducted with a microflexLT MALDI TOF Mass Spectrometer (Bruker Daltonics) in positive ion mode. External calibrations were performed using protein standards II. Mass spectrum raw data was recorded with flexControl and analyzed with flexAnalysis (Bruker Daltonics).

Small-angle neutron scattering (SANS)

SANS experiments were performed with Quokka [11]

installed at the Australian Nuclear Science and Technology Organization (ANSTO, Lucas Heights, NSW, Australia). The wavelength and the sample-to-camera distance were 6.0 Å and 6.0 m, respectively, covering the Q range from 0.012 Å^{-1} to 0.10 Å^{-1}. The obtained SANS intensity profiles were corrected for background, empty cell, buffer solution scattering, and transmittance. Utilizing the incident beam flux, the SANS intensity profiles were converted to absolute intensities (cm^{-1}).

Fourier transform infrared (FT-IR) spectroscopy

The IR spectra were measured with FT/IR-4600 (Jasco, Tokyo, Japan) equipped with a triglycine sulfate detector, Ge/KBr beam splitter and ATR PRO ONE. The sample solution with the volume of 3.0 µL was placed on the center of diamond prism and immediately measured. Spectra were recorded in the wavenumber covering from 500 to 4000 cm^{-1} with the resolution of 4 cm^{-1} at a scan rate of 2 mm/min. To secure high S/N ratio of observed spectrum, the spectrum was finally acquired after averaging of spectra from 32 scans using the Spectra Manager (Jasco).

Evaporator

To collect the D_2O from the used 100% D_2O containing M9 medium (used 100% D_2O M9 medium), used 100% D_2O M9 medium was evaporated with the Rotavapor® R-300 (Buchi, Flawil, Switzerland) equipped with the vacuum pump V-300 (Buchi), the heating bath B-305 (Buchi) and the circulating water pump LTB-125 (AS ONE Co., Osaka, Japan). The circulating water of which temperature was set to 4°C, was connected to the reflux tube of the evaporator. Flask filled with 100% D_2O M9 medium was immersed in the heating bath at 42°C and rotated at 140 rpm. The internal pressure was initially set at 200 mbar and then reduced to 100 mbar step-wisely. After confirming the absence of sudden boiling, the internal pressure was gradually reduced to 30 mbar.

Software

VolumeCalc1 calculates the numbers of exchangeable and non-exchangeable H atoms with a sequence of one- or three-letter abbreviation of amino acid residues as an input file (ASCI file). For the calculation, VolumeCalc1 obtained the number of non-exchangeable hydrogen atoms in each amino acid residue from the report of Jacrot [12]. In addition, VolumeCalc1 also provides for the expected SLD of a concerned protein corresponding to the degree of deuteration of protein and the volume fraction of D_2O in the buffer.

D_2O/H_2O ratio calculator (d2or) is a Python script using the Numpy library. The peaks at around 1200 cm^{-1} (Peak 1) and 1450 cm^{-1} (Peak 2) [13] correspond to the frequency of bending vibration of D-O-D bonding and that of H-O-D
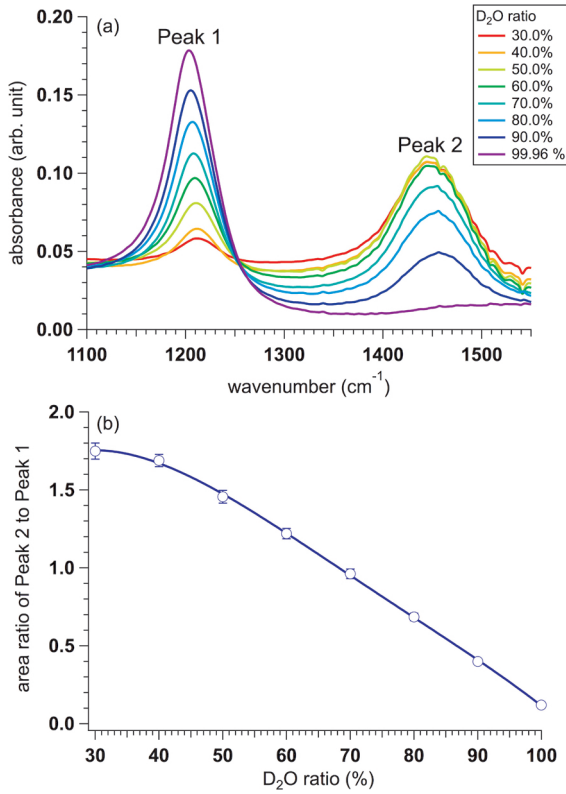
Figure 3 (a) IR spectra from D$_2$O/H$_2$O ratio of 30.0% (v/v) (orange line), 40.0% (v/v) (lemon line), 50.0% (v/v) (light green line), 60.0% (v/v) (green line), 70.0% (v/v) (cyan line), 80.0% (v/v) (light blue line), 90.0% (v/v) (blue line) and 99.96% (v/v) (purple line), respectively. (b) Area ratio of Peak 2 to Peak 1 as a function of D$_2$O/H$_2$O ratio (circle) and solid line corresponds to fit with a polynomial equation.

bonding, respectively (Fig. 3 (a)). By changing the volume fraction of D$_2$O, we measured IR spectra and calculated the integrated intensity from 1150 cm^{-1} to 1250 cm^{-1} (area 1) and that from 1400 cm^{-1} to 1500 cm^{-1} (area 2). From the ratio of area 2 to area 1 as a function of volume fraction of D$_2$O, the calibration curve was determined (Fig. 3 (b)). With this calibration curve, the D$_2$O/H$_2$O ratio of a concerned buffer can be computed by supplying the ratio of area 2 to area 1 as an input parameter.

Software is available from following our website (http://www.rri.kyoto-u.ac.jp/NSBNG/activity.html).

Protocols

Preparation of 100% and partially deuterated protein samples

Purpose

As described in the introduction, contrast $\Delta\rho_{component}$ is a crucial parameter that determines "scatteringly visibility" (or "scatteringly invisibility") of solute in the solution. The forward scattering intensity (I_0) is related to $\Delta\rho_{component}$ through the following equation.

$$I_0 = NV^2(\rho_{component} - \rho_{solvent})^2 = NV^2\Delta\rho_{component}^{\;2}, \qquad \text{eq. (1)}$$

where N and V correspond to the number density and volume of component (solute), respectively. Referring to Figure 1, the SLDs of h- and pd-proteins are scatteringly invisible in 40% (v/v) D$_2$O solvent and 100% D$_2$O solvent, respectively. Taking advantage of their properties, the combination of hydrogenated (h-) and 100%-deuterated (100d-) proteins is utilized for CM-SANS. And the combination of h- and pd-proteins is utilized for iCM-SANS.

In this section, we introduce the procedures for the preparation of recombinant 100d- and pd-proteins with the *E. coli* expression system.

Preparations for pre-culture and culture of E. coli for 100d- and pd-proteins

Prior to the pre-culture of *E. coli* for 100d-protein, the following procedures are needed.

0. H$_2$O should be sterilized with autoclaving.

1. Referring to the ingredients summarized in Table 1, 5 times concentrated LB broth (5× LB broth) is prepared. This is utilized for the pre-culture of both 100d- and pd-proteins.

2. Referring to the ingredients in Table 2, 10 times concentrated M9 medium (10× M9 medium) is prepared. This is used for the culture of pd-protein.

3. Referring to the ingredients in Table 3, the additive solution for M9 medium with the volume of 1 L is prepared. To avoid the contamination of H$_2$O to M9 medium, this solution is applied to freeze-drying. After freeze-drying, it is stored at –20 °C.

4. The solutions of appropriate inducer (ex. isopropyl

Table 1   Ingredients of 5× LB broth

5× LB broth		1× concentration
bacto-tryptone	50.0 g	10.0 g/L
bacto-yeast extract	25.0 g	5.0 g/L
NaCl	50.0 g	10.0 g/L
NaOH		adjust to pH 7.5
D. W	up to 1 L	
Total	1 L	*autoclave

Table 2   Ingredients of 10× M9 medium

10× M9 medium		1× concentration
NH$_4$Cl	10.0 g	18.7 mM
KH$_2$PO$_4$	20.0 g	14.7 mM
Na$_2$HPO$_4$	63.4 g	44.7 mM
NaCl	5.0 g	8.6 mM
H$_2$O	up to 1 L	
Total	1 L	*autoclave

Table 3   Ingredients of additive solution for 1 L M9 medium

Additive solution for 1 L M9 medium		final concentration
2 M MgSO$_4$	1.0 mL	2.0 mM
1 M CaCl$_2$	100.0 μL	100.0 μM
0.1 M FeCl$_3$	216.0 μL	21.6 μM
50 mg/mL Thiamin	48.0 μL	2.4 mg/L
1 mg/mL Biotin	1.2 mL	1.2 mg/L
Total	2.6 mL	*freeze-dry

β-D-thiogalactopyranoside) and antibiotic (ex. Ampicillin) are prepared in H$_2$O. These solutions are sterilized with a filter having the pore size of 0.22 μm. To avoid the contamination of H$_2$O to M9 medium, the filtered solutions are applied to freeze-drying. After freeze-drying, they are stored at –20°C.

5. Flask for the culture, the filtration device, the culture tubes and so on are autoclaved. They are dried at 100°C for at least 4 days to avoid the contamination of H$_2$O.

Culture for 100d-protein

Firstly, the adaptation of *E. coli* to the D$_2$O environment is indispensable for avoiding long, and variable lag time of growth [14]. The volume fraction of D$_2$O in the culture solution must be step-wisely increased at the stage of pre-culture, hence four steps are needed for the culture for 100d-protein. In the following, we briefly summarize the procedures at each step and further detailed procedures are shown in Figure 4.

Step 1: 1st pre-culture

Referring to the ingredients in Table 4, LB broth containing 30% D$_2$O (30% D$_2$O LB broth) with the volume of 5 mL is prepared. Then, 30% D$_2$O LB broth is inoculated from the glycerol stock solution of *E. coli*. The bacterial cells are cultured in 30% D$_2$O LB broth for over 12 h at 37°C.
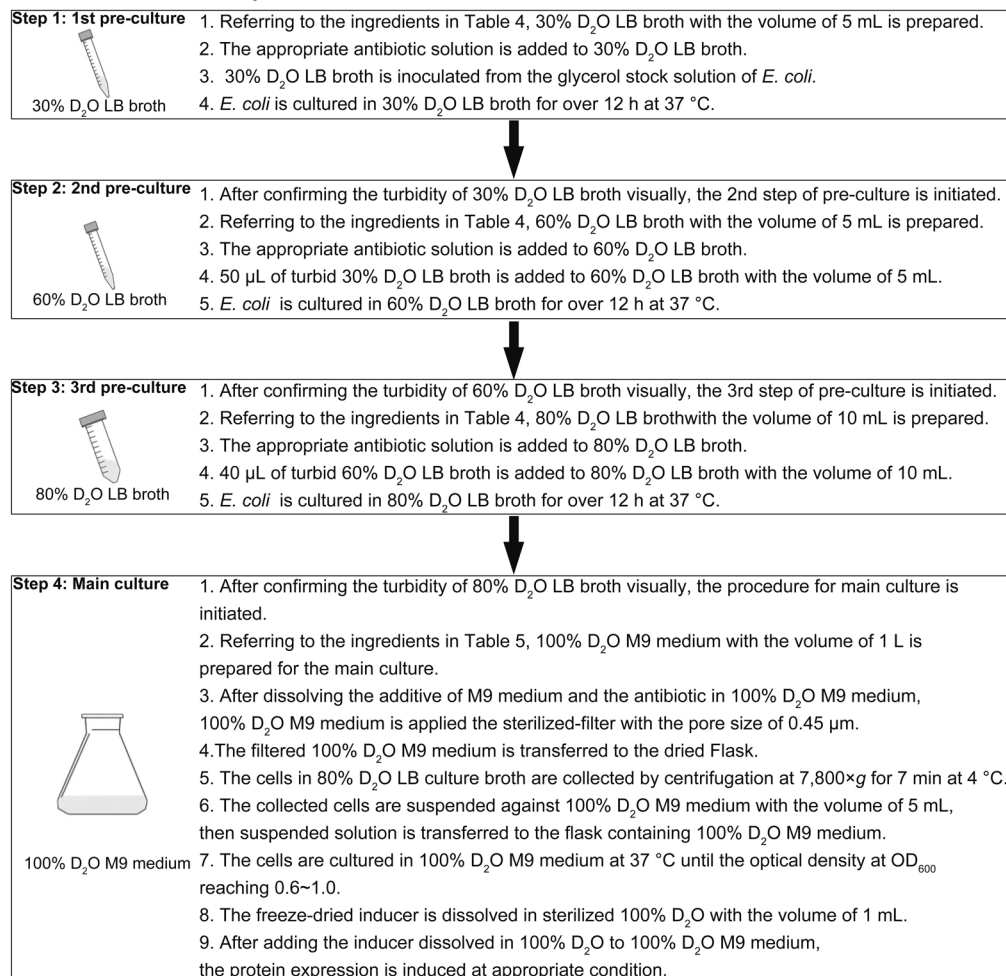
Culture for 100d-protein

Figure 4   The schematic procedures for the cultivation of 100d-protein.

Table 4   Ingredients of 30%, 60% and 80% D_2O LB broth

30% D_2O LB broth	
5× LB broth	1.0 mL
filter-sterilized D_2O	1.5 mL
sterile H_2O	2.5 mL
antibiotic (in H_2O)	
Total	5.0 mL

60% D_2O LB broth	
5× LB broth	1.0 mL
filter-sterilized D_2O	3.0 mL
sterile H_2O	1.0 mL
antibiotic (in H_2O)	
Total	5.0 mL

80% D_2O LB broth	
5× LB broth	1.0 mL
filter-sterilized D_2O	4.0 mL
antibiotic (in H_2O)	
Total	5.0 mL

Step 2: 2nd pre-culture

Referring to the ingredients in Table 4, LB broth containing 60% D_2O (60% D_2O LB broth) with the volume of 5 mL is prepared. Then, 50 μL of turbid 30% D_2O LB broth is added to 60% D_2O LB broth. *E. coli* is cultured in 60% D_2O LB broth for over 12 h at 37°C.

Step 3: 3rd pre-culture

Referring to the ingredients in Table 4, LB broth containing 80% D_2O (80% D_2O LB broth) with the volume of 10 mL is prepared. Then, 40 μL of turbid 60% D_2O LB broth is added to 80% D_2O LB broth. *E. coli* is cultured in 80% D_2O LB broth for over 12 h at 37°C.

Step 4: Main culture

Referring to the ingredients in Table 5, 100% D_2O M9 medium with the volume of 1 L is prepared for the main culture. After dissolving the additive of M9 medium and

the antibiotic in 100% D_2O M9 medium, 100% D_2O M9 medium is applied to the sterilized-filter. The cells in 80% D_2O LB culture broth are collected by centrifugation and the collected cells are suspended against 100% D_2O M9 medium. Then, the suspended solution of the cells is cultured in 100% D_2O M9 medium at 37°C until the optical density of 600 nm (OD_{600}) reaching 0.6~1.0. Finally, the protein expression is induced at the appropriate condition after adding the inducer.

Culture for pd-protein

Three steps are needed for the culture for *p*d-protein. In the following, we briefly summarize the procedures at each step and further detailed procedures are presented in Figure 5.

Step 1: 1st pre-culture

Referring to the ingredients in Table 4, 30% D_2O LB broth with the volume of 5 mL is prepared. Then, 30% D_2O LB broth is inoculated from the glycerol stock solution of *E. coli*. The bacterial cells are cultured in 30% D_2O LB broth for over 12 h at 37°C.

Step 2: 2nd pre-culture

Referring to the ingredients in Table 4, 60% D_2O LB broth with the volume of 10 mL is prepared. Then, 100 μL of turbid 30% D_2O LB broth is added to 60% D_2O LB broth. *E. coli* is cultured in 60% D_2O LB broth for over 12 h at 37°C.

Step 3: Main culture

Referring to the ingredients in Table 6, 75% D_2O M9 medium with the volume of 1 L is prepared for the main culture. After dissolving the additive of M9 medium and the antibiotic in 75% D_2O M9 medium, 75% D_2O M9 medium is applied to the sterilized-filter. The cells in 60% D_2O LB culture broth are collected by centrifugation and the collected cells are suspended against 75% D_2O M9 medium. Then, the suspended solution of the cells is cultured in 75% D_2O M9 medium at 37°C until OD_{600} reaching 0.6~1.0. Finally, the protein expression is induced

Table 5   Ingredients of 100% D_2O M9 medium

Ingredient	weight/volume	final concentration
NH_4Cl	1.0 g	18.7 mM
KH_2PO_4	2.0 g	14.7 mM
Na_2HPO_4	6.34 g	44.7 mM
NaCl	0.5 g	8.6 mM
deuterated D-glucose	2.0 g	2.0 mg/mL
D_2O	1 L	
freeze-dried antibiotic	appropriate weight	appropriate concentration
freeze-dried additive	Refer to Table 3	
Total	1 L	*filter sterilize

Culture for *p*d-protein

Step 1: 1st pre-culture

30% D$_2$O LB broth

1. Referring to the ingredients in Table 4, 30% D$_2$O LB broth with the volume of 5 mL is prepared.
2. The appropriate antibiotic solution is added to 30% D$_2$O LB broth.
3. 30% D$_2$O LB broth is inoculated from the glycerol stock solution of *E. coli*.
4. *E. coli* is cultured in 30% D$_2$O LB broth for over 12 h at 37 °C.

Step 2: 2nd pre-culture

60% D$_2$O LB broth

1. After confirming the turbidity of 30% D$_2$O LB broth visually, the 2nd step of pre-culture is initiated.
2. Referring to the ingredients in Table 4, 60% D$_2$O LB broth with the volume of 10 mL is prepared.
3. The appropriate antibiotic solution is added to 60% D$_2$O LB broth.
4. 100 µL of turbid 30% D$_2$O LB broth is added to 60% D$_2$O LB broth with the volume of 10 mL.
5. *E. coli* is cultured in 60% D$_2$O LB broth for over 12 h at 37 °C.

Step 3: Main culture

75% D$_2$O M9 medium

1. After confirming the turbidity of 60% D$_2$O LB broth visually, the procedure for main culture is initiated.
2. Referring to the ingredients in Table 6, 75% D$_2$O M9 medium with the volume of 1 L is prepared for the main culture.
3. After dissolving the additive of M9 medium and the antibiotic in 75% D$_2$O M9 medium, 75% D$_2$O M9 medium is applied the sterilized-filter with the pore size of 0.45 µm.
4. The filtered 75% D$_2$O M9 medium is transferred to the dried Flask.
5. The cells in 60% D$_2$O LB culture broth are collected by centrifugation at 7,800×*g* for 7 min at 4 °C.
6. The collected cells are suspended against 75% D$_2$O M9 medium with the volume of 5 mL, then suspended solution is transferred to the flask containing 75% D$_2$O M9 medium.
7. The cells are cultured in 75% D$_2$O M9 medium at 37 °C until the optical density at OD$_{600}$ reaching 0.6~1.0.
8. The freeze-dried inducer is dissolved in sterilized 75% D$_2$O with the volume of 1 mL.
9. After adding the inducer dissolved in 75% D$_2$O to 75% D$_2$O M9 medium, the protein expression is induced at appropriate condition.

Figure 5　The schematic procedures for the cultivation of *p*d-protein.

Table 6　Ingredients of 75% D$_2$O M9 medium

Ingredient	weight/volume	final concentration
10× M9 medium	100.0 mL	
deuterated D-glucose	1.5 g	1.5 mg/mL
hydrogenated D-glucose	0.5 g	0.5 mg/mL
D$_2$O	750.0 mL	
H$_2$O	150.0 mL	
freeze-dried antibiotic	appropriate weight	appropriate concentration
freeze-dried additive	Refer to Table 3	
Total	1 L	*filter sterilize

at the appropriate condition after adding the inducer.

Following the procedures of culture for 100d-protein, deuterated synechococcus KaiA (SyKaiA) was obtained with an amount of *ca*. 5 mg after appropriate purification steps. Following the procedures of culture for *p*d-protein, *p*d-αB-crystallin (αB-cry) was obtained with an amount of *ca*. 6 mg after appropriate purification steps.

Efficient determination of the degree of deuteration of protein and D$_2$O/H$_2$O ratio in buffer

Purpose

As given in eq. (1), both $\rho_{\text{component}}$ and ρ_{solvent} affect the observable I_0 value. To realize a successful iCM (or CM) SANS study, both the degree deuteration of protein and the D$_2$O/H$_2$O ratio in the buffer should be determined accurately. In this section, we firstly introduce the procedure that can efficiently determine the degree of deuteration of protein through MALDI-TOF MS. As a next step, we also introduce the procedure that can efficiently determine the D$_2$O/H$_2$O ratio in the buffer through FT-IR spectroscopy.

Efficient determination of the degree of deuteration of protein with MALDI-TOF MS

Because the nonvolatile ingredients in the buffer such as

Tris and NaCl sometimes retards the ionization of a sample, the desalting of a sample is normally required prior to MALDI-TOF MS measurement. On the other hand, we have succeeded to obtain a mass spectrum with high S/N even from the non-desalting sample with the following two technical tips. The first one is the adjustment of the molar ratio of a protein sample solution and saturated sinapinic acid one of 1 to 1000 in the MALDI-TOF MS sample. The second one is the preparation of a protein sample solution at the concentration of 5.0~10.0 mg/mL. Considering the above-described experimental requirements, SyKaiA solution at the concentration of 6.0 mg/mL and αB-cry at the concentration of 8.0 mg/mL were utilized for the preparation of MALDI-TOF MS samples.

The buffer containing D_2O is normally utilized for neutron scattering, hence it is desirable for determining the degree of deuteration of protein in the buffer used for neutron scattering. On the other hand, it was reported that the exchange rates from H atoms to D atoms of the exchangeable H atoms of amino acid residues in D_2O were different, reflecting the variation of the local environment of amino acid residues in the protein [15]. It makes the coexistence of H and D atoms in the exchangeable H atoms of protein, hindering the accurate determination of mass of 100d- (or pd-) protein. As a result, the degree of deuteration of 100d- (or pd-) protein cannot be determined accurately from a single mass spectrum of 100d- (or pd-) protein. To overcome such a problematic situation, we have to consider an alternative procedure.

Since the number of non-exchangeable H atoms in the protein is not affected by the D_2O/H_2O ratio in the buffer, the number of non-exchangeable D atoms in the deuterated protein is usable for the determination of the degree of deuteration of protein. We then adopt the following procedures.

1. Both deuterated and hydrogenated proteins must be dialyzed against the buffer with the same D_2O/H_2O ratio for the same length of time.
2. MALDI-TOF MS measurements are performed for both samples.
3. Because the peak shift between 100d- (or pd-) and h-protein in the MS spectra corresponds to the number of non-exchangeable D atoms in the deuterated protein, the peak shift value between two samples ($\Delta m/z$) is calculated.
4. The degree of deuteration of protein can be calculated from dividing $\Delta m/z$ by the total number of non-exchangeable H atoms in the h-protein.

Figure 6 shows the MS spectra of hydrogenated and deuterated SyKaiA and those of hydrogenated and partially deuterated αB-cry. There are several peaks in the mass spectra. We adopted the peaks with the lowest m/z (indicated by solid arrows) for the calculation of the degree of deuteration of protein because they have less effect from the solvents. In the present work, the averaged $\Delta m/z$ values

from three different sample lots were utilized for further analysis. The numbers of total non-exchangeable H atoms in h-protein and $\Delta m/z$ values of SyKaiA and αB-cry are summarized in Table 7 and the degrees of deuteration of SyKaiA and αB-cry are calculated to 101.0±1.5% and 70.4±1.2%, respectively. It implies that the expected degrees of deuteration are approximately realized by the above-described cultivation procedures. It should be stressed that the degree of deuteration of protein can be determined within one hour from our protocol.

To check whether this 70.4% deuterated αB-cry is "scatteringly invisible" in 100% D_2O solvent or not, SANS measurements were performed with this αB-cry in 0%, 40% and 100% D_2O solvent (Fig. 7 (a)). With increasing the volume fraction of D_2O, the scattering intensity decreased and 70.4% deuterated αB-cry was almost scatteringly invisible in 100% D_2O. With this data, we also plotted $(I_0/c)^{0.5}$, which is comparable to $\Delta\rho_{component}$ in eq. (1), as a function of volume fraction of D_2O (Fig. 7 (b)). It was revealed that $(I_0/c)^{0.5}$ was equal to 0 at the volume fraction of 98.8±1.5% D_2O, supporting its scattering invisibility in 100% D_2O buffer.

To assess the tolerance of deviation from the exact degree of deuteration that can be matched in 100% D_2O solvent (exact degree of deuteration point), we also calculated the expected I_0 value as a function of the degree of deuteration of αB-cry in 100% D_2O. As shown by the solid arrow in Figure 8, it was revealed that 73.2% deuterated αB-cry is exactly scatteringly invisible in 100% D_2O solvent. Assuming the case that the degree of deuteration of pd-αB-cry was 5% deviated from 73.2%, the expected I_0 value of 68.2% (or 78.2%) deuterated αB-cry is computed to 2.5×10^{-3} cm^{-1}. This value approximately corresponds to 1/200 of I_0 value of h-αB-cry in 100% D_2O solvent, implying the fulfillment of the condition of scattering invisibility. Hence, it is considered that less than 5% deviation from the exact degree of deuteration point is demanded for conducting iCM-SANS study.

Efficient determination of D_2O/H_2O ratio in the buffer

Prior to neutron scattering, the sample is normally dialyzed against buffer including D_2O. During the dialysis process, it is not possible to avoid the contamination of H_2O from the ambient air absolutely. It prohibits the preparation of strictly controlled D_2O/H_2O ratio in the buffer, hence we also have to grasp the real D_2O/H_2O ratio in the buffer for neutron scattering. Here, we also introduce the efficient method for determining the D_2O/H_2O ratio in the buffer by FT-IR spectroscopy. As we have already explained in Materials and Methods, the ratio of the peak area at around 1450 cm^{-1} (Peak 2) to that of 1200 cm^{-1} (Peak 1) is usable for the determination of D_2O/H_2O ratio in the buffer.

To inspect whether the salt in the buffer affects the IR
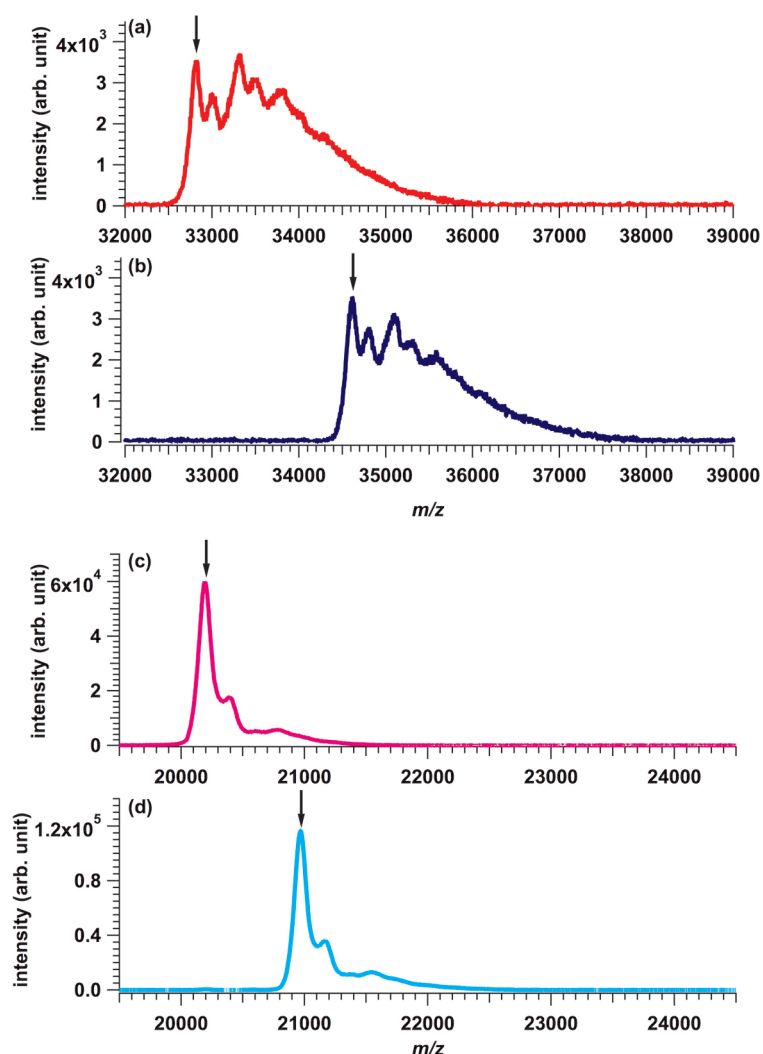
Figure 6   Mass spectra of hydrogenated (a) and deuterated (b) SyKaiA. Mass spectra of hydrogenated (c) and deuterated (d) αB-cry.

Table 7   Numbers of non-exchangeable H atoms in h-protein, $\varDelta m/z$ values and the degrees of deuteration of SyKaiA and αB-cry

	number of non-exchangeable H atoms in h-protein	$\varDelta m/z$	degree of deuteration (%)
SyKaiA	1747	1764.5±26.2	101.0±1.5
αB-cry	1093	773.2±13.0	70.4±1.2

spectrum or not, we prepared D$_2$O buffer comprising of 100 mM Tris/HCl (pH 7.4/pD 7.0), 150 mM NaCl and 90% D$_2$O. The IR spectra from this 90% D$_2$O (blue line), 99.96% D$_2$O (green line) and D$_2$O buffer (red line) are shown in Figure 9. The D$_2$O/H$_2$O ratio of the buffer was calculated to 89.7±0.5%, meaning that the D$_2$O/H$_2$O ratio is not affected by the salt in the buffer. Hence, D$_2$O/H$_2$O ratio of the buffer can be correctly determined through our procedure. With this technique, the D$_2$O/H$_2$O ratio in the buffer can be determined within five minutes.

Recycling of D$_2$O
Purpose

Depending upon the measurement type, the sample volume ranging from 280 μL to 6.0 mL is normally necessary for conducting the neutron scattering. It also means that a large amount of expensive deuterated chemical reagents are required for the sample preparation. Then, we also have to consider the method for reducing the cost for sample preparation. Since 100% D$_2$O is not necessary for the preparation of *p*d-protein, recycling of
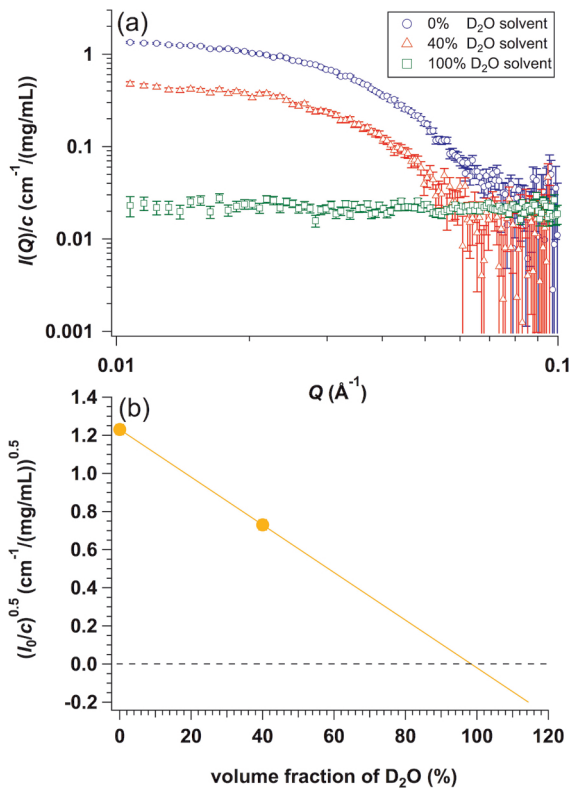
Figure 7 (a) SANS intensity profiles as a function of Q of pd-αB-cry in 0% (blue circle), 40% (v/v) (red triangle) or 100% (green square) D$_2$O solvent. (b) $(I_0/c)^{0.5}$ as a function of volume fraction of D$_2$O. The solid line corresponds to the result of fit with a linear function.
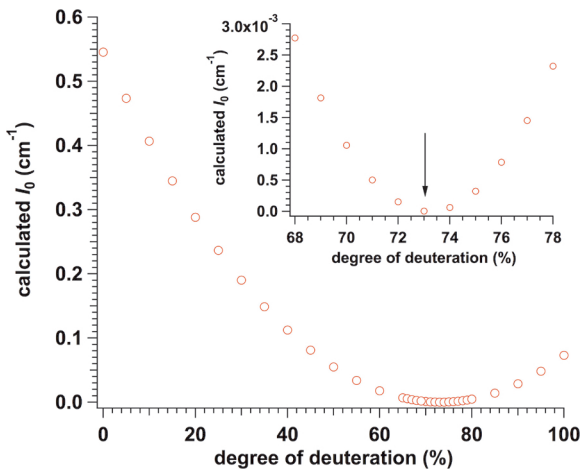
Figure 8 The calculated I_0 value as a function of the degree of deuteration level of αB-cry in 100% D$_2$O. Inset indicates its magnified plot at around the exact degree of deuteration point (=73.2%), indicated by the solid arrow.

D$_2$O from D$_2$O containing M9 medium would contribute to the reduction of the cost for the preparation of pd-protein. In this section, we introduce the procedures for recycling
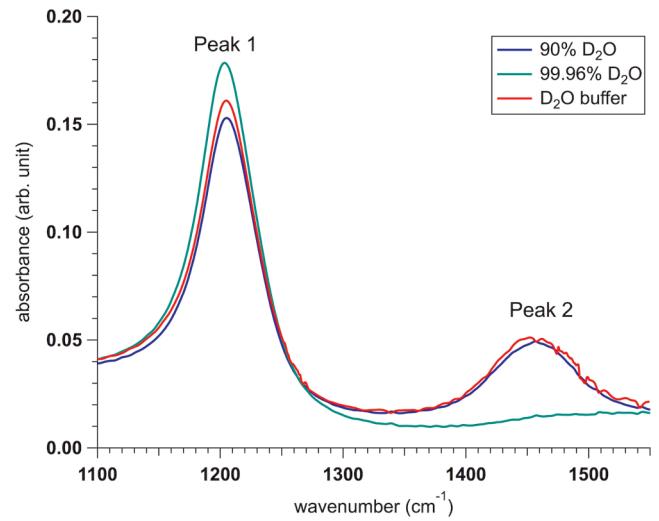
Figure 9 IR spectra of 90.0% (v/v) D$_2$O (blue line), 99.96% (v/v) D$_2$O (green line) and D$_2$O buffer (red line).

of D$_2$O from the used 100% D$_2$O M9 medium with an evaporator.

Procedure for recycling of D$_2$O

To remove the contaminants from the used 100% D$_2$O M9 medium, the used medium is firstly centrifuged at 7,500×g for 15 min over prior to the commencement of evaporation. After the centrifuge, the supernatant is transferred to a flask of an evaporator. For efficient evaporation, the volume of the supernatant should not exceed 60% of the volume of the flask. Following the procedure described in Materials and Methods, the evaporation of used 100% D$_2$O M9 medium was commenced. Distillation of D$_2$O solvent with the volume of 300 mL was completed within 1–2 h. After distillation, a viscous residual material remain at the bottom of the flask (Fig. 10 (a)). Then, the recycled D$_2$O is transferred from the receiver flask to a dry medium bottle. By adding 1–2 dispensing spoons of activated charcoal to the medium bottle, the bottle cap is tightly sealed with Parafilm (Fig. 10 (b)). Just prior to reuse, activated charcoal is removed from the recycled D$_2$O by filtering with the pore size of 0.22 μm. If there is no loss during the evaporation process, about 95% of the initial volume could be recollected. The D$_2$O/H$_2$O ratio of recycled D$_2$O was calculated to be 99.5±0.2% (Fig. 10 (c)), supporting the successful recycling of used D$_2$O from our protocol.

Considering both the D$_2$O/H$_2$O ratio and collected volume of recycled D$_2$O, it is possible to realize approximately 50% cost reduction for the preparation of pd-protein. Compared to the required sample volume for SANS (280~560 μL), the required sample volume for quasielastic neutron scattering (>1 mL) is still a big load for normal users. Hence, the cost reduction of sample preparation of deuterated protein will offer more
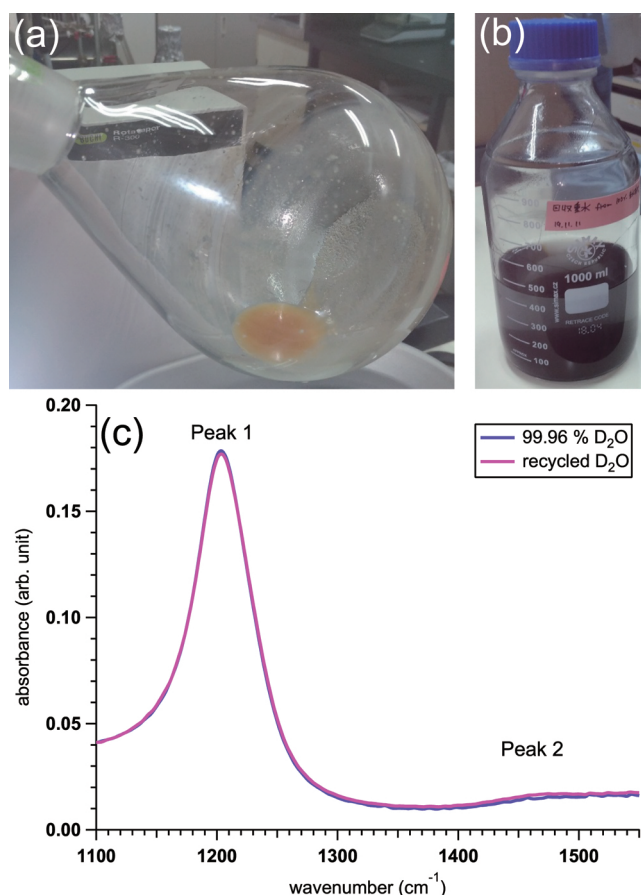
Figure 10 (a) Viscous residual material left after evaporation. (b) Recycled D_2O with activated charcoal in the medium bottle. (c) IR spectra of 99.96% (v/v) D_2O (purple line) and recycled D_2O (pink line) from 100% D_2O M9 medium.

opportunity for studying the dynamics of the target domain in the complex or target protein in the multi-component system with quasielastic neutron scattering.

Future perspective

Recently, more elaborate protein deuteration techniques such as selective isotopic labeling of specific amino acid residue [16], specific region [17] and specific domain in multi-domain [18] are under progress to realize the observation of functionally relevant structure and dynamics of specific sites in the protein. As a next research target, we also have started to prepare the selectively deuterated domain in the multi-domain protein assisted by segment ligation technique [19]. When the efficient segment ligation technique coupled with deuteration one will be established, this protocol will be reported in near future.

Conclusion

The combination of deuterated protein and neutron scattering enables to selectively visualize the target domain in the protein complex or the target protein in the multi-component system. Despite of its unique and notable property, following three obstacles mainly hampered the normal users to conduct the neutron scattering studies on deuterated protein. The first one is the requirement of the deuterated sample of which the degree of deuteration is controlled. The second one is the difficulty for the efficient determination of the degree of deuteration of the sample. The third one is the requirement of a large amount of expensive deuterated reagents. To overcome the above-described problems, the protocol of protein deuteration technique was presented in this report. In the first section, the procedures for the preparation of 100d- and *p*d-protein were presented. In the second section, the procedures concerning the efficient determination of the degree of deuteration of protein and the D_2O/H_2O ratio in the buffer were introduced. In the third section, the procedure for recycling of D_2O from the used 100% D_2O M9 medium was summarized.

It is strongly expected that this basic protocol will offer more opportunity to perform the neutron scattering studies on deuterated protein by normal users.

Conflicts of Interest

The authors declare no competing interests.

Author Contributions

A. O., R. I., Y. Y., M. Y. -U., H. Y., M. Shimizu, R. U., K. K., and M. Sugiyama contributed to the establishment of the protocol for the preparation of 100d- and *p*d-protein. A. O., and N. S. performed MALDI-TOF MS measurements on 100d-, *p*d- and h-protein and analyzed the mass spectra. R. I., and M. Sugiyama performed SANS measurements on *p*d-αB-cry solutions and analyzed SANS intensity profiles. K. M. performed FT-IR spectroscopy measurements on the D_2O buffer and analyzed the spectra. A. O., R. I., T. S., and N. S. contributed to the establishment of the protocol for recycling of D_2O with an evaporator. M. Sugiyama and R. I. created the program of "VolumeCalc1" and "d2or". R. I., and M. Sugiyama designed the research and all of the authors wrote the paper.

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