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Application News

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Spectrophotometric Analysis

Protein Analysis Using FTIR - Secondary Structure Analysis of Bovine Serum Albumin Using Curve Fitting -

Proteins have local three-dimensional structures that are called secondary structures such as α-helices, β-sheets, β-turns, and random coils. These structures are created by the hydrogen bonding of C=O groups and N-H groups of peptide bonds within or in between polypeptide chains. The infrared absorption related to such secondary structures appears as a single broad peak at about 1650 cm^{-1} due to the overlapping of multiple absorption bands. This broad peak is referred to as the "amide I band" and is caused by the stretching vibrations of C=O groups in peptide bonds. By analyzing the amide I band, we can obtain information about the secondary structure of proteins.

A method for determining the individual peaks of overlapping absorption bands is curve fitting (peak splitting). Curve fitting expresses the waveform of each absorption band as a calculated spectrum using approximate curves such as Lorentzian or Gaussian curves and optimizes the peak information (position, intensity, and full width at half maximum) of the approximate curves to minimize the difference between the actually measured spectrum and the calculated spectrum.[1)]

This article introduces an example analysis of the secondary structure of bovine serum albumin (BSA) using curve fitting.

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Sample Preparation

Commercially available BSA was prepared and diluted to 2 mM using water (H_2O). Since the amide I band overlaps with the absorption of H_2O, heavy hydrogen (D_2O) is often used instead. However, there are differences in the form of proteins and hydrogen bonds between D_2O and H_2O, and H_2O is considered to be more close to the natural state.[2)]

Measurement of BSA

The MicromATR™ ATR measurement accessory shown in Fig. 1 was used for measurement with a 9 reflection ATR crystal. The ATR method can obtain spectra by simply pressing a sample against the crystal. In addition, cleaning after measurement is simple compared to the transmission method. By using the 9 reflection ATR crystal, measurement sensitivity is increased compared with a single reflection crystal. Although the 9 reflection ATR crystal can only be used with liquids, the necessary sample amount is minute at 30 µL. The measurement conditions are listed in Table 1 and the difference spectrum (with the intensity of the vertical axis magnified) obtained by subtracting the absorption of H_2O from the infrared spectrum of BSA is shown in Fig. 2. The spectrum was measured with four times zero filling. This is to increase the number of Fourier transform data points and thereby obtain a smoother spectrum so that we can focus on the narrow wavenumber range of the amide I band. In obtaining the difference spectrum to remove the H_2O absorption from the amide I peak, the subtraction was made so that the waveform range from 1900 to 1700 cm^{-1} will be flat (indicated by the blue arrow in Fig. 2). The optical system was purged with dry air in advance since the amide I peak overlaps with the peak of water vapor.

Fig. 1 MicromATR ATR Measurement Accessory

Table 1 Measurement Conditions

Instrument	: IRTracer™-100, MicromATR (9 reflection ATR crystal)
Resolution	: 4 cm^{-1}
Accumulation	: 100
Apodization function	: Sqr-Triangle
Zero filling	: 4 times
Detector	: DLATGS

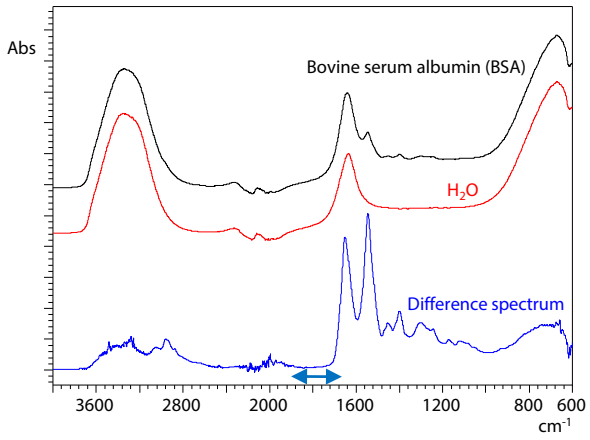

Fig. 2 Infrared Spectra and Difference Spectrum of BSA and H_2O

Second Derivative Spectrum

Curve fitting requires advanced setting of initial values such as the waveform of the absorption band and the number of bands. In determining the number of bands, the second derivative spectrum is used. In this example, we counted the wavenumber of negative peaks in the range from 1700 to 1480 cm^{-1} from the second derivative spectrum shown in Fig. 3 and determined the number of bands.

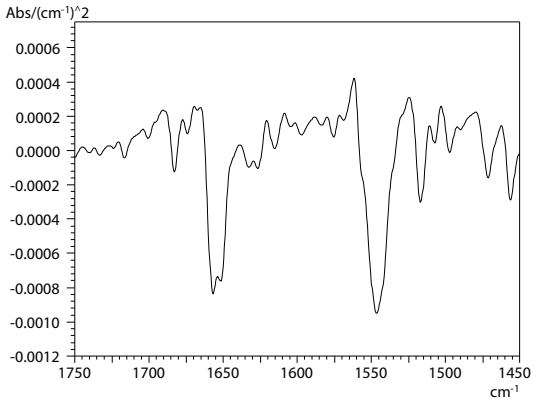

Fig. 3 Second Derivative Spectrum of BSA

Curve Fitting

Based on the information from the second derivative spectrum, we performed curve fitting on the difference spectrum shown in Fig. 2 in the range from 1760 to 1480 cm^{-1}.

Table 2 lists the conditions for curve fitting. Fig. 4 shows the infrared spectrum before curve fitting, the individual peaks determined through curve fitting, and the spectrum synthesized from the individual peaks. If the curve fitting accuracy is favorable, the measured spectrum and the synthesized spectrum should match well.

Table 2 Conditions for Curve Fitting

Peak curve type	: Lorentzian function
Baseline	: Offset 1Pt
Range	: 1760 to 1480 cm^{-1}
Maximum error	: 0.01 %

Secondary Structure Analysis of Proteins

We performed peak detection on the waveform that comprises the amide I band in the range from 1700 to 1600 cm^{-1} and determined peak wavenumbers and corrected areas. The results are listed in Table 3. Based on a reference[2], we assigned secondary structure types to each of the wavelengths and determined the ratio of each type. The resulting ratios were 23.31 %, 37.14 %, 27.56 %, and 12.00 % for α-helices, β-sheets, β-turns, and random coils, respectively.

Table 3 Peak Analysis Results of Curve Fitting

Peak Wavenumber	Secondary Structure	Corrected Area	Area Ratio
1685.78	β-turn	0.221	2.50 %
1679.52	β-turn	0.335	3.80 %
1673.25	β-turn	0.48	5.44 %
1667.47	β-turn	0.617	6.99 %
1662.17	β-turn	0.779	8.83 %
1656.87	α-helix	0.988	11.19 %
1652.05	α-helix	1.069	12.11 %
1646.75	Random coil	1.059	12.00 %
1640.96	β-sheet	1.078	12.21 %
1634.70	β-sheet	1.108	12.55 %
1626.99	β-sheet	1.092	12.37 %

Conclusion

We successfully analyzed the secondary structure of proteins in an aqueous solution by analyzing BSA and employing curve fitting for the amide I band. Analysis can be done easily with a small amount of sample by using FTIR.

References:
1) Mitsuo TASUMI (author and editor), "Introduction to Experimental Infrared Spectroscopy: Fundamentals and Practical Methods" John Wiley and Sons, Inc.
2) Jilie KONG, Shaoning YU. Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. Acta Biochim Biophys Sin 2007, 39(8): 549–559

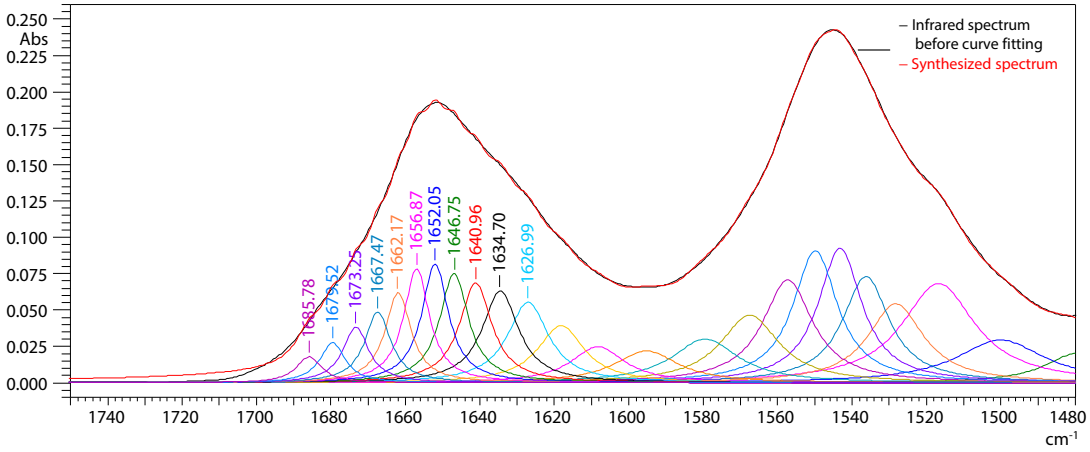

Fig. 4 Individual Peaks and the Synthesized Spectrum Obtained by Curve Fitting

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