Application Note Booklet

CD SPECTROMETER

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CD SPECTROMETER

CONTENTS

Measurement of protein water solution in vacuum UV region

Introduction

CD spectroscopy is an essential tool for structural analysis of proteins. CD spectra reflecting the secondary structure of protein are usually observed from UV region below 260 nm to the vacuum ultraviolet region, however, the water itself used as a solvent has strong absorption in the vacuum UV region. In case of light water (H_2O), its absorption becomes drastically higher at around 175 nm and even the absorption of heavy water (D_2O), at around 170 nm. Therefore, in order to obtain the highly accurate SSE results, the measurement keeping high signal to noise ratio (S/N) down to such wavelength limit for water is required.

The JASCO's new J-1500 CD spectrometer allows the researchers to carry out the measurement with high S/N ratio in the vacuum UV region, by incorporating the several latest technologies such as digital lock-in detection in electrical system, high-throughput optics and highly effective nitrogen gas purging system based on computational fluid simulation.

In this application note, the CD measurement of protein water solution (light water: H_2O) down to the vacuum UV region was performed in order to carry out the SSE, by using of the J-1500 CD spectrometer and multivariate SSE program (Model JWMVS-529).

Keywords: Secondary structure estimation (SSE), CD multivariate SSE, improvement of estimation accuracy of β-sheet structure

Measurement Conditions

Apparatus:	J-1500 CD spectrometer	Data acquisition interval:	0.1 nm
Response:	2 sec	Spectral bandwidth (SBW):	1 nm
Scan speed:	50 nm/min	Accumulation:	4 times
Sample concentration:	1 mg/mL (H_2O)	Cell pathlength:	0.1 mm (cylindrical quartz cell)

Results

CD spectra of human serum albumin (Helix rich), concanavalin A (β-sheet rich) and trypsin inhibitor (Random rich) in light water solution (H_2O) are shown in Fig. 1. CD spectrum reflecting each specific secondary structure was obtained for each protein down to 174 nm with high S/N ratio.

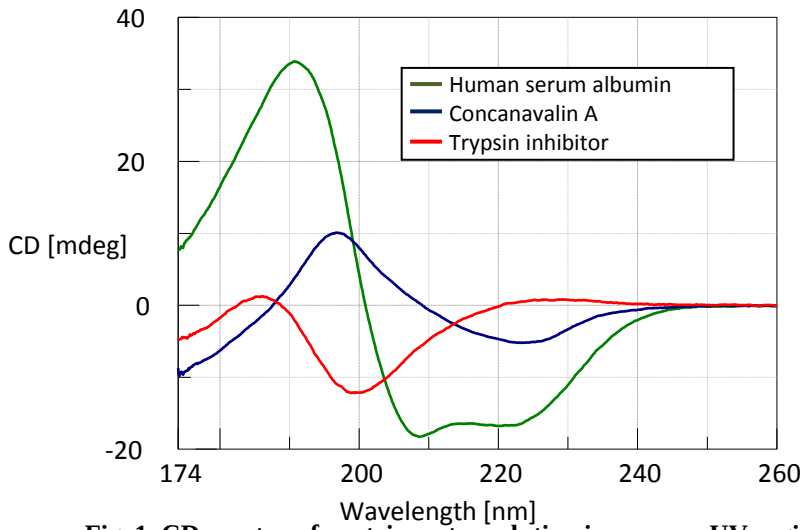


Fig. 1. CD spectra of protein water solution in vacuum UV region

Secondary structure estimation (SSE)

SSE was carried out for spectrum of each protein by JASCO's multivariate SSE program (JWMVS-529) and the results are shown in Table 1 together with each corresponding SSE result by X-ray crystal structural analysis and CDSSTR method for comparison. Regarding the SSE by CD spectrum, it is generally known that the estimation of b-sheet structure is less accurate than that of Helix structure, however in Table 1, as you can clearly see, the better estimation of b-sheet structure was obtained by using JASCO's multivariate SSE program, referring to the results by X-ray. This improvement was achieved by applying PLS/PCR multivariate analysis method and calibration model generated by CD spectra of 26 kinds of standard protein in the range from 260 to 176 nm.

Table 1 SSE results by three different methods

		Helix (%)	β-Sheet (%)	Turn (%)	Random (%)
Human serum albumin	X-ray [1)]	71.8	0.0	8.2	20.0
	JASCO [2)]	70.6	0.0	9.4	20.0
	CDSSTR [3)]	71.1	0.0	6.9	22.9
Concanavalin A	X-ray	3.8	46.4	10.5	39.2
	JASCO	9.7	42.7	10.4	37.2
	CDSSTR	6.1	35.3	12.0	46.6
Trypsin inhibitor	X-ray	1.7	33.1	10.5	54.7
	JASCO	1.0	35.8	14.6	48.6
	CDSSTR	5.1	17.5	16.2	59.5

1) The SSE results by X-ray crystal structural analysis are all referred to the data of RCSB PROTEIN DATA Bank (PDB).

(protein)	(PDB ID)
Human serum albumin:	1bm0
Concanavalin A:	2ctv
Trypsin inhibitor:	1ba7

Helix: (α-helix) + (3/10-helix)
β-Sheet: β-strand
Turn: turn
Random: other

2) In JASCO's multivariate SSE program (JWMVS-529), PLS/PCR method is applied to:

Human serum albumin:	PLS
Concanavalin A:	PLS
Trypsin inhibitor:	PCR

3) In CDSSTR method, the reference spectra "SP22X[H, 3/10, S, T, P2, U]178-260 nm" are used:
Helix: (α-helix) + (3/10-helix)
β-Sheet: β-sheet
Turn: turn
Random: (poly(Pro)II Structure) + (unordered)

Measurement of protein film samples in vacuum UV region

Introduction

CD spectroscopy is considered as an essential tool to analyze the structure of protein samples. CD spectra reflecting the secondary structure of protein are usually observed from UV region below 260 nm to the vacuum ultraviolet region. Nowadays, it has been reported as an example that by CD instrument utilizing synchrotron radiation the protein film sample was successfully measured down to around 140 nm*. CD analysis by using synchrotron radiation is a very effective method to measure CD in vacuum ultraviolet region, however, it is not easy since it requires a very special facility.

JASCO Model J-1500, having the most updated electric system using the latest digital lock-in detection capability, the further improved optical system with higher light through-put and N_2 purge function with higher efficiency which was developed based on flow simulation technology, enables to achieve high S/N CD measurement even in vacuum ultraviolet region. This note shows the analysis example of protein film sample in vacuum ultraviolet region by using J-1500 CD spectrometer

Keywords: Vacuum ultraviolet, CD, protein, film

Measurement condition

Instrument:	J-1500 CD spectrometer	Data interval:	1 nm
Response:	4 sec	Spectral bandwidth :	1 nm
Scan speed:	20 nm/min	Accumulation:	4 times

Results

Protein sample in aqueous solution was dropped on the quartz plate and then evaporated to form a film on the quartz plate. For 4 different kinds of protein film samples, CD and absorbance were measured in 250-163 nm region. Obtained CD and absorbance spectra are shown in Fig. 1. CD spectra of Myoglobin with rich Alpha-helix, Lysozyme with a-helix and b-sheet, Concanavalin A with rich b-sheet and Trypsin inhibitor with rich random structure reflect the structural characteristics of each sample.

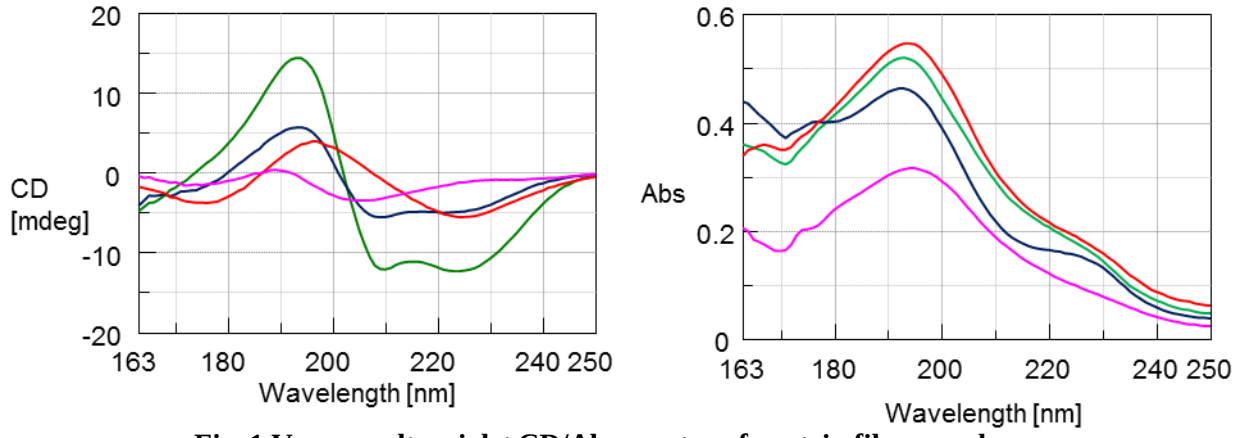


Fig. 1 Vaccum ultraviolet CD/Abs.spectra of protein film samples

Reference

* Modern Techniques for Circular Dichroism and Synchrotron Radiation Circular Dichroism spectroscopy, B. A. Wallace and R. W. Janes (Eds.), IOS Press, 2009, p 43.

Measurement of α-pinene gas in vacuum UV range

Introduction

J-1500 CD spectrometer is the system optimized to the most suitable CD measurement by high-efficiency of the nitrogen purge function that is realized by fluid simulation calculation, the optical system improved for higher throughput and the electrical system using latest digital lock-in detection. By this system, the CD spectra can be obtained with high S/N ratio even in the vacuum UV range or for the sample with high absorption, enabling as a result to improve the accuracy of protein secondary structural analysis or the measurement of the samples with small g value that have been very difficult until now.

The measurement of CD spectra in the vacuum UV range of (1*R*)-(+)-α-pinene gas and (1*S*)-(-)-α-pinene gas is reported as below.

Keywords: Vacuum ultraviolet CD, Gas measurement, α-pinene

Measurement conditions

Instrument:	J-1500 CD spectrometer		
Measurement wavelength range:	245 - 163 nm		
Data sampling interval:	0.1 nm	Response:	1 second
Spectral bandwidth:	1 nm	Scanning speed:	20 nm / min
Accumulation:	1 time	Cell:	Cylindrical quartz cell (optical pathlength 10 mm)

Results

The vacuum ultraviolet CD spectra of (1*R*)-(+)-α-pinene gas and (1*S*)-(-)-α pinene gas are shown in Fig. 1. As shown, the mirror symmetrical CD spectra with high S/N ratio were obtained in the range down to as low as 163 nm, and the sharp peaks specific to the gas sample were also observed.

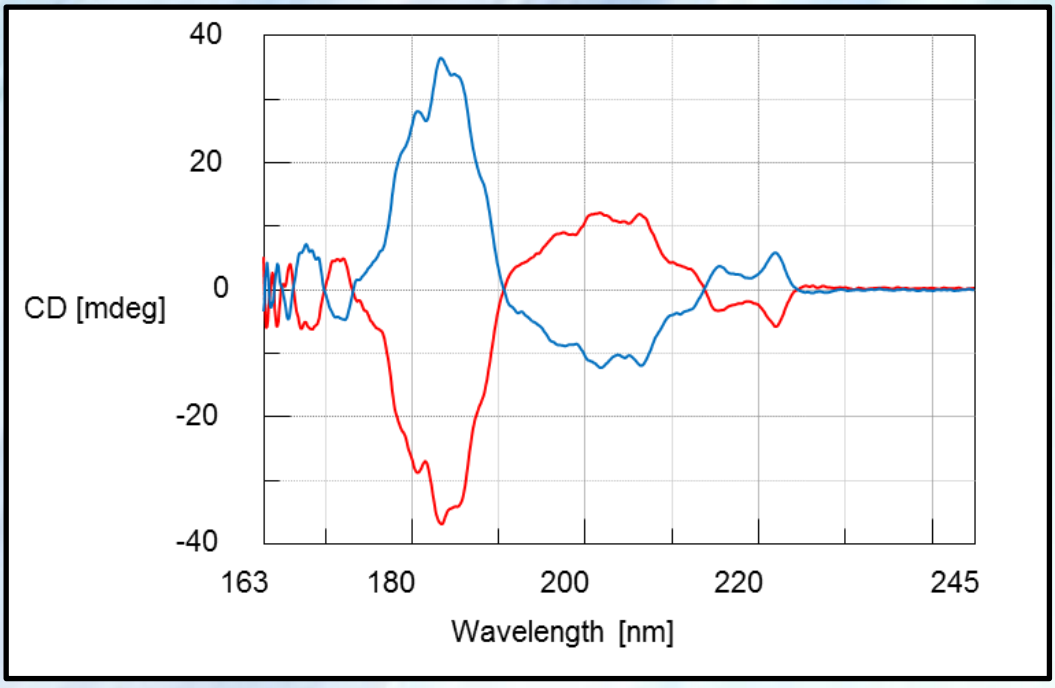


Fig. 1 Vacuum UV CD spectra of α-pinene gas

Measurement of microscale sample
-Measurement of hemoglobin and DNA derived from bovine thymus-

Introduction

CD measurement method is an essential tool for structural analysis of biological molecule such as protein or nucleic acid. In case of using the cell with the optical pathlength of 1mm in CD measurement, it is possible to measure the microscale sample as low as 400 µL in volume and 0.1 mg/mL in concentration, however for the precious samples, there may be the case that it is difficult to get even such amount of samples.

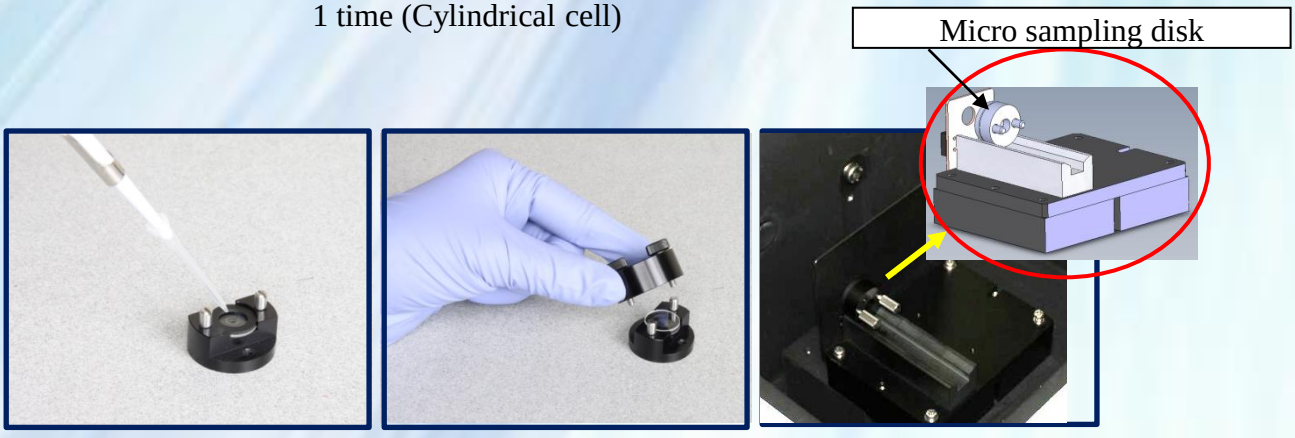
J-1500 CD spectrometer enables the measurement of CD spectra with high accuracy and high S/N ratio of even microscale sample using the micro cell with small acceptance surface, due to the improved optical system for higher throughput and the electrical system using the latest digital lock-in detection.

In this application data, CD measurement of hemoglobin and DNA derived from bovine thymus was implemented using micro sampling disk for microscale sample and J-1500 CD spectrometer. The measurement was performed with sample volume of 3 µL, and secondary structural analysis was performed for CD spectra of hemoglobin.

Keywords: Microassay, Micro sampling disk, Biological molecule

Measurement condition

Measurement unit:	J-1500 CD Spectrometer Micro sampling disk, Cylindrical cell		
Data sampling interval:	0.1 nm	Response:	2 seconds
Spectral bandwidth:	1 nm	Scan speed:	100 nm/min
Optical pathlength:	0.2 mm	Concentration:	0.5 mg/mL
Accumulation:	9 times (Micro sampling disk) 1 time (Cylindrical cell)		



1. Drop of sample　　2. Assembling of disk　　3. Setting to sample compartment

*By simply dropping the sample in the central part of the disk, the sample is surrounded by water repellent, and so the sample can be retained in optical axis even when the disk assembly is placed at vertical position in sample compartment.

Fig. 1 Procedure of micro sampling disk

Measurement results

CD spectra of hemoglobin and DNA derived from bovine thymus by using of micro sampling disk and normal cylindrical cell are shown in Fig. 2 and Fig. 3. Accurate CD spectra with high S/N ratio were obtained in the range down to 180 nm even with sample volume of only 3 µL.

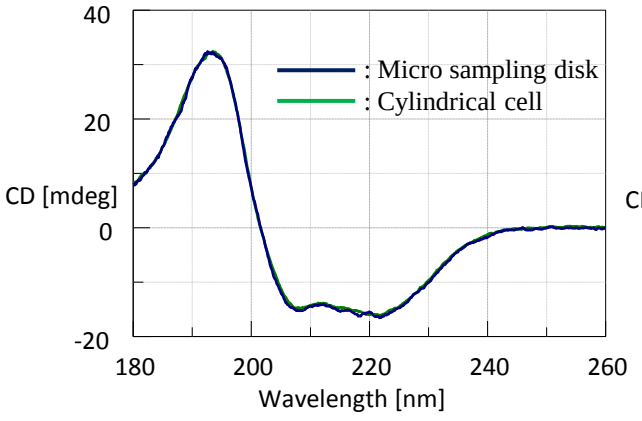


Fig. 2 CD spectra of hemoglobin

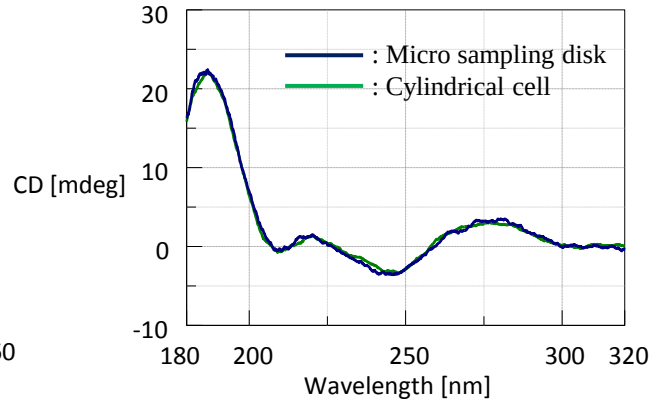


Fig. 3 CD spectra of DNA derived from bovine thymus

Secondary structural analysis

For CD spectra of hemoglobin measured by using the micro sampling disk and cylindrical cell, the secondary structural analysis by JWMVS-529 CD multivariate SSE analysis program was performed. The analysis results comparing with the result by X-ray crystal structural analysis are shown in Table 1. The secondary structural analysis result by CD spectra obtained using micro sampling disk was in good agreement with the result by X-ray crystal structural analysis as well as the result by CD spectra obtained using normal cylindrical cell.

Table 1 Comparison of secondary structural analysis

		Helix (%)	β -Sheet (%)	Turn (%)	Random (%)
Hemoglobin	X-ray	78.0	0.0	6.3	15.7
	Micro- sampling disk	73.8	0.0	10.9	15.3
	Cylindrical- cell	72.2	0.0	12.0	

1) The results of X-ray crystal structural analysis refer to DSSP data included to RCSB PROTEIN DATA Bank (PDB).

Hemoglobin:	2qss
Helix:	(α-helix) + (3/10-helix)
β -sheet:	β-strand
Turn:	turn
Random:	other

2) JWMVS-529 CD multivariate SSE analysis program used here for analysis was by using PCR method.

CD multivariate SSE analysis program JWMVS-529

Introduction

The structure of protein or peptide is closely related with its function, and CD measurement method is widely used for structural analysis of those molecules in the field of protein or peptide applied to medicines. CD spectra show the shape reflecting the abundance ratio of secondary structure of protein or peptide, and secondary structural analysis using CD spectra is to analyze the abundance ratio of the secondary structure of protein or peptide from CD spectra.

JWSSE-513 protein secondary structural analysis program is the program by using Classical Least Squares (CLS) method, including the reference spectra of Yang[1] and Reed[2]. Yang's reference spectra are extracted from CD spectra of protein, and best suited to protein secondary structural analysis. On the other hand, Reed's reference spectra are extracted from CD spectra of peptide, and suitable to the secondary structural analysis of peptide because of less effect by CD derived from side chain of aromatic amino acid often seen in protein. In case of secondary structural analysis of peptide, JWSSE-513 protein secondary structural analysis program with Reed's reference spectra is effective.

In addition, JASCO added JWMVS-529 CD multivariate SSE analysis program as a new lineup. This program includes CD spectra (176 - 260 nm) of 26 proteins, which were created by JASCO and also calibration model based on those spectra. In this program Partial Least Squares (PLS) method which is the latest multivariate analysis method and Principal Component Regression (PCR) method are adopted. In PLS and PCR method, the spectra are compressed to a few potential factors, and the concentration is indicated based on such potential factors. Then abundance ratio of secondary structure is calculated so that the residual error of the concentration may be the minimum. This enables the improvement of the analysis accuracy of b-sheet structure which has no specific strong CD peaks.

Some functions for validating the analysis result and verification function of created calibration model are also included.

Keywords: Secondary structural analysis, PLS/PCR method, Validation of analysis result

Features

- PLS and PCR methods which are much more precise multivariate analysis methods as compared with CLS method that has been used for traditional protein secondary structural analysis
- Possible to verify the calibration model by cross validation
- Possible to edit the ratio of secondary structure and also reference spectra
- Possible to validate the analysis result by F-test
- Verification of recalculation and result of calculation (GLP/GMP compliant)
- CFR Part11 compliant
- CD spectra (176 – 260 nm) of 26 proteins and calibration model based on those spectra

Results of the cross validation for 26 proteins are shown in Fig. 1.

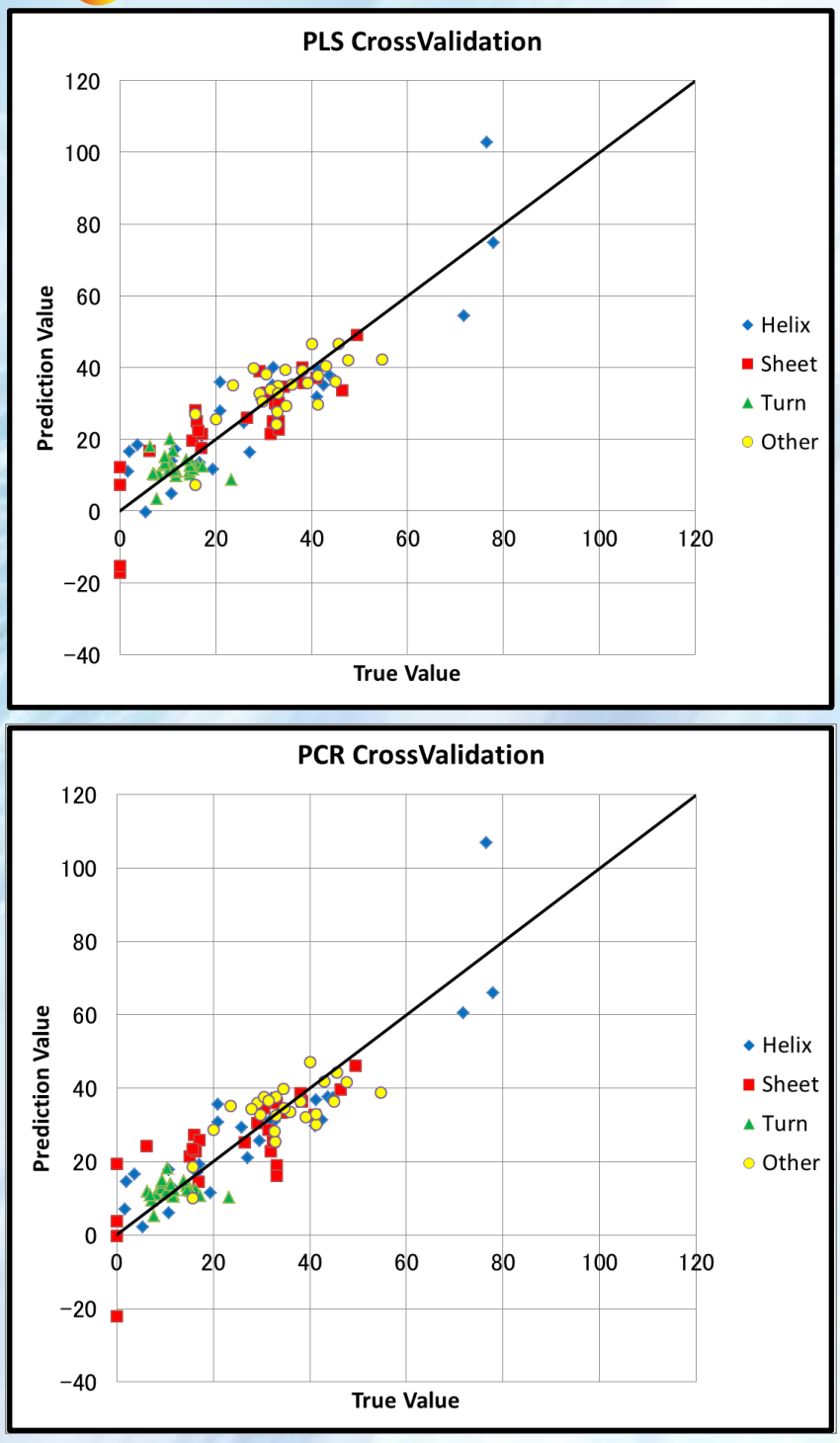


Fig. 1 Results of cross validation by PLS method and PCR method

<Reference>
(1) Jen Tsi Yang, Chuen-Shang C. Wu, and Hugo M. Martinez, *Methods in Enzymology*, **130**, 208-269, (1986)
(2) J. Reed, and T. A. Reed, *Anal. Biochem.* **254**, 36-40, (1997)

Secondary structural analysis of Lysozyme

Introduction

It is well known that secondary structures of proteins are sensitive to changes of pH value of solution, temperature of it, and host molecules located about the proteins. This feature causes the chemical reaction in the organism, where appropriate conformations constructed by several secondary structures. Therefore, to know weights of secondary structures of proteins is one of the keys to reveal the appropriate conformation in the organism. Concerning to the secondary structural analysis, helix, b-sheet, turn, and random structures are important factures, so JASCO has been produced the secondary structural analysis program package JWSSE-480, which based on Dr. Yang's concept describing CD spectra by using CD reference of such four structures [1]. Here, we introduce the application JWMVS-529 and show the several functions of it.

Measurement and calculation

We employed phosphoric buffer (pH=7.0) as solvent in order to avoid large absorption bellow 200 nm. The solution was set under the room temperature. The CD spectrum of lysozyme was obtained by using J-1500 spectropolarimeter. After the measurement, we carried out the structural analysis using four structures, helix, b-sheet, turn, and random structures, which are installed on JWMVS-529 program package.

Result

Fig.1 and Table.1 show the CD spectrum of lysozyme and the calculated weights of the secondary structures, respectively. CD_R and CD_N, depicted in Table.1 are the weights of the secondary structures under the contribution including other structures and neglecting the effects. The RMS values are very small in both cases, but CD_R is much smaller than CD_N. This small variation cases the similarity between the calculated and experimental spectra (Fig.1). And also, the smaller RMS value causes the good agreement with the X-ray result. Considering these facts, it is suggested that lysozyme in pH-7.0 buffer is constructed by mainly helix, random, and b-sheet, where the weights' ratio is 9:6:4 and other structures.

Table 1. Calculation result of Lysozyme by JWMVS-529 (%)

	helix	sheet	turn	random	RMS
CD_N)1	59.4	6.6	0.	34.0	5.3
CD_R)2	46.7	22.8	0.	30.5	3.4
X-ray[1]	41.0	16.0	37.0	20.0	-

1) Normalized 2) Re-normalized

[1] Yang et al., *Anal. BioChem.* **91**, 13-31 (1978).

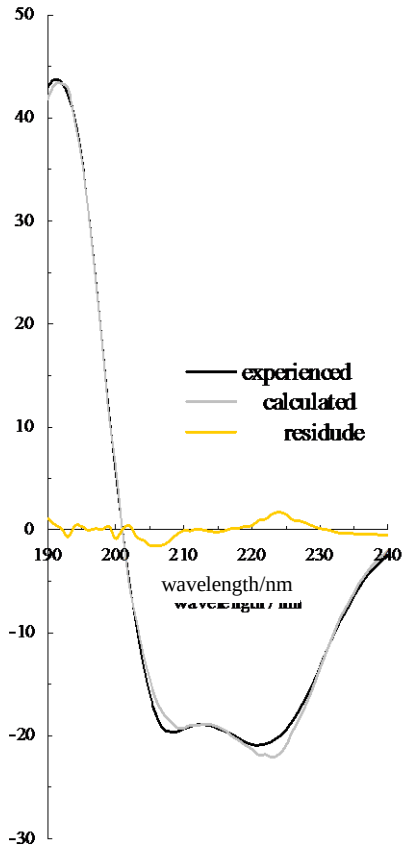


Fig. 1 Comparison of experience and calculated spectrum

Measurement of alanine powder by using of DRCD method with Multi-probe function

Introduction

Generally, a sample for CD measurement needs to be a liquid (solution) sample, while recently there has been increasing requirements of CD measurement for hardly soluble sample or the sample whose structure may change in a solution. Such sample has to be measured in the solid state as it is. Although the transmission method can be applied to the CD measurement for such solid samples, there needs sample preparation such as making a pellet and also, if the sample dilution is required, it is not easy to recover the sample. For solving those difficulties in such transmission method, diffuse reflection (DR) CD method has been recommended. [1), 2)] This application note illustrates the DRCD measurement by using of model DRCD-575 (See Fig. 1.) optimized for the J-1500 CD spectrometer with the multi-probe function.

DRCD is a method, in which the sample powder is located at the measurement point for diffuse reflection in integrating sphere and the CD is measured by utilizing diffuse reflection light (See Fig. 2.) and therefore, the DRCD can be effectively applied to the powder sample. However, in CD measurement of such solid sample, the influence of LD (linear dichroism) derived from the optical anisotropy of solid sample may have to be taken into consideration in order to minimize the CD artifact. The model J-1500 CD spectrometer allows the users to estimate the CD artifact easily by an incorporated quad digital lock-in amplifier allowing multi-probe function which enables the simultaneous measurement of CD and LD. As an example of the DRCD measurement, L- and D-alanine powder were used as sample.

Keywords: powder sample, amino acid, DRCD method

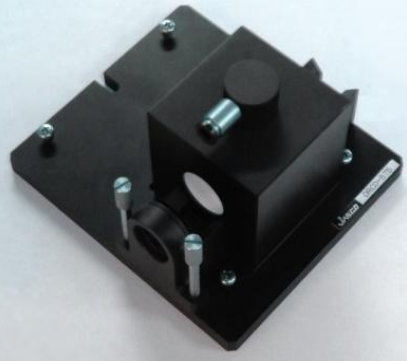


Fig. 1 Model DRCD-575 Powder CD Unit

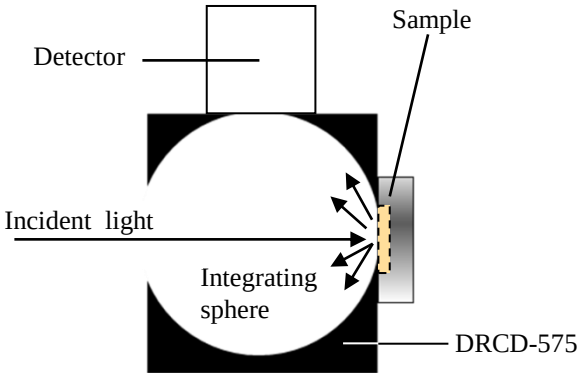


Fig. 2 Optical layout, DRCD-575

DRCD-575

The DRCD can be measured by placing the powder sample at the position for diffuse reflection measurement (opposite end of inlet port of incident light) and by locating the detector in close contact with the integrating sphere at 90 degrees side from the incident light axis. Also, the diffuse transmission measurement can be carried out when the sample is set at an inlet port of incident light and the diffuse plate (white plate) is located at the sample position for diffuse reflection measurement. In the diffuse transmission measurement, the dilution of sample may be often required, while the very small amount of sample can be measured.

Measurement

In order to minimize the influence from optical anisotropy, the alanine powder was well ground by using of mortar and then the simultaneous CD and LD measurement was performed by using of multi-probe function of model J-1500 CD spectrometer.

System configuration

P/N: 7000-J006A	J-1500-450ST CD Spectrometer
P/N: 7069-J034A	PML-534 FDCD PMT Detector
P/N: 7069-J025A	FLM-525 N_2 gas flow meter
P/N: 7069-J075A	DRCD-575 Solid state(powder) CD measurement unit

*1) This application can be applied to the J-1500-150ST (P/N: 7000-J005A)
*2) N_2 gas supply and regulator are required separately.
*3) Water-circulator for cooling 450W Xe source needs to be prepared locally.
*4) Powder CD unit can be applied to the transmission measurement using KBr pellet method.
*5) Cell holder for powder sample is a standard item in DRCD-575.

Measurement parameters

Wavelength range:	300-200 nm	Photometric mode:	DRCD, DRLD
CD/LD sensitivity:	10000 mdeg/1.0 delta OD	Data interval :	0.1 nm
Scan speed:	100 nm/min	Spectral bandwidth (SBW):	2 nm
Response:	1 sec	Number of accumulation:	5

Results

The DRCD and DRLD spectra of L- and D-alanine powder are shown in Fig. 3. In the CD mode, L- and D-alanine spectra obtained are in the mirror image showing the same intensity of plus and minus sign respectively. In the LD mode, the signal intensity of each L and D spectrum obtained is less than +/- 0.005 delta OD that is low enough. Such results indicate that the influence from optical anisotropy is considered to be in negligible level.

It is confirmed that the DRCD-575 Powder CD unit and J-1500 with multi-probe function can be used for the measurement of DRCD of alanine sample sufficiently powdered. It is expected that such powerful measurement technique will be able to expand the application of solid sample CD to the metal complex or super molecule.

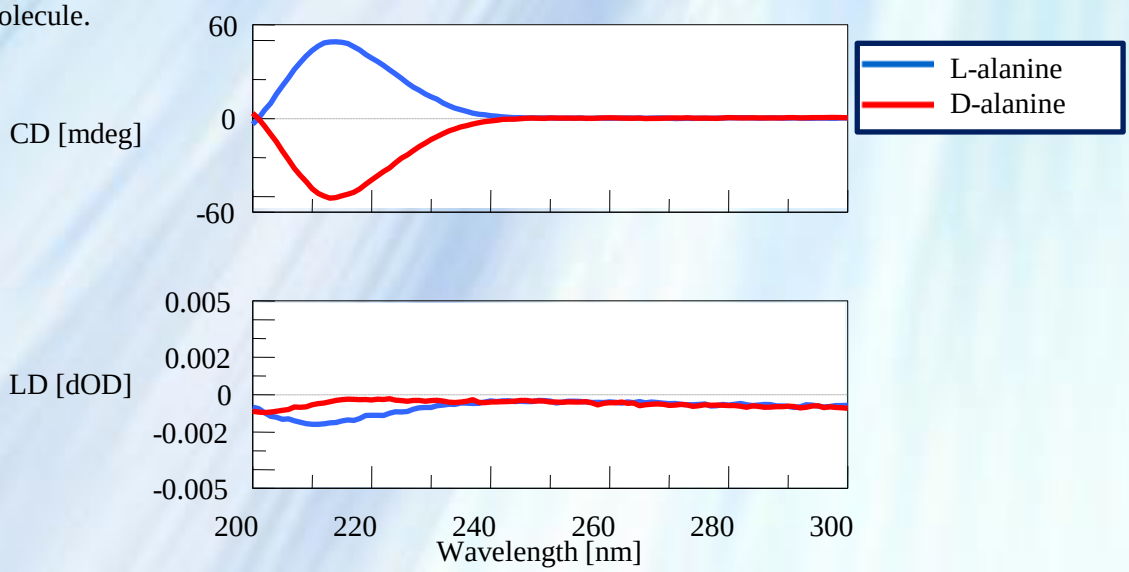


Fig. 3 CD and LD spectra of L- and D-alanine (accumulated)

Reference

Ettore Castiglioni and Paolo Albertini, *CHIRARITY*, **2000**, *12*, 291-294.
Huibin Qiu, Yoshihira Inoue and Shunai Che, *Angew. Chem. Int. Ed.* **2009**, *48*, 3069-3072.

J-1500-PAL System High throughput CD Spectral Measurement

-Application to biomedicines, evaluation of pH dependency of human serum albumin structure-

Introduction

R&D of biomedicines utilizing active ingredient derived from protein is increasing day by day. However, such biomedicines are more sensitive against environmental change such as temperature, pH, and salt concentration than those of ordinary pharmaceuticals produced from low molecular compounds. And such sensitivity will be the possible cause of deactivation of biomedicines in production process.

The protein structure and its activity are related closely, while CD measurement can easily provide the information regarding the change of protein structure in small amount of sample. Therefore, the CD measurement is widely used in the quality control of biomedicines including proteins.

For such pharmaceutical lab, a fully automated high throughput CD spectral measurement system has been developed by JASCO in order to meet the demand for a great number of sample analyses in the short period. This system consists of model J-1500 CD spectrometer and a liquid handler, CTC PAL enabling the automation of sample pretreatment, injection and washing.

In this report, this automated system has been applied to the evaluation of pH dependency of human serum albumin (HAS) structure.

Keywords: Biomedicines, Quality control, Automated measurement/High throughput screening

System configuration

	Model	Description	P/N	Remarks
Main unit	J-1500-450	CD Spectrometer	7000-J006A	
Accessory	FLM-525	N_2 gas flow meter	7069-J025A	
	PTC-517	Peltier Thermostatted Rectangular Cell Holder	7069-J017A	
	MCB-100	Mini water circulation bath	6970-J010A	For water-cooling of peltier
		Rectangular quartz cell, 10 mm path		For CD
	CTC-PAL	Liquid handler		

1) N_2 gas cylinder and regulator will be required additionally.
2) The 450W Xe source is recommended for offering higher S/N in vacuum UV region. A relevant water-circulator for cooling xenon lamp will be additionally required.
3) Please contact your local JASCO for the detailed configuration of CTC-PALPAL

Measurement parameters

Measurement program: [Spectra Measurement]
Reagent 1: 0.05 mg/mL human serum albumin (HAS) aqueous solution
Reagent 2: pH adjuster (pH1.3 - pH12.7, adjusted by diluted sulfuric acid and diluted sodium hydroxide aqueous solution)

Reagent 1 and reagent 2 were mixed into the ratio 1:4 and, the mixed reagent was injected into 10 mm rectangular cell placed in sample compartment of J-1500. All the sampling procedure such as mixing of reagents, CD spectral measurement, washing of cell and drying of cell have been pre-programmed so that a fully automated and unattended measurement can be carried out.

Data acquisition interval:	0.5 nm	Response:	1 sec
Band width:	1 nm	Scan speed:	100 nm/min
Optical path length:	10 mm	Accumulation:	2 times
Concentration:	0.01 mg/mL	Amount of HAS used for measurement: 30 µg	

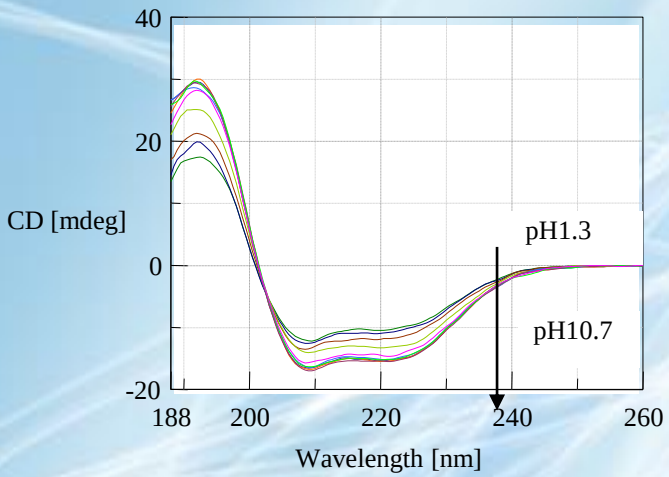


Fig. 2 CD spectra of HSA
(pH: 1.3, 2.2, 3.1, 4.1, 5.4, 6.7, 7.5, 8.4, 9.3, 10.7)

Fig. 1 J-1500-PAL High Throughput system

Results

Fig. 2 shows the CD spectra of human serum albumin in each pH. As you can see, the CD spectra of HAS changed according to structural change by pH. Fig. 3 shows the change of intensity of CD peak at 222 nm (alpha-helix structure) against pH change. It indicates that in the pH range from 5 to 10, alpha helix rich structure is maintained. However, in the acidic conditions (below pH5) and basic conditions (over pH10), the CD intensity was decreased and, it suggests that the denaturation of HAS happened.

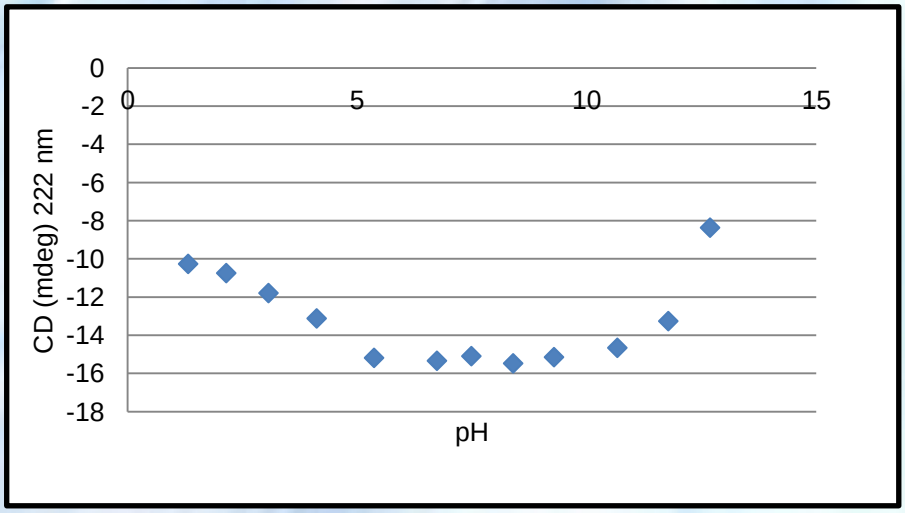


Fig. 3 pH dependency of CD intensity at 222 nm
(pH: 1.3, 2.2, 3.1, 4.1, 5.4, 6.7, 7.5, 8.4, 9.3, 10.7, 11.8, 12.7)

Conclusion

As demonstrated, the CD measurement can be the very effective procedure for quality control of biomedicines. The JASCO J-1500-PAL system will help the pharmaceutical labs who have a great number of samples to be measured.

Measurement of warfarin

Introduction

HPLC is a more effective method for quantitative estimation of trace amounts of compounds and for mixture separation/purification. HPLC is also used for analysis of medicinal products and in food science. In these fields, samples often have optical activity allowing analysis of enantiomeric excess and the separation/purification of the enantiomers.

JASCO has developed both dedicated CD detectors for HPLC (CD-4095) as well as an HPLC flow cell for the J-1500/J-1700 CD Spectrometers. The J-1500/J-1700 provide high sensitivity, high resolution, and wide dynamic range, allowing high accuracy detection down to 200 nm.

In this report, the CD measurement of warfarin was carried out using the J-1500 in a static mode as well as coupled to an HPLC system for on-line CD detection of the HPLC eluent.

Keywords: Medicinal products, LCCD, Chiral analysis

Sample preparation

In the CD spectral measurements, R-(+)-warfarin and S-(−)-warfarin are dissolved using a mixture of pH 2.0 aqueous phosphoric acid and acetonitrile, the mixture ratio is 1:1.

In the CD chromatographic measurement, racemic warfarin is dissolved using a similar mixture of pH 2.0 aqueous phosphoric acid and acetonitrile, the mixture ratio is 1:1.

Measurement parameters

1. CD spectrum measurement

(Measurement parameters)

Wavelength range:	210 - 400 nm	Data interval:	0.1 nm
Response:	2 sec	Spectral bandwidth (SBW):	1 nm
Scan speed:	100 nm/min	Number of accumulations:	1
Optical pathlength:	1 mm	Concentration of sample:	200 µg/mL

2. CD chromatogram measurement

(Measurement parameters)

Column: CHIRALCEL OD-RH column (4.6 mm I.D. x 150 mm L, 5 µm)

Wavelength range:	220 nm, 263 nm	Data interval:	1 sec
Response:	1 sec	Bandwidth:	1 nm
Optical pathlength:	10 mm	Concentration of sample:	200 µg/mL
Injection volume:	10 µL		
Mobile phase:	pH 2.0 aqueous phosphoric acid / acetonitrile (40/60)		
Flow rate:	0.5 mL/min		
Column temp.:	room temperature		

Results

1. CD spectrum measurement

Fig. 1 shows the CD and absorption spectrum of R-(+)-warfarin and S-(−)-warfarin. CD peaks are observed at 220, 263, 306 nm, which can be used as the detection wavelength for the CD chromatogram.

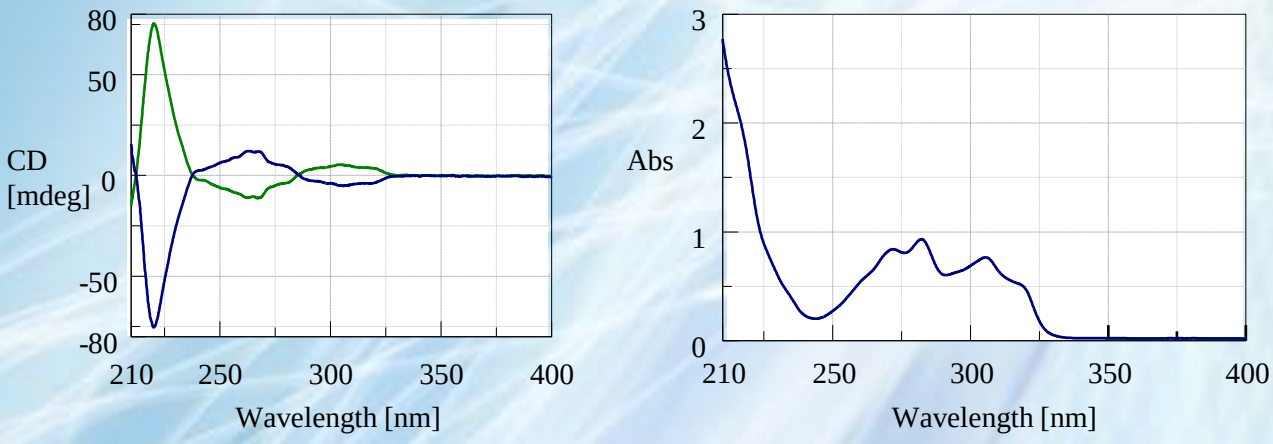


Fig. 1 CD and absorption spectra of warfarin

R-(+)-warfarin
S-(−)-warfarin

2. CD chromatogram measurements

Fig. 2 shows the CD and absorption chromatograms of *R*-(+)-warfarin and S-(−)-warfarin at 220 and 263 nm. The measurement was carried out using the Time Course Measurement mode. The measurement is initiated by the trigger signal of the manual or auto injector. The peak of R-(+)-warfarin is observed at 457 seconds and the peak of S-(−)-warfarin is observed at 587 seconds.

The CD and absorption signals at shorter wavelengths are generally greater providing higher sensitivity for measurement detection. As shown, the CD peak area detected at 220 nm is six times larger than the peak at 263 nm and the absorption peak area detected at 220 nm is twice as large than the absorption peak at 263 nm. The area ratio of R-(+)-warfarin and S-(−)-warfarin is 1:1.

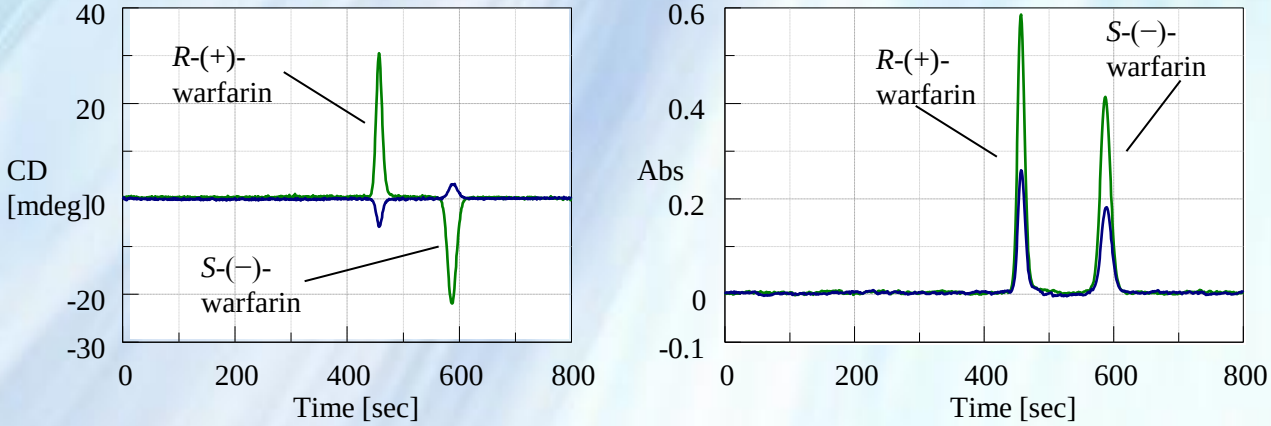


Fig. ~~2 CD~~ and absorption chromatograms of warfarin

chromatogram detected at 220 nm
chromatogram detected at 263 nm

Thermal Denaturation Measurement of Minute Sample Volumes

Introduction

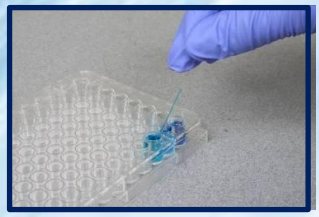
Circular Dichroism (CD) measurement is an effective tool for secondary structure analysis and the thermal denaturation analysis of proteins and nucleic acids. A rectangular cell of 1 mm optical path length is generally used for Far UV measurement, requiring approximately 200 µL of sample volume. There has been great interest in an apparatus and technique to carry out CD measurements on very low volumes in the case of precious samples where only a tiny amount can be purified.

JASCO now offers a new capillary cell and capillary jacket for thermal ramping analysis of sample volumes less than 10 µL. Measurement is simple and the capillary cells are inexpensive and disposable.

Keywords: microassay, capillary jacket, temperature controlled CD measurement, denaturation

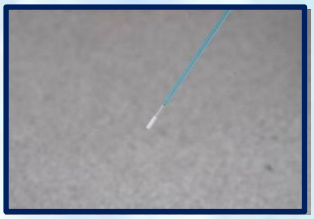
Sample preparation

1 mg/mL ribonuclease A aqueous solution is drawn into the capillary cell with a 0.5 mm optical pathlength and the capillary base is sealed. The cell is inserted in the capillary jacket for the CD measurement. A 0.5 mg/mL ribonuclease A solution using a rectangular cell of 1 mm optical path length is also measured for comparison.



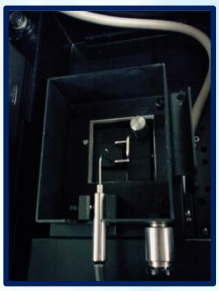
Drawing sample* into capillary

Sealing the sample



Ready for measurement

Insert the cell in capillary jacket



Set the capillary jacket in Peltier Thermostatted Single Cell Holder

*The above pictures are using a colored sample for visibility, not the ribonuclease A solution.

Measurement conditions

Measurement wavelength:	222 nm	Data sampling interval:	0.2ºC
Response:	8 second	Spectral bandwidth:	1 nm
Rising temp. rate:	1ºC /min		

Results

Fig.1 shows the thermal denaturation of ribonuclease A. Analysis using the JASCO JWTDA-519 Denatured Protein Analysis software calculates a denaturation temperature of 59.4ºC for the capillary cell and is in accordance with 59.7ºC for the rectangular cell. This result shows that the microassay for the capillary cell can be carried out with high accuracy.

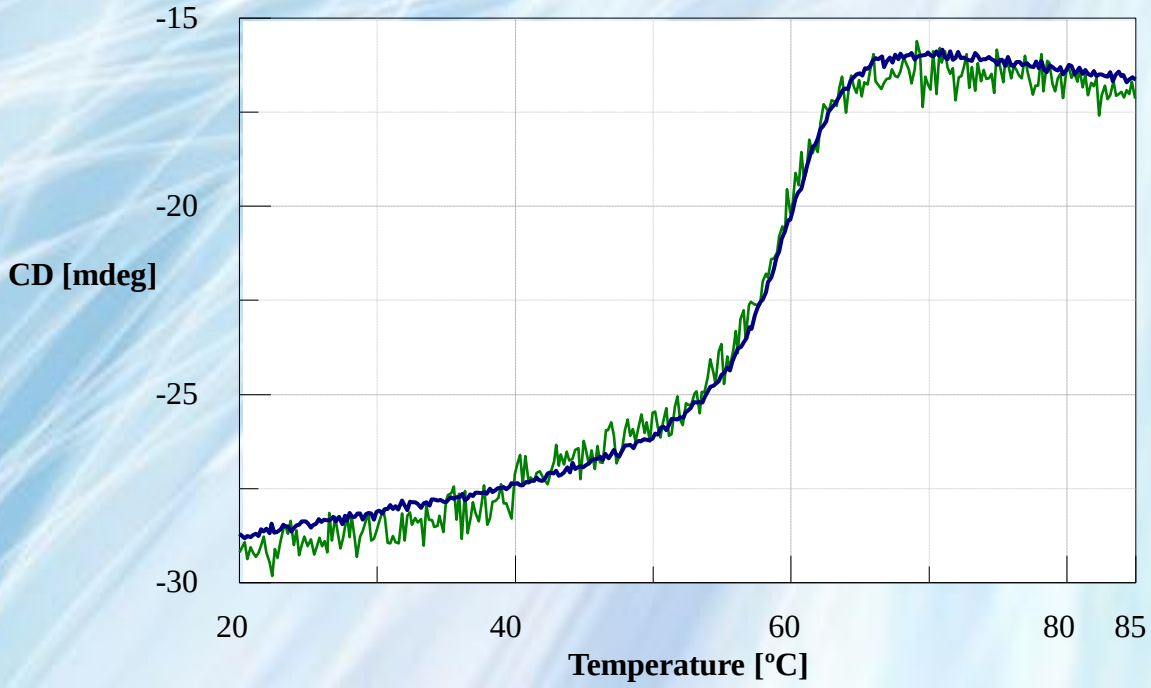


Fig. 1 Temperature control data of ribonuclease A

capillary cell

1 mm rectangular cell

NOTE:

JASCO also offers the MSD-462 Micro Sampling Disk for spectral scanning measurements on sample volumes of 2uL to 10 µL. The MSD-462 applications are shown in the following Application Notes: 260-CD-0011 and 260-CD-0019.

CD measurement at NIR region by J-1500

-Measurement of nickel tartrate and limonene-

Introduction

In the NIR region, observed CD spectra (NIR-VCD) are a result of the combination and overtone vibrational modes of the O-H and C-H transitions as well as the CD spectra (NIR-ECD) derived from the d-d transitions of metallic proteins and metal complexes. NIR-ECD spectra of the metal protein and metal complexes are known to be sensitive to quite detailed structures including the conformation of metal ligands as well as the configuration around the central metal, which is used for structural analysis of these molecules.[1)] For NIR-VCD, theories and calculation methods to interpret the NIR-VCD spectra have been introduced, and comparison analysis with many of the spectra has been accomplished. [2)]

The J-1500 CD Spectrometer can be used for data collection in the NIR region (up to 1600cm^{-1}) by replacing the detector and light source. In this configuration the instrument can be used for NIR-VCD measurement of the vibration transitions and NIR-ECD measurements of molecules including metal complexes, as well as ECD spectra in the UV region, primarily used for measurement of biological samples. Examples of the NIR-CD spectra of a nickel tartrate solution[3)] and limonene[2)] by the J-1500 CD Spectrometer are shown below.

Keywords: NIR-ECD, NIR-VCD, metal complex

Sample preparation

1. Nickel tartrate solution

Nickel sulfate solution of 0.24 M and sodium-potassium tartrate solution of 0.36 M mixed in a 1:1 volume ratio to prepare the nickel tartrate solution.

2. Limonene

Liquid (R)-(+) and (S)-(-) limonene in a 10 mm pathlength cylindrical cell.

Measurement condition

1. Nickel tartrate solution

Light source:	UV/Vis: Xe lamp	NIR: Halogen lamp (option)
Detector:	UV/Vis: PMT	NIR: InGaAs (option)
Measurement range:	UV/Vis: 235 - 940 nm	NIR: 940 - 1600 nm
Band width:	UV/Vis: 1 nm	NIR: 16 nm
Data interval:	UV/Vis: 0.1 nm	NIR: 1 nm
Path length:	UV/Vis: 10 mm	NIR: 0.5 mm

Scan speed: 200 nm/min
Response: 1 second
Accumulation: 1
Gain: 100x (InGaAs detector only)

2. Limonene

Light source: Halogen lamp (option)
Detector: InGaAs (option)
Measurement range: 1100 – 1350 nm
Measurement mode: CD/DC, UV single (Abs)
Band width: 16 nm
Data interval: 0.1 nm
Scan speed: 100 nm/min
Response: 2 seconds
Accumulation: 16
Gain: 100x

Results

1. Nickel tartrate solution

CD spectrum from the UV region to the NIR region of the nickel tartrate solution is shown in Figure 1. The vertical axis is converted to Molar ellipticity (Mol. Ellip) because the path length in the NIR region is different from the spectrum collected in the UV/Vis range. The spectrum can be measured with high sensitivity up to 1600 cm^{-1}, although any light absorptions from H_2O is observed in the region greater than 1400 nm.

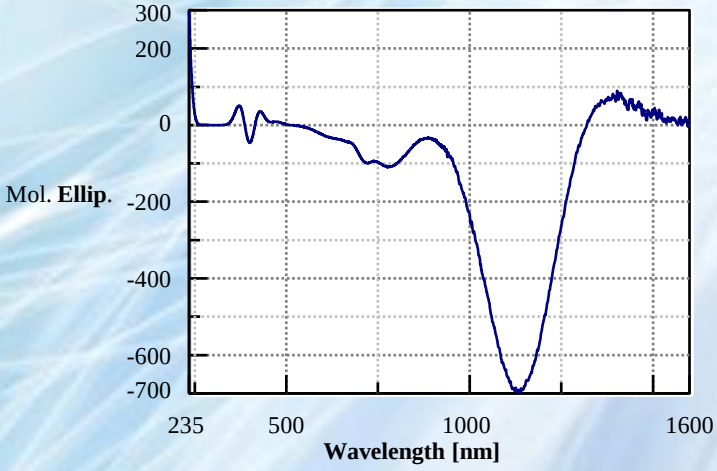


Fig. 1 CD spectrum of the nickel tartrate solution
Concentration of the nickel tartrate [3] : 0.1188 M in H_2O

2. Limonene

Absorption and CD spectra derived from the double overtone of the C-H vibrational transition of the (R)-(+) and (S)-(-)-limonene are shown in Figure 2. The limonene of a racemic form was used for the blank of the CD spectrum. The limonene was measured with pathlengths of 10 mm and 2 mm because there is no appropriate solvent to serve as the blank for the liquid limonene at room temperature in the absorption spectrum. The difference spectrum (absorbance equivalent to the 8 mm path length) is multiplied by 1.25 and converted to the absorbance equal to a 10 mm path length. The very weak CD signal below 1 mdeg can thus be measured with high-sensitivity.

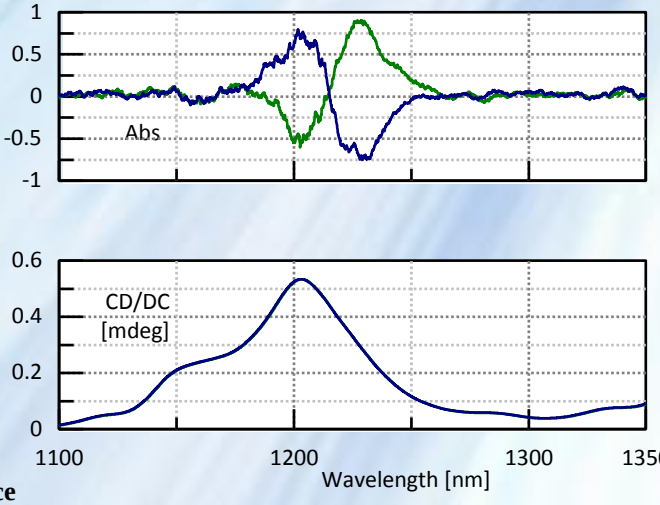


Fig. 2 CD spectrum of the limonene
(R) (+) - limonene
(S) (-) - limonene

Reference

(1) William A. Eaton, Graham Palmer, James A. Fee, Tokuji Kimura, and Walter Lovenberg, *Proc. Nat. Acad. Sci. USA*, 68, 12, 3015-3020, (1971)

(2) Sergio Abbate, Ettore Castiglioni, Fabrizio Gangemi, Roberto Gangemi, and Giovanna Longhi, *CHIRALITY*, 21, Issue 1E E242-E252, (2009)

(3) T. Konno, H. Meguro, T. Murakami, and M. Hatano, *CHEMISTRY LETTERS*, 953-956, (1981)

Lysozyme N-acetyl Glucosamine Interaction

Introduction

The change in the protein's CD spectrum reflects precisely the change in protein structure. The absorption derived from peptide bonds is seen in a shorter wavelength region than 240 nm. Therefore, by measuring the CD spectra in such wavelength region, the information regarding secondary structure of proteins can be acquired. On the other hand, absorption due to side chains such as aromatic amino acids exists in the region of 210-220 nm, while there are absorption bands in the longer-wavelength regions (which does not overlap with the absorption band of peptide bonds) than 240 nm as well. For this reason, in order to study side chains, a CD spectrum in the longer-wavelength region than 240 nm, which does not overlap the absorption of peptide bonds, is usually measured.

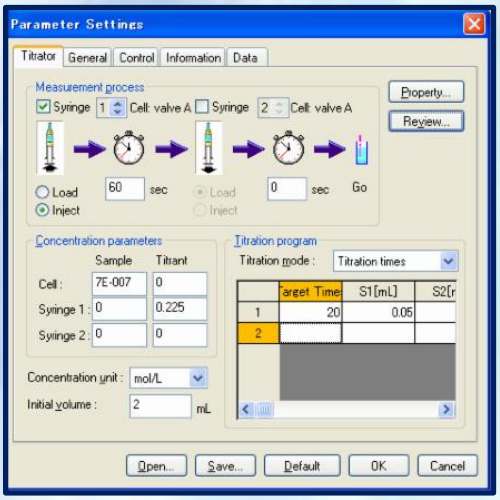
In this application, as an example, the interactions between lysozyme and its inhibitor, N-acetyl-(D+)-glucosamine (NAG), were measured by using the Circular Dichroism spectrometer.

[Measurement Conditions]

While performing titration of NAG into Lysozyme water solution (0.07 mM), CD spectra in the region of 260-320 nm were obtained.

ATS-530 Automatic titrator

Number of syringe: 2 sets
Syringe volume: 2.5 ml



NAG (225mM) with 0.05ml increment was titrated slowly by utilizing the ATS-530 Automatic Titration System. Furthermore, measurement was implemented by using 10mm cells, maintaining a temperature at 20 ºC through the Peltier type constant temperature cell holder.

ATS-530 Automatic Titration System
Number of Syringe: 2
Volume of Syringe: 2.5 mL

[Measurement Result]

The CD spectra of lysozyme, lysozyme+NAG, and NAG are shown in Figure 1. The CD peaks of lysozyme in water solution were positive at 293.5 nm, 289 nm and 283.2 nm. By adding NAG, an increase of CD intensity in the region of 300-270 nm was observed, while in this wavelength region, the CD due to NAG was scarcely observed.

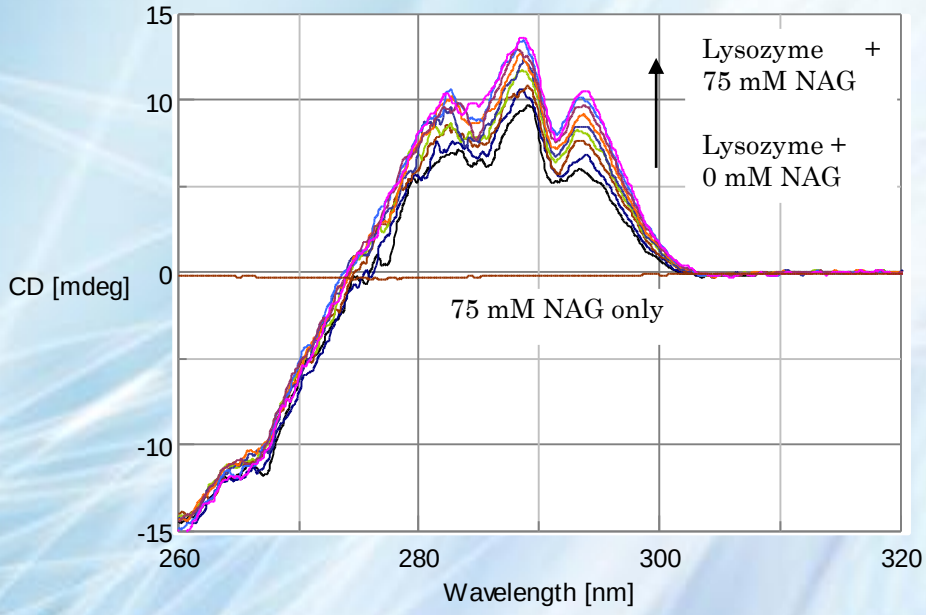


Figure 1: CD Spectra of Lysozyme, Lysozyme+NAG, NAG

In Figure 2, the change of CD spectra using NAG titration is shown using a bird's-eye view; in Figure 3, the change of CD intensity at 293.5nm is shown.

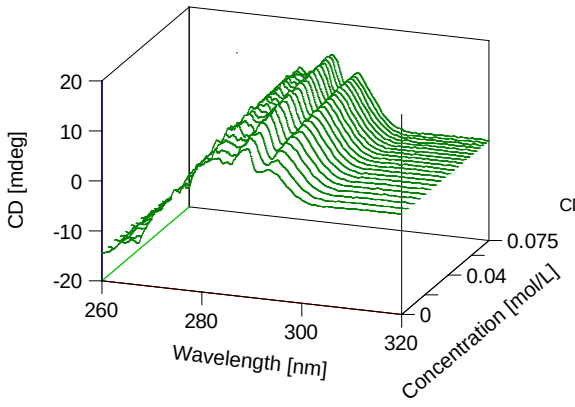


Figure 2: Change in CD Spectra using NAG Titration

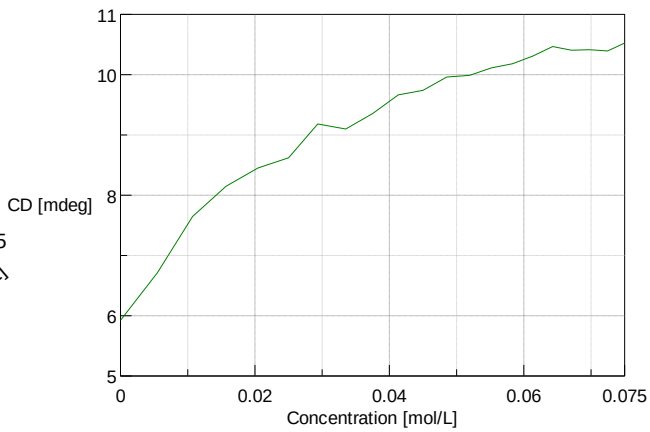


Figure 3: Change in CD value at 293.5nm by NAG Titration

According to the result by the X-ray analysis, it is evident that in the substrate bond region of lysozyme there exists triptophan resudude. From this, it can be interpretted that the increase in CD intensity is due to the interaction of triptophan resudude and NAG.

By conducting such CD measurements, the change in condition of the side chain of enzyme molecules during interactions between an enzyme and substrate can be recognized, and when the substrate bond region is known, the type of CD-related side chain can be estimated.

FDCD-465 Highly-Sensitive FDCD Measurement Accessory

Introduction

Fluorescence Detected Circular Dichroism(FDCD) is a method to measure the difference in intensities of fluorescence that arise when optically active sample is excited by left-hand and right-hand circularly polarized light. FDCD gives equivalent information to absorption CD while utilizing the superior sensitivity and selectivity of fluorescence detection. When FDCD is actually measured, CD value can be calculated from the results of FDCD measurement by the following equation.

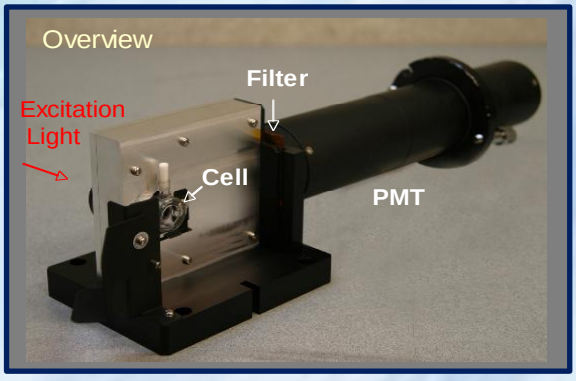
$$\Delta\varepsilon = \varepsilon_l - \varepsilon_r = (3.032 \times 10^{-5}) \times S \times (1 - 10^{-A}) / (cd10^{-A}) \text{ ---------- (1)}$$

where;

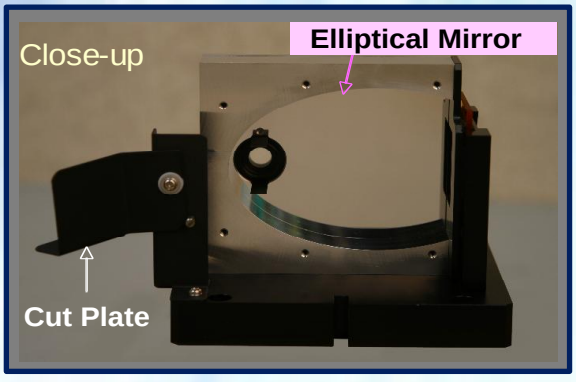
A: absorbance of sample, c: molar concentration of sample,
d: cell length(cm), S: FDCD value measured(mdeg)

Principle of Measurement

FDCD-465 is a highly sensitive FDCD measurement accessory combining both a cylindrical cell and an elliptical cylinder mirror. This accessory is designed to collect all radiated fluorescence light emitted in circumferential direction from the cell, resulting not only in the improvement of light collection efficiency, but also the ability to eliminate the effect from artifacts caused by fluorescence anisotropy. [2)3)]



FDCD-465 Outer view



Inside of FDCD-465

FDCD-465 is designed to minimize the influence from artifacts due to fluorescence anisotropy, however, using solvents with high viscosity such as glycerol may cause such an influence to some degree. In order to eliminate such an influence, the FDCD-465 is provided with Balancing Mask as described below.

The fluorescence light in F-y direction does not reach the detector due to the blockage caused by the cell itself. At this time, the intensity of fluorescence of (F+x + F-x) becomes slightly larger than the (F+y + F-y), creating the possible cause of the artifact. By using the Balancing Mask, the balance of intensity of these fluorescence can be maintained to eliminate the artifacts even when utilizing solvents with high viscosity.

Mask (mm)

[FDCD measurement of ammonium d-10-camphorsulfonic acid water solution]

Figure 1 displays FDCD, CD, and UV spectrum of ammonium *d*-10-camphorsulfonic acid (d-10-ACS) water solution of 0.0024M(0.06%w/v). Since the FDCD is an excitation spectrum, the excitation and emission spectra need to be obtained by a spectrofluorometer beforehand. The fluorescence peak, when d-10-ACS water solution was excited by 285 nm, was at 427 nm. Here, J-820, FDCD-465, L38 excitation light cut filter, and 1cm cell were used to obtain FDCD spectrum in the range of 350-220 nm.

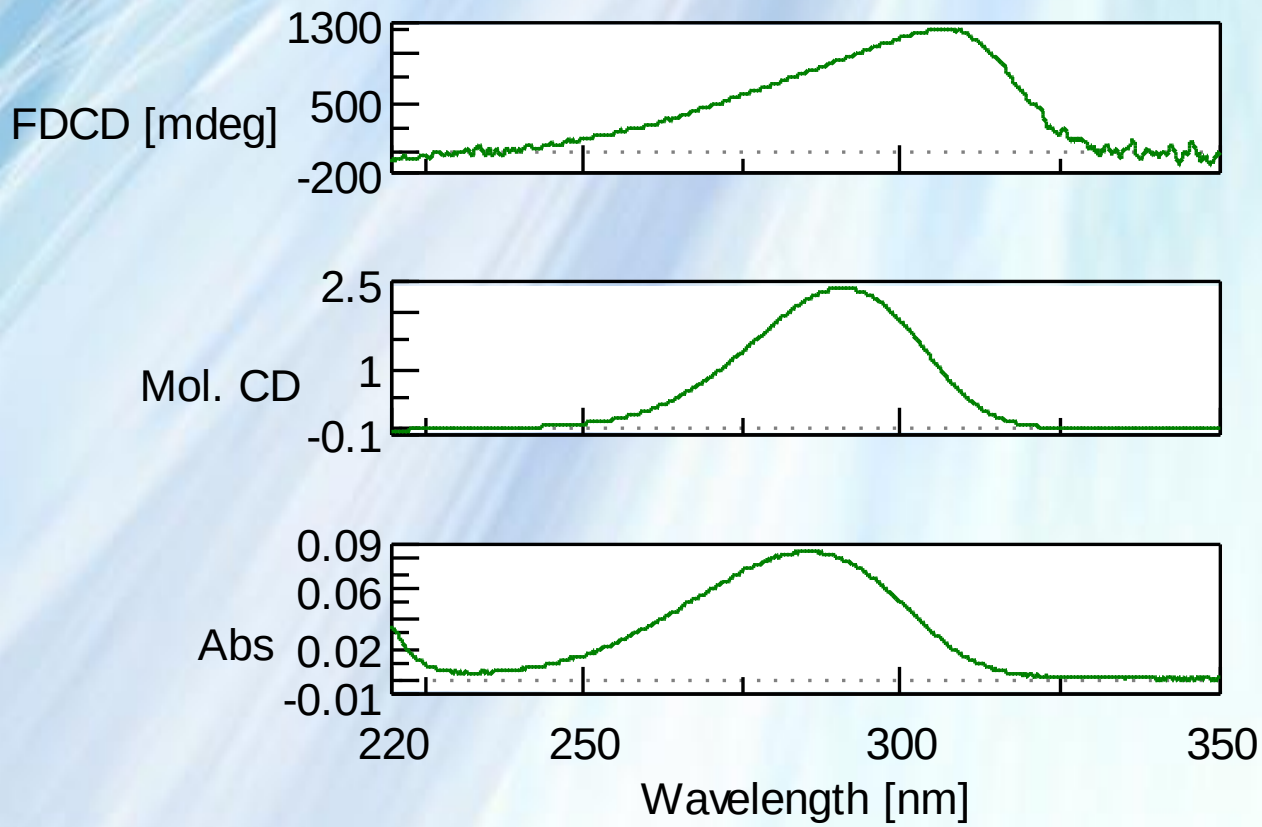


Fig1. d-10-ACS (0.0024M) water solution's FDCD (top), CD (middle),UV (bottom) spectrum

Based on the obtained FDCD, CD, and UV spectrum, utilizing the equation(1), CD spectrum converted from FDCD was compared with the measured CD spectrum. As shown in Figure 2, the CD spectrum obtained from FDCD was in good agreement with the measured CD spectrum within an experimental error.

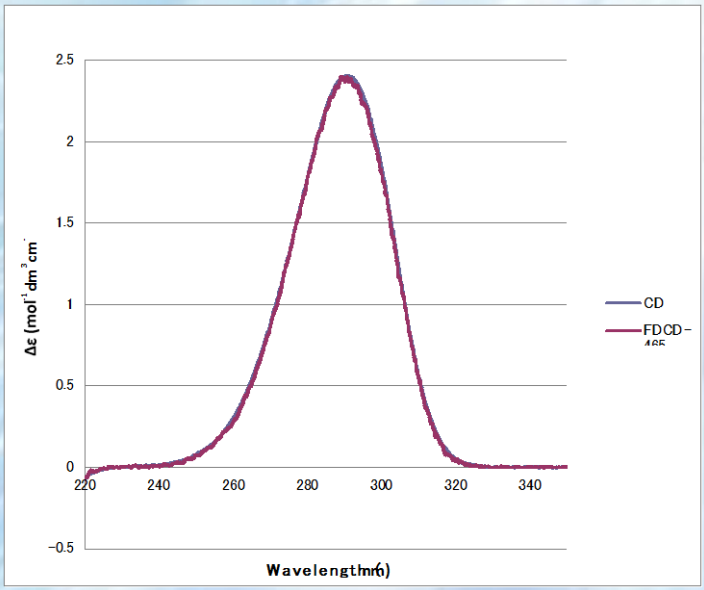


Fig 2. The CD spectrum obtained from FDCD was in good agreement

[Comparison of sensitivity in FDCD and CD measurement]

Figure 3 and Figure 4 show FDCD spectrum and CD spectrum of (1*S*,2*S*)-trans- cyclohexanediol bis (6-methoxy-2-naphthoate)/ acetonitrile solution with very low concentration conditions, respectively.

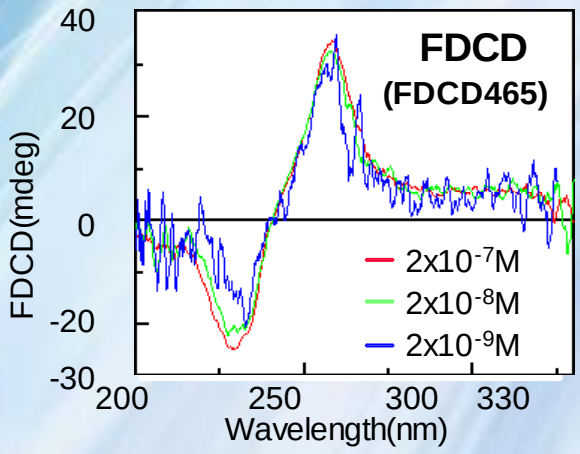


Fig 3. FDCD Spectrum

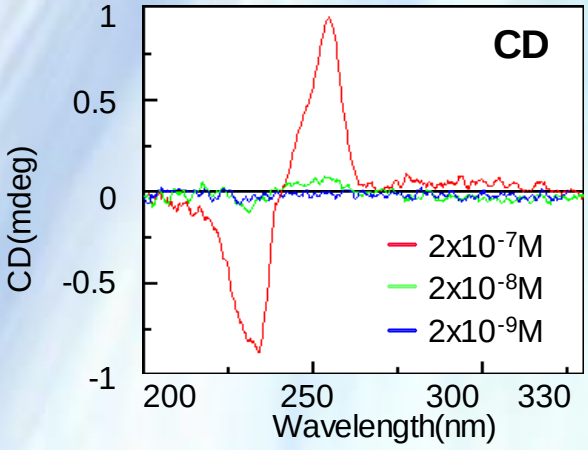


Fig4. CD Spectrum

Measurement Conditions

FDCD: SBW 4nm, 16 times as accumulation (2×10^{-7}M)

CD: SBW 2nm, 8 times as accumulation (2×10^{-7}M)

In the actual FDCD data obtained, the difference in intensity of fluorescence excited by right-hand circularly polarized light and left-hand circularly polarized light is normalized against the intensity of total fluorescence. Therefore, as shown in Figure 3, the FDCD value (mdeg) displays a constant value, regardless of sample concentration.

In the CD measurement, as shown in Figure 4, CD signals were observed with 2×10^{-7}M, but CD signal could not be observed with 2×10^{-8}M. On the other hand, by using the FDCD-465 for measurement, signals were clearly observed with lower concentration of 2×10^{-9}M.

As explained, if the sample is suitable for FDCD measurement, much higher sensitivity such as several tenfold to a hundred times can be achieved than standard CD measurement.

Information regarding (1*S*,2*S*)-trans-cyclohexanediol-bis-(6-methoxy-2naphthoate) has been provided by Tatsuo Nehira, Hiroshima University, and Katsunori Tanaka, Osaka University.

<Reference>

1) D. H. Turner, I. Tinoco Jr, M. Maestre,
2) T. Nehira, K. Tanaka, T. Takakuwa, C. Ohshima, H. Masago, G. Pescitelli, A.Wada, and N. Berova, *Applied Spectroscory*,
3) K. Tanaka, G. Pescitelli, K. Nakanishi, and N. Berova,
4) T. Nehira, *Monatshefte für Chemie*
5) J.-G. Dong, A. Wada, T. Takakuwa, K. Nakanishi, and N. Berova,
(1997)
6) K. Muto, H. Mochizuki, R. Yoshida, T. Ishii, and T. Handa,
7) T. Nehira, C. A. Parish, S. Jockusch, N. J. Turro, K. Nakanishi, and N. Berova,
121, 8681, (1999)

Unfolding of Concanavalin A by the Trifluoroethanol

Introduction

The Stopped Flow method is used to analyze reactions occurred during the duration of several milliseconds and several seconds meanwhile the CD measurement provides valuable information regarding the protein's secondary structures and environment of aromatic amino acids. Therefore, CD measurement with Stopped-flow method provides one of the best techniques in analyzing the protein's Unfolding and Refolding process.

Concanavalin A (derived from Jack bean) in natural state has abundant b-sheet structures, however, it is known that its structure changes into the one with rich a-helix under trifluoroethanol (TFE) and its Unfolding process has been reported also.

In this application, the model SFS-562T High-Speed Stopped-flow System was used to measure the Unfolding process of Concanavalin A under TFE.

CD Spectra of Concanavalin A

The Concanavalin A's in pH2 hydrochloric acid gives a CD spectrum specific to β-sheet structure. In contrast, in a solution with 50% of TFE added it has a CD spectrum specific to α-helix structures. As seen in Figure 1, a big change from abundant β-sheet structure to abundant α-helix structure can be identified.

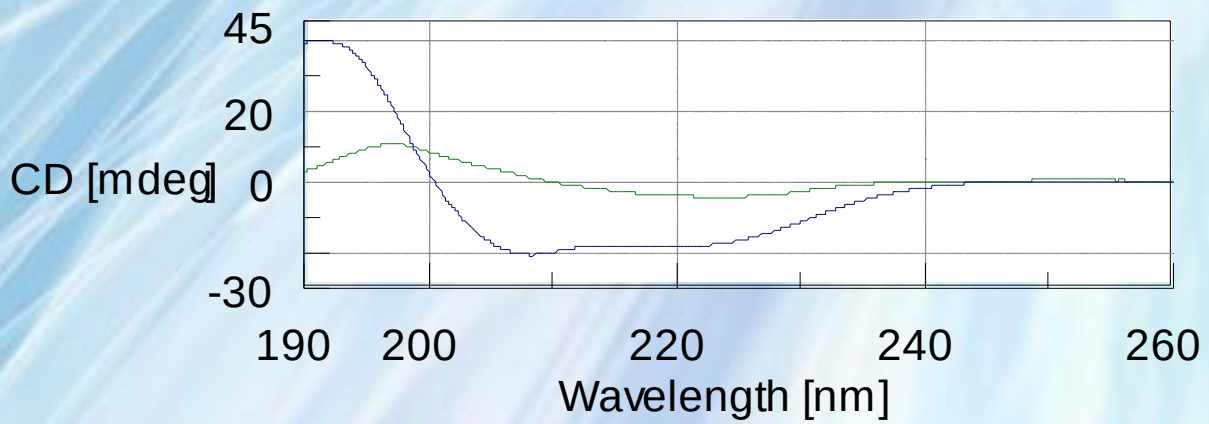


Fig1. CD Spectrum of Concanavalin A

Green: CD spectrum of Concanavalin A in pH2 hydrochloric acid
Blue: CD spectrum of Concanavalin A in solution with 50% TFE
(Both are Concanavalin A: 0.1 mg/ml; cell path length: 1 mm)

[Measurement Results]

<Stopped Flow Measurement>

Concanavalin A (0.2 mg/ml, in pH2 hydrochloric acid) was mixed with TFE with a ratio of 1:1 and its Unfolding process was measured using the Stopped Flow method. The CD value at 220 nm showed an increase in negative side, and change from abundant β-sheet structure to abundant α-helix structure was observed. By analyzing the reaction as a two-step reaction (A --> B --> C model) through the use of the Reaction Speed Calculation Program, unsurpassed fitting of the spectra was obtained (Figure 2).

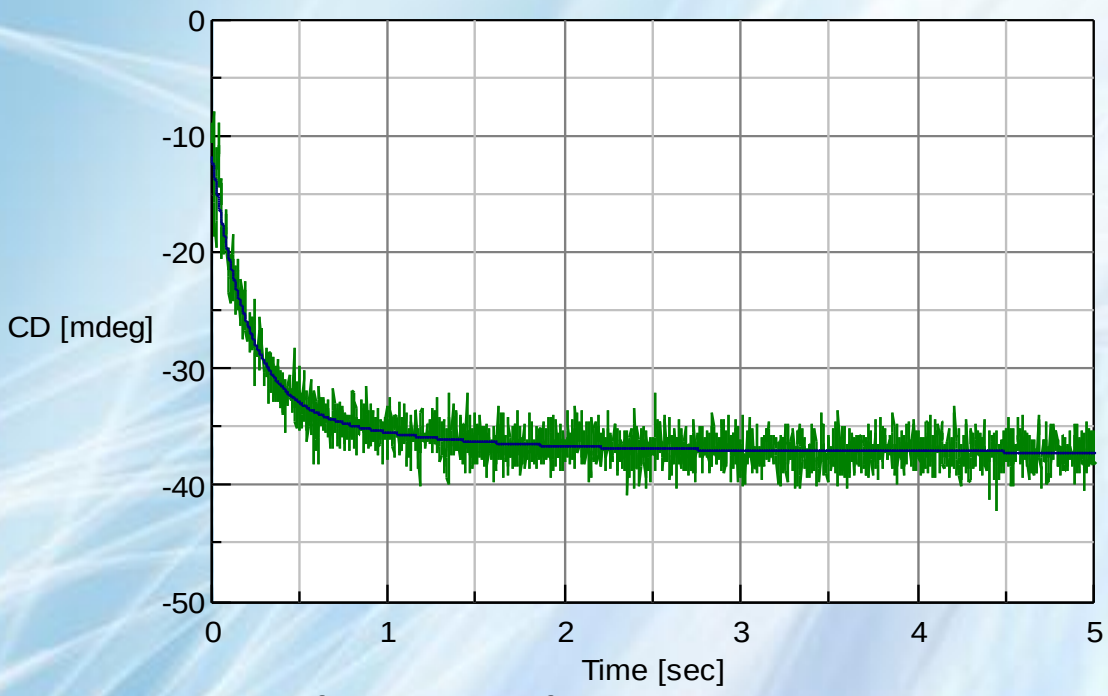


Figure 2: Unfolding process of Concanavalin A in TFE and analysis result

[Measurement Conditions]

Syringe 1:	0.2 mg/mL, Concanavalin A, in hydrochloric acid (pH2)
Syringe 2:	TFE
Mixing ratio:	100 µL:100 µL
Total flow rate:	10 ml/sec
Cell path length:	2 mm
Wavelength for measurement:	220 nm
Data pitch:	2 msec
Band width:	1 nm
Accumulation:	4 times

[Result of Analysis]

Reaction speed equation:	Y(t)=20.5925*exp(-t/0.189295)+ 4.73648*exp(-t/0.903939)
Step 1 time constant:	0.189295 [s]
Step 1 speed constant (k1):	5.28275 [s^{-1}]
Step 2 time constant:	0.903939[s]
Step2 speed constant (k2):	1.10627 [s^{-1}]

<Reference>

(1) Qi Xu and Timothy A. Keiderling, (2005) *Biochemistry*, p.44, 7976-7987

Measurement of complex forming reaction of Nickel Sulfate and Rochelle salt

Introduction

Stopped-flow CD measurement technique is well known method to analyze unfolding and refolding process of Protein and also observe complex forming reaction. Because Transition metal complex have typically absorbance in region from Visible to N-IR range.

We introduce this measurement about complex forming reaction of Nickel Sulfate and Rochelle Salt in using of high speed stopped flow systems consists of J-1500 and SFS-562T.

<CD spectra of mix solution including Nickel Sulfate and Rochelle Sault>

Fig. 1 shows that CD spectrum of the mixed solution sample which consists of 0.24M Nickel Sulfate solution and 0.36M Rochelle Salt solution as 1:1 mixture ratio. It can show CD signal in broad range from UV/Vis to N-IR.

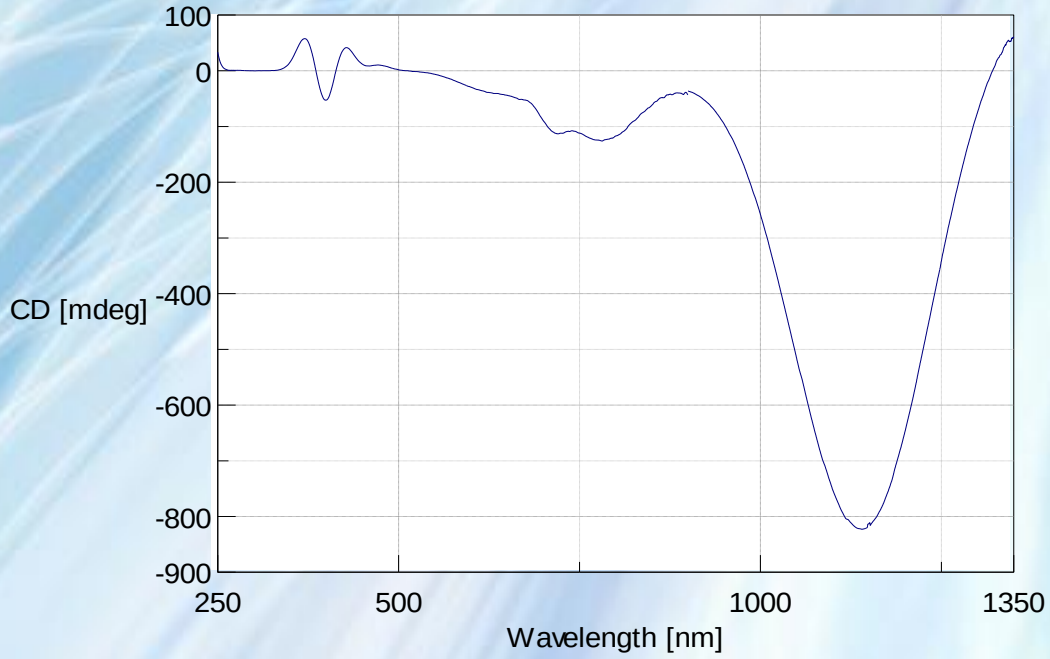


Fig. 1: CD spectra of mix solution including Nickel Sulfate and Rochelle Salt
Cell path :10 mm Instrument range: 250-900 nm (J-1500) 900-1350 nm (J-730)

<Stopped- Flow measurement>

The complex forming process about the above sample is measured with CD stopped flow system. Following Fig.2 shows the CD spectra in NIR-region 720nm and Fig.3 shows the one in N-IR region 1000 nm.

It shows in Fig.4 that complex forming reaction is finished within 100 msec and shapes of both data in 720 nm, 1000 nm is match after data normalization, which mean both data in 720 nm and 1000 nm indicates same reaction process.

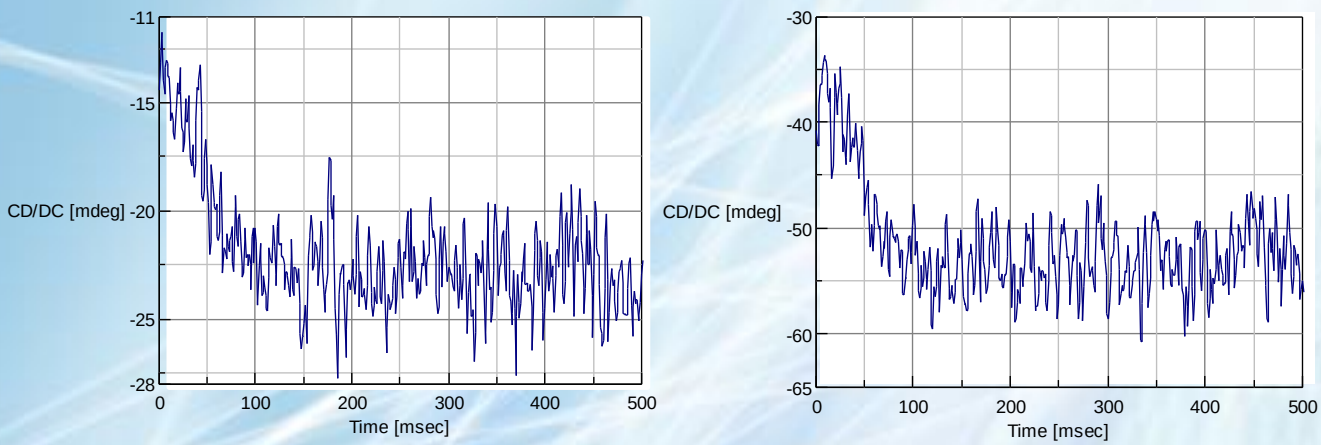


Fig. 3: Complex forming reaction of Nickel Sulfate and Rochelle salt in 1000 nm.

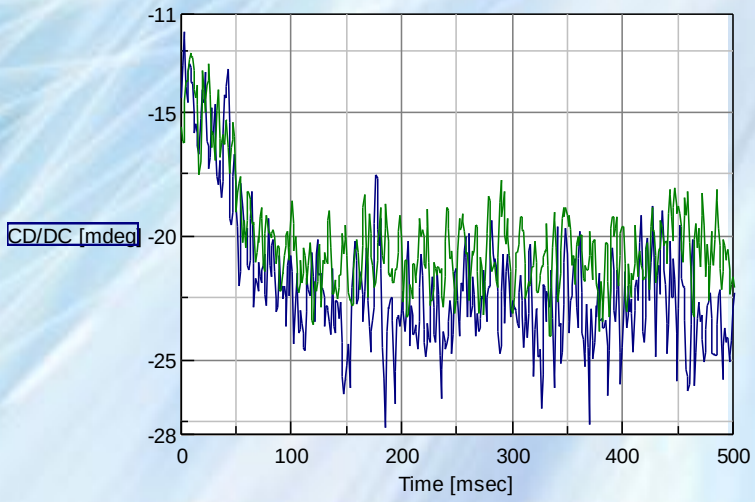


Fig. 4: Stopped flow data comparison between 720 nm (Blue) and 1000 nm (Green)

<Measurement Condition>

Syringe 1 : 0.24M Nickel Sulfate Solution
Syringe 2 : 0.36M Rochelle Salt Solution
Mixture ratio : 100 µL: 100 µL
Total flow rate : 5 ml/sec
Cell length : 2 mm
Measurement range :720 nm (SBW5 nm, Data acquisition 75 times)
1000 nm (SBW10nm, Data acquisition 50 times)
Data pitch : 1 msec
Response : 2 msec

<Reference>

(1) Hiroyuki Miyake, Hideki Sugimoto, Hitoshi Tamiaki and Hiroshi Tsukube, (2005) *Chem. Commun.*, 4291-4293
(2) Hiroyuki Miyake, Hiroshi Kamon, Ikuko Miyahara, Hideki Sugimoto, and Hiroshi Tsukube, (2008) *J. Am. Chem. Soc.*, **130**, 792-793

Introduction of Temperature/Wavelength Scan Program

Introduction

CD spectrum and fluorescence spectrum are used in a complementary manner in the structural analysis of the proteins. The thermodynamic parameters of the protein obtained by the measurement with variable temperature provide the important knowledge for the thermal stability of the protein.The Temperature/Wavelength Scan program for JASCO CD spectrometers allows to obtain the data of CD with the temperature change for the protein and DNA required to calculate the denaturation temperature (Tm), enthalpy change (DH), and entropy change (DS). In addition such program also enables temperature interval measurements of CD and fluorescence spectra.

The obtained data can be processed on the [Spectrum Analysis] and [Interval Analysis] program, which also enables to show the 3D representation. The secondary structure analysis and the calculation of thermodynamic parameters of the protein can be implemented by using optional [Thermal Denaturation Analysis] and [Protein SSE] program.

This report introduces the measurement of Lysozyme (0.03 mg/mL) using Temperature/Wavelength Scan program as an example.

Key words: CD spectra measurement, Fluorescence spectra measurement, Melting temperature, Proteins

Results:

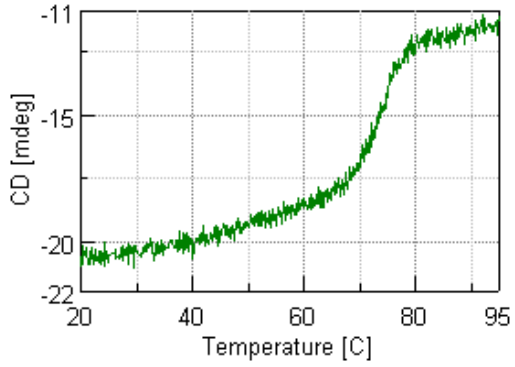


Fig. 1 Thermal denaturation curve

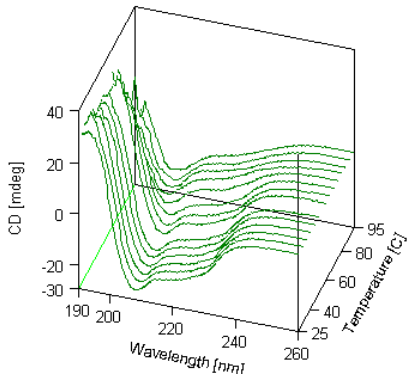


Fig. 2 CD Spectra with temperature interval

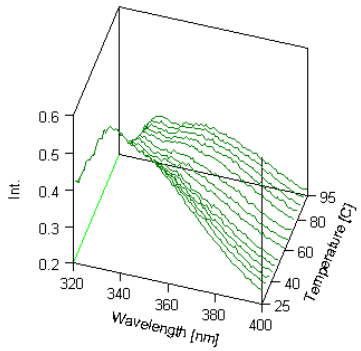


Fig. 3 Fluorescence spectra with temperature interval

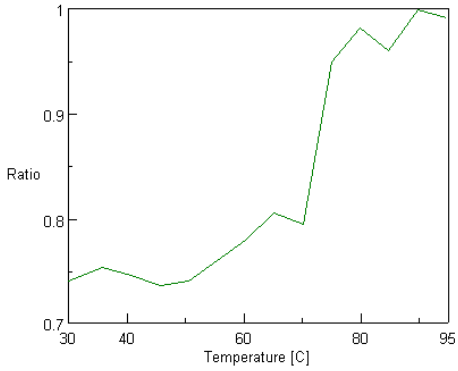


Fig. 4 Fluorescence intensity ratio of 357/338 nm

One-drop CD spectra measurement in using of Micro Sampling Disc

Introduction

CD spectroscopy is one of indispensable measurement method for protein structure analysis as same as NMR or X-ray crystallography. And small volume or low concentration sample measurement is common request in this market recently. JASCO has developed new technology and application to meet such small-volume or low-concentration sample measurement condition finally. In this note, we like to show several measurement results by One-drop CD spectra measurements in using of Micro sampling disk (MSD).

Keywords: One-drop measurement, Protein, Circular Dichroism

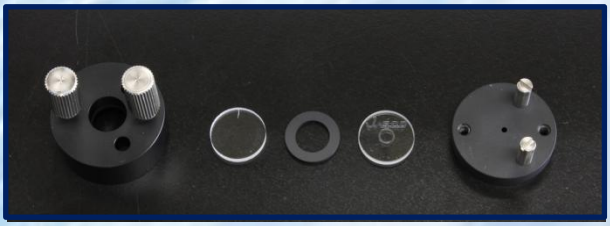


Fig. 1 MSD components

1. Add the sample to the MSD **2. Clamp the MSD** **3. Place the MSD in the sample chamber**

Fig. 2 How to use the MSD

Advantages

1. One-drop CD measurement
   10 µL (1 mm path length), 2 µL (0.2 mm path length)
2. Easy handling
   Hydrophobic treatment keeps samples centered
3. Variable path length
   Spacers are attached for 1 or 0.2 mm path length
4. Artifact free
   Windows allow for artifact-free measurements
5. Alignment free
   JASCO CD spectrometers use a parallel light beam
6. Highly reproducible baseline

Results

The following are comparisons between the MSD and a conventional cell. CD spectra that show secondary structures (between 260 nm and 190 nm) can be measured completely in less than 3 minutes using the MSD.

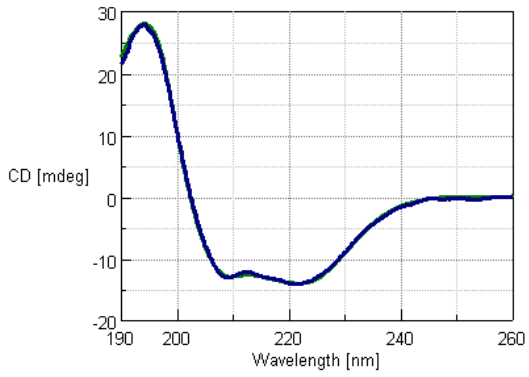


Fig. 3 Hemoglobin

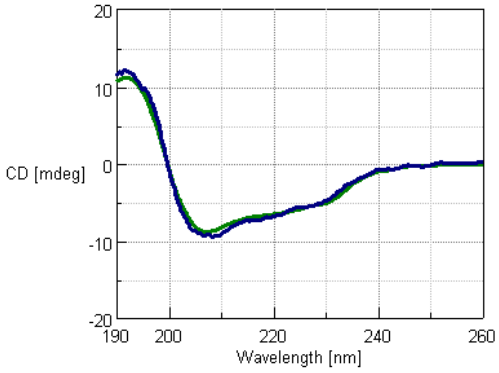


Fig. 4 Lysozyme

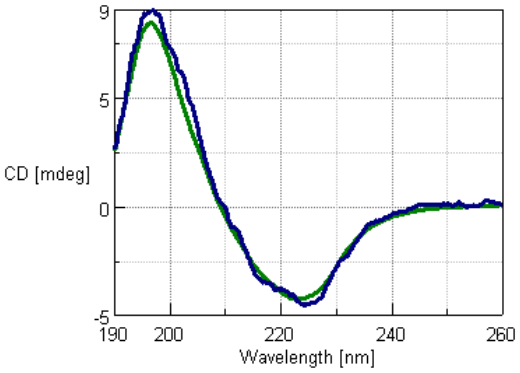


Fig .5 Concanavalin A

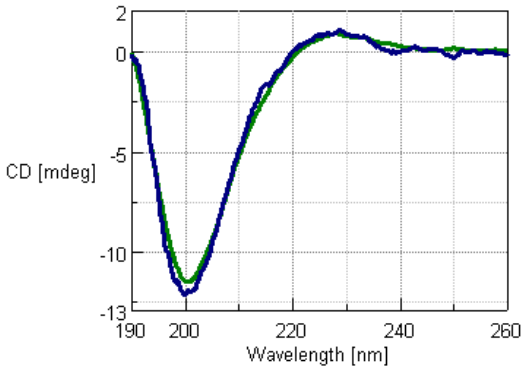


Fig. 6 Trypsin Inhibitor

MSD: **Conventional cell:**

Measurement parameters

Path length: 1 mm
Sample concentration: 0.1 mg/mL
Bandwidth: 1 nm
Data interval: 0.1 nm
Scan speed: 100 nm/min
Response: 2 sec
No of scans: 4 times (MSD), 1 time (Conventional cell)
Measurement time: 2.8 min (MSD), 42sec (Conventional cell)

One-drop CD spectra measurement of organic compounds and metal-complexes using Micro sampling disk

Introduction

CD spectra measurement can be used for spectra measurement of organic compounds and metal-complexes. In this note, we show several CD spectra data concerning organic compounds having an absorption in the UV region and metal-complexes having an absorption in the UV/Vis region.

Keywords: One-drop measurement, Circular Dichroism, Organic compound, Metal-complex

Results

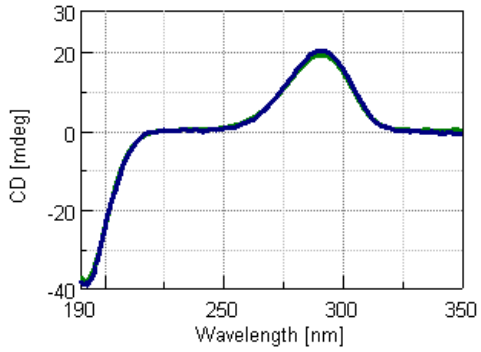


Fig. 1 (1S)-(+)-10-Camphorsulfonic acid, ammonium salt

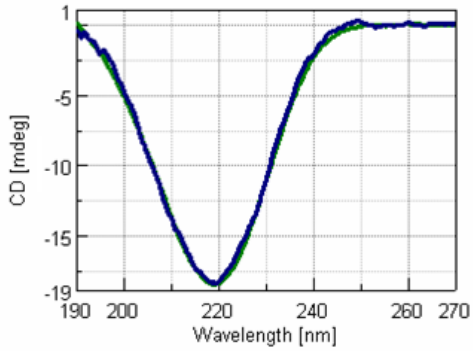


Fig. 2 D-Pantolactone

Micro sampling disk (MSD) : Conventional cell:

Sample volume: 10 µL(MSD), 400 µL (Conventional cell) Path length: 1 mm,
Bandwidth: 1 nm Data Interval: 0.1 nm Scan speed: 100 nm/min
Response: 2 sec Accumulation: 4 times (MSD), 1 time (Conventional cell)

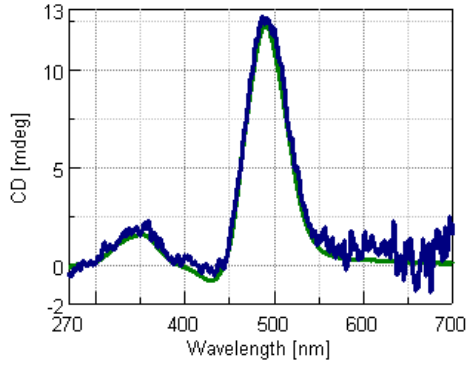


Fig. 3 $2(+)_D$-$[Coen_3]Cl_3NaCl$-$6H_2O$

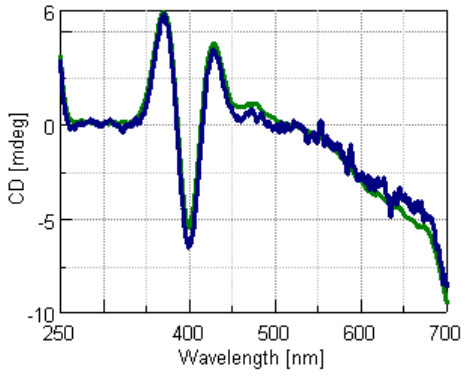


Fig. 4 (0.24M) $NiSO_4$+(0.36M) Rochelle salt

Micro sampling disk (MSD) : Conventional cell:

Sample volume: 10 µL(MSD), 400 µL (Conventional cell) Path length: 1 mm,
Bandwidth: 1 nm Data Interval: 0.1 nm Scan speed: 200 nm/min
Response: 2 sec Accumulation: 9 times (MSD), 1 time (Conventional cell)

Advanced application of CD measurement for pharmaceutical research
-Analyzing the interaction of human serum albumin and 3,5-diiodosalicylic acid-

Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma. HSA binds with pharmaceutical compounds and other in vivo substances, and plays an important role in the transport of these substances to target organs. Several studies have reported on the binding affinity of HSA and its interaction with various compounds [1)-3)].

Herein, the measurement of CD induced by the interaction of HSA titrated with 3,5-diiodosalicylic acid and subsequent analysis of the dissociation constant is presented.

Keywords: Proteins, Induced CD, Pharmaceuticals

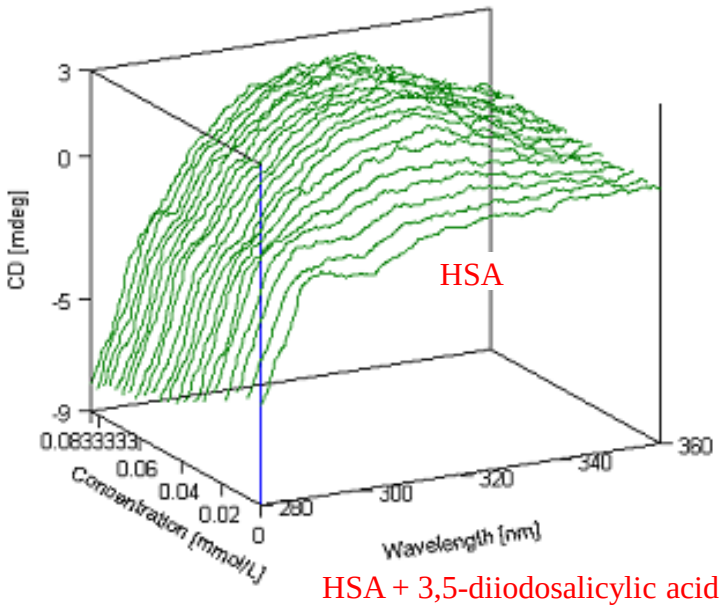
<Measurement conditions>

Sample:	HSA, 0.0228 mM, 100 mM acetate buffer (pH 6.3), 2 mL
Titration:	3,5-diiodosalicylic acid, (0.25 mM), 50 mL, 20 times
Measurement range:	360 to 260 nm

<Measurement system>

J-1500 CD Spectrometer
PTC-510 Peltier thermostatted cell holder
ATS-530 Automatic titrator

Chirality resulting from an achiral substance interacting with a chiral substance is called induced CD. Though, 3,5-diiodosalicylic acid is achiral, it exhibits chirality through interaction with HSA. The resulting CD spectra show a positive peak around 320 nm. (Fig. 1)



HSA does not show a CD signal around 320 nm, but the induced CD from the interaction of HSA and 3,5-diiodosalicylic acid increases with additional titration of 3,5-diiodosalicylic acid. (Fig. 2)

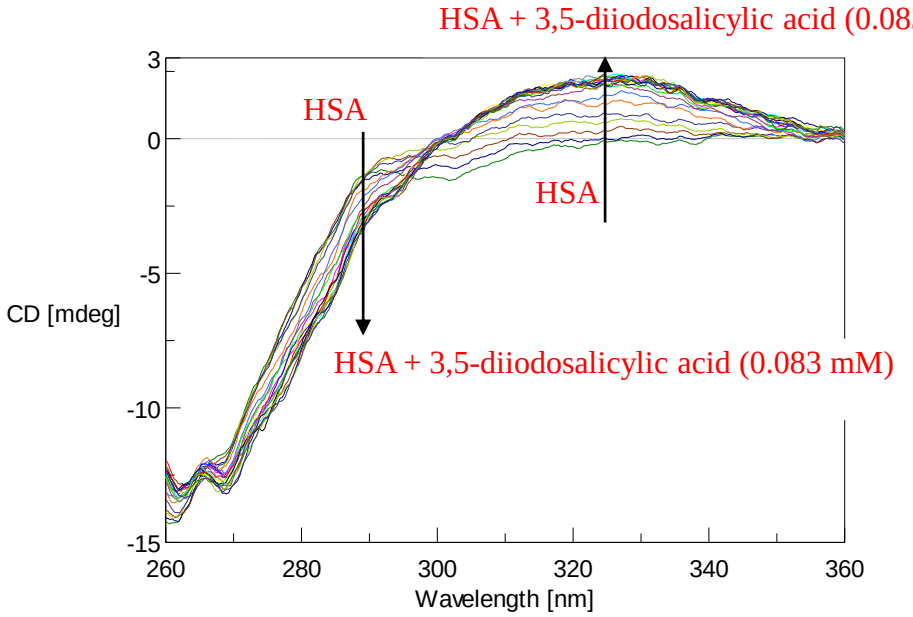


Fig. 2 2D-CD spectra of HSA titrated with 3,5-diiodosalicylic acid
The arrow represents increasing volume of 3,5-diiodosalicylic acid

A plot of the increase of the induced CD signal (at 320 nm) versus the increase in 3,5-diiodosalicylic acid (Fig. 3) and the Hill plot (Fig. 4) are shown below.

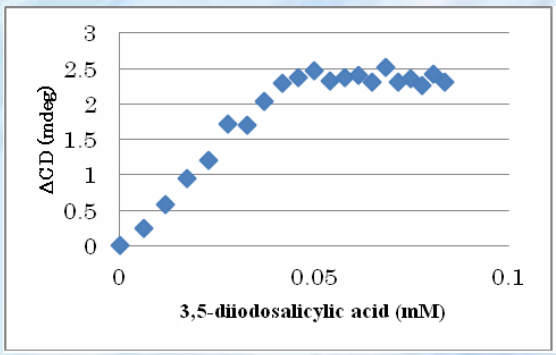


Fig. 3 Increase of induced CD (320 nm)

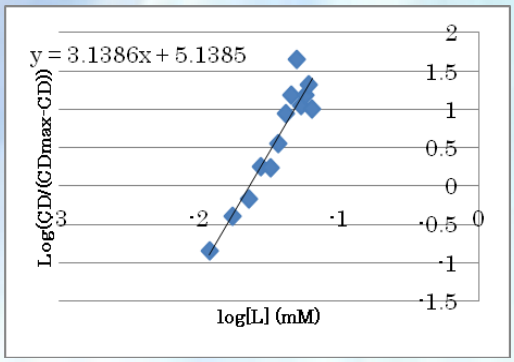


Fig. 4 Hill plot

The estimated dissociation constant (K_d), which is the concentration at which 50% of the 3,5-diiodosalicylic acid is bound with HSA is 0.023 mM. The Hill coefficient is approximately 3.1, indicating a positive cooperative reaction.

<References>

(1) R. Brodersen, *J. Biol. Chem.* (1977) **252**, 14, 5067-5072

(2) Ulrich KRAGH-HANSEN, *Biochem. J.* (1983) **209**, 135-142

(3) S. S. Sinha, R. K. Mitra and S. K. Pal, *J. Phys. Chem. B.* (2008) **112**, 4884-4891

Change of fluorescence anisotropy spectrum of a-lactalbumin by denaturation

Introduction

CD spectrum measurement is one of the leading techniques in protein structure analyses, while fluorescence spectra and fluorescence anisotropy spectra can give complementary information to CD spectra. CD spectra provide information about the secondary structure of proteins, while fluorescence spectra and fluorescence anisotropy spectra provide information about the local environment of the fluorophore, such as the tryptophan residue. In particular fluorescence anisotropy spectra provide information about rotational movement which cannot be obtained by fluorescence spectra alone.

JASCO J-1500 CD spectrometer enables measurement of CD spectra, absorption spectra, Ex/Em spectra and fluorescence anisotropy spectra, thereby, allowing not only secondary structure estimation but also the analysis of protein-ligand binding and rotational movement of protein.

Here, the change of fluorescence anisotropy spectrum of a-lactalbumin by denaturation[1)] using J-1500 is explained.

Keywords: Fluorescence anisotropy spectra, α-Lactalbumin, Denaturation

<System>

J-1500 CD spectrometer
LD-403 LD attachment
CDF-426 CD/Fluorescence Measurement unit (Peltier type) with Polarizer (optional)

<Samples>

0.02 mg/mL a-lactalbumin, 0.1 mM EDTA in H_2O
0.02 mg/mL a-lactalbumin, 0.1 mM EDTA in 3.4 M GuHCl

<Parameters>

Measurement range:	310 to 245 nm	Scan speed:	100 nm/min
Response:	2 sec	Data interval:	0.1 nm
Ex Bandwidth:	7 nm	Cutoff filter:	UV34

<Fluorescence anisotropy spectrum of a-lactalbumin >

Fluorescence anisotropy spectra of native-state α-lactalbumin in H_2O and unfolded α-lactalbumin in 3.4 M GuHCl are shown in Fig. 1. These two spectra show a peak maximum at 267 nm and two peak minima at 283 nm and 291 nm originating from the tryptophan residue[2)]. Denaturation of α-lactalbumin clearly results in a decrease in fluorescence anisotropy originating from the tryptophan residue.

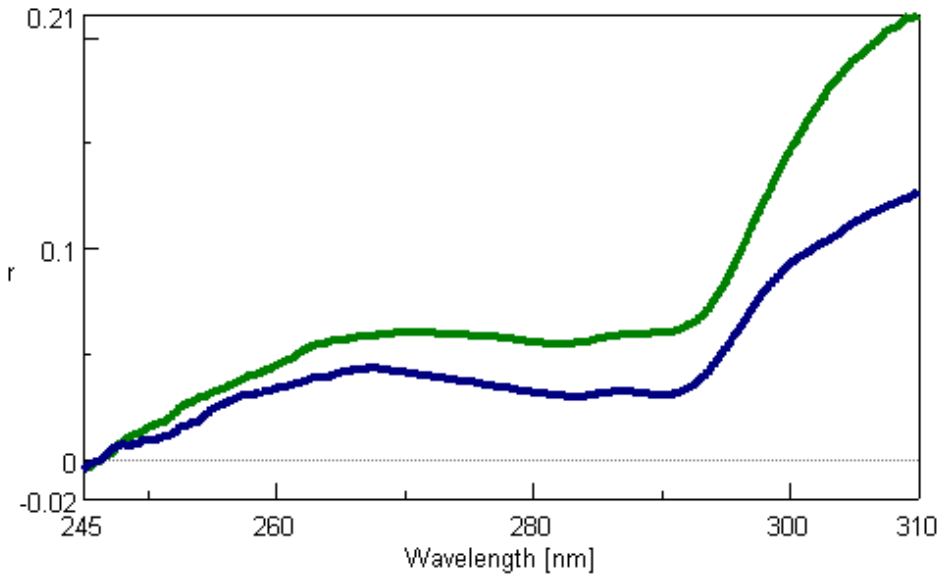


Fig. 1 Change of fluorescence anisotropy spectrum of α-lactalbumin by denaturation

0.02 mg/mL a-lactalbumin, 0.1 mM EDTA in H_2O:
0.02 mg/mL a-lactalbumin, 0.1 mM EDTA in 3.4 M GuHCl:

<References>

(1) Denis Canet, Klaus Doering, Christopher M. Dobson, and Yves Dupont, *Biophysical Journal*, **80**, 1996-2003, (2001)

(2) Protein fluorescence. *In* Principles of Fluorescence Spectroscopy. J. R. Lakowicz, editor. Kluwer Academic/Plenum Publishers, New York. 446-487.

Thermal denaturation analysis of super thermostable cellulase

Introduction

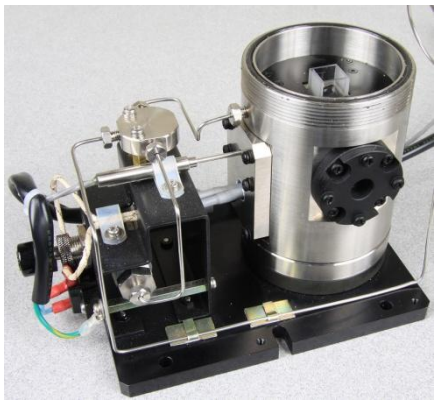
The thermodynamic properties and secondary structures of proteins reveal important information on their functions. Differential scanning calorimetry (DSC) and CD spectroscopy are usually used to measure the thermal denaturation of proteins. CD spectroscopy has advantages such that CD spectra can be measured using lower concentrations of proteins than DSC and can also be measured at various pHs and in a wider range of solvent conditions. However, CD spectra cannot be measured at the temperature above 100ºC because the boiling point of water is 100ºC.

JASCO has developed the TC-700/700PC type, high pressure-resistant/high temperature measurement sample chamber for high temperature measurements above 100ºC. TC-700 enables measurement of CD spectra at temperatures up to 170ºC by pressurizing the sample solution to 1 MPa using commercially available high-pressure N_2 gas. Using TC-700, the thermal denaturation of super thermostable proteins, such as those originating from thermophiles, can be measured.

Herein, thermal denaturation analysis of super thermostable cellulase using TC-700PC is explained. Super thermostable cellulase maintains its activity at temperatures up to 90ºC. Research on enzymes, such as super thermostable cellulase, may lead to the development of methods for the production of bioethanol from agricultural waste rather than from important crops, such as corn or sugarcane.

Keywords: Thermostable proteins, Thermal stability, Agricultural waste

<Specifications of TC-700/700PC>



TC-700 Sample chamber

Temperature Controller

Available cells:	Rectangular cells (1, 2, 5 and 10 mm) (1, 2 and 5 mm cells need corresponding cell spacers)
Temp. Control Region:	RT to 170ºC
Accuracy:	+/- 0.5ºC
Method:	PID control, 100 W heater Manual control (TC-700) PC control (TC-700PC)
Temp. Sensor:	Platinum temperature sensor

<CD spectra measurement>

Measuring conditions

Conc.:	0.01 mg/mL	Pressure:	0.8 MPa	Temp.:	45, 80, 100 and 120ºC
Response:	2 sec	Sensitivity:	Standard	Wavelength:	260-195 nm
Data interval:	0.1 nm	Scan speed:	100 nm/min	Cell pathlength:	10 mm
Bandwidth:	1 nm				

1.2 mg/mL super thermostable cellulase in 20 mM Tris-HCl buffer (pH 8.0) was diluted with distilled water and measured.

Results

The CD spectra of super thermostable cellulase measured at 45, 80, 100 and 120ºC are shown in Fig. 1. The thermal denaturation is observed at temperature of only over 100ºC.

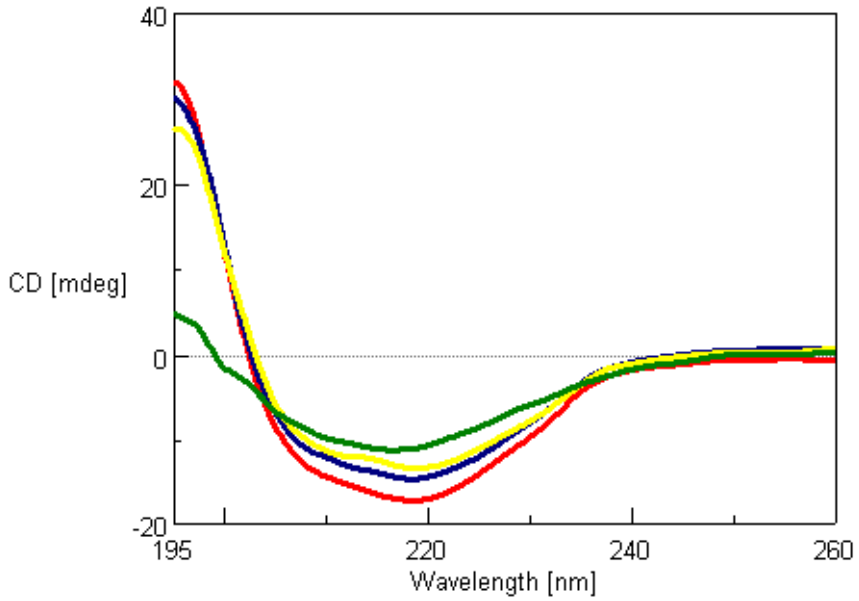


Fig. 1 CD spectra of super thermostable cellulase
Red line: 45ºC, Blue line: 80ºC, Yellow line: 100ºC, Green line: 120ºC

<CD spectra measurement>

Measuring conditions

Conc.:	0.025 mg/mL	Pressure:	0.8 MPa	Temp. range:	80-120ºC
Temp. interval:	0.1ºC	Temp. slope:	1ºC/min	Bandwidth:	1 nm
Cell pathlength:	10 mm	Wavelength:	220 nm		

Results

The thermal denaturation of super thermostable cellulase was monitored at 220 nm. The result of the analysis using the [Denaturation Analysis] program is shown in Fig. 2. There is a sharp decrease in the CD value at temperatures over 100ºC. The melting temperature (T_m) of super thermostable cellulase is shown to be 106.3ºC.

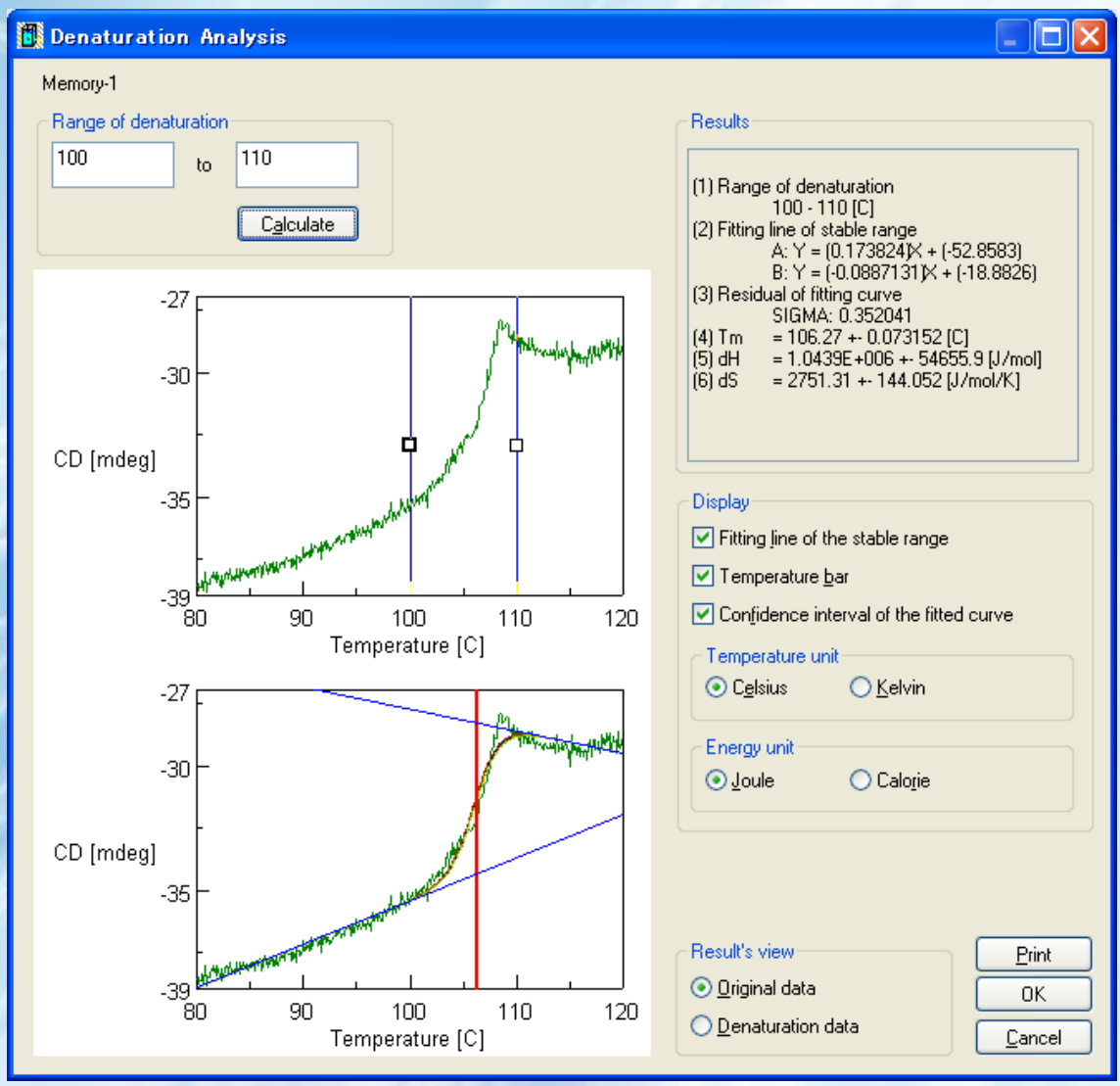


Fig. 2 Thermal denaturation analysis results of super thermostable cellulase

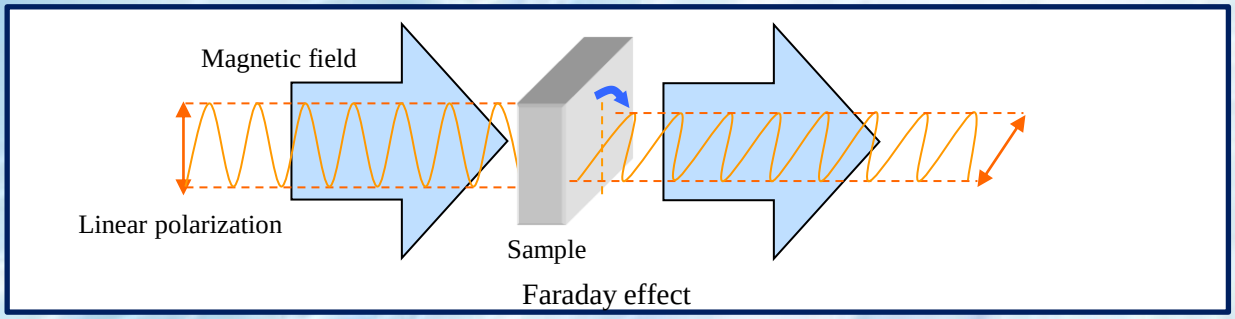
<Reference>

(1) S. Uchiyama, A. Ohshima, S. Nakamura, J. Hasegawa, N. Terui, S. J. Takayama, Y. Yamamoto, Y. Sambongi, and Y. Kobayashi, *J. Am. Chem. Soc.* **2004**, *126*, 14684-14685.

Magnetic CD measurement
-PM-491 Permanent Magnet (small type), 1.6 Tesla-

Introduction

When a linear polarization is passed through the material in the magnetic field parallel to the direction of the magnetic field, the polarization plane is rotated. This phenomenon is termed the Faraday effect after the discoverer, which is regarded as optical activity induced by magnetic field.

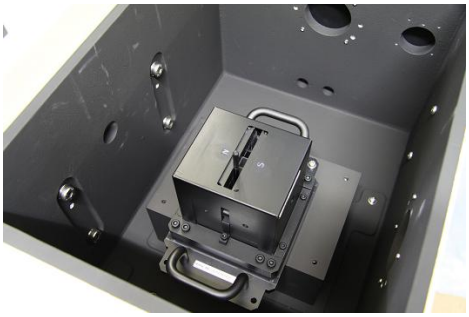


ORD and CD are used for the observation of the Faraday effect as well as optically active materials. These experiments are named Magnetic Optical Rotary Dispersion (MORD) and Magnetic Circular Dichroism (MCD), respectively, due to phenomenon induced by the magnetic field. Although both the MORD and MCD can be measured by setting the magnet in the sample compartment for the ORD and CD respectively, MCD is widely used today due to simple measurement and high-sensitivity.

Previously, to generate a strong magnetic field over 1 Tesla, a large electrical magnet was used. The electrical magnet cannot be easily set in the sample compartment due to a weight of over 60 kg although it has an advantage that measurements can be performed while changing the intensity of the magnetic field. The PM-491 permanent magnet (small type) can provide a strong magnetic field of 1.6 Tesla. It is also available for the observation of small MCD signals such as a low-concentration sample. In addition, the PM-481 magnet can be easily set in the sample compartment due to its small size. The direction of the magnetic field can be changed by a simple switch to the reverse direction.

MCD is a phenomenon which is observed for all materials in principle. The normal CD spectra provides the configuration and conformational information, whereas the MCD spectra provides the electronic state information.

Measurement examples for the neodymium glass, cytochrome C and cobalt chloride (II) 6 hydrate are shown in the following pages.



PM-491 Permanent magnet, 1.6 Tesla

Intensity of magnetic field: 1.6 Tesla

Cell length: 5 mm (option: 1 mm, 2 mm)

Keywords
Faraday effect, Magnetic Circular Dichroism, Magnetic Optical Rotary Dispersion, cytochrome C, Soret

MCD of neodymium glass

Neodymium glass is optically inactive, however it illustrates MCD spectra from the UV/Vis to the NIR range using the generated magnetic field. Not only do the spectra demonstrate strong sharp MCD signals (Fig. 1) which have strong optical absorptions but also the small MCD signals due to weak optical absorptions are displayed (Fig. 2) due to the extremely strong magnetic field of 1.6 T. MCD spectra can also separate sharp peaks which cannot separates in the absorption spectra. In this application note (please see the "supplemental explanation" in the last page), if the direction of the magnetic field is the same as that of the measurement light, it is defined as the magnetic field in the forward direction. If the direction of the magnetic field is different from that of the measurement light, it is defined as the magnetic field in the inverse direction. By changing the direction of the magnetic field, the code of the MCD spectra is also reversed.

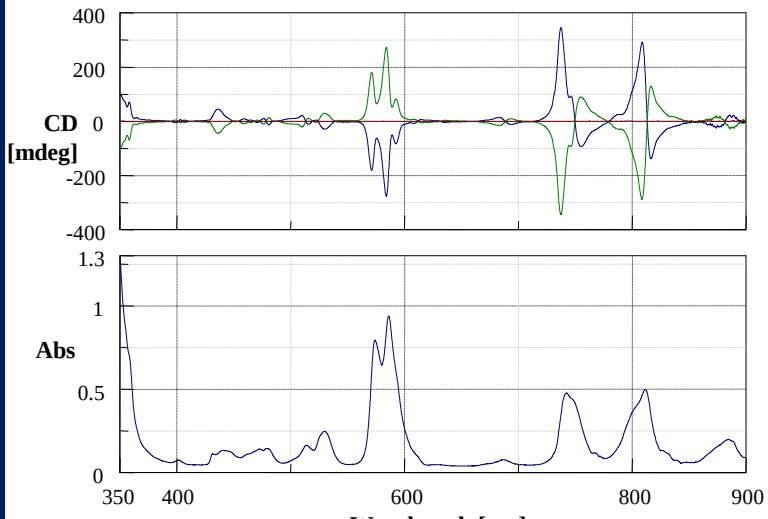


Fig. 1 MCD and absorption spectra of a neodymium glass sample (900 - 350 nm)
Blue line: Magnetic field in the forward direction
Green line: Magnetic field in the inverse direction
Red line: without magnetic field

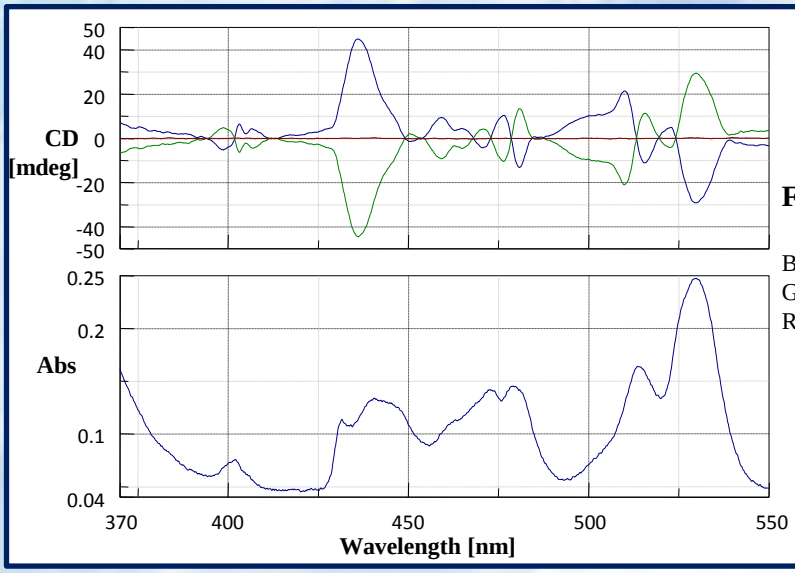


Fig. 2 MCD and absorption spectra of the neodymium glass (550 - 370 nm)
Blue line: Magnetic field in the forward direction
Green line: Magnetic field in the inverse direction
Red line: without magnetic field

MCD of Fe (III) cytochrome c (derived from equine heart)

MCD is widely used for the structural analysis of hemoprotein samples, and several studies of myoglobin[1], hemoglobin[2], cytochrome b_5[3, 4], cytochrome c[3, 5, 6], cytochrome P-450[7] and horseradish peroxidase[8] are reported. The measurement examples for the MCD spectra in the Soret band of the Fe (III) cytochrome C solution are shown below.

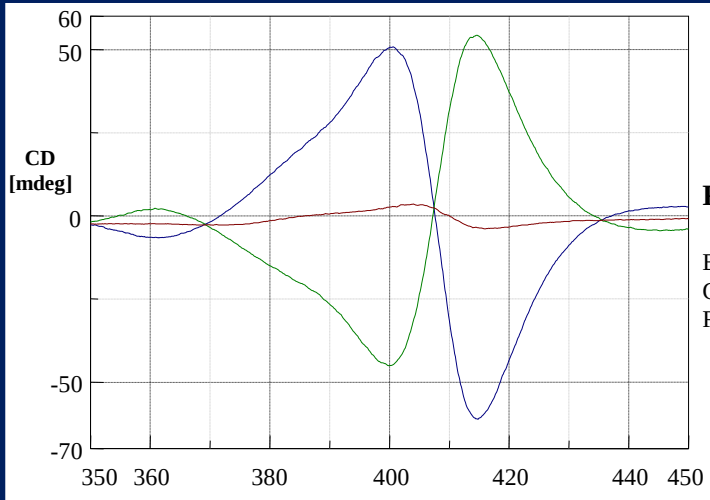


Fig. 3 MCD spectra and CD spectrum of the Fe (III) cytochrome C

Blue line: Magnetic field of forward direction
Green line: Magnetic field of inverse direction
Red line: without magnetic field (CD spectra)

If the CD spectrum obtained with the non-magnetic-field condition is subtracted from the MCD spectra, the symmetric MCD spectra can be observed.

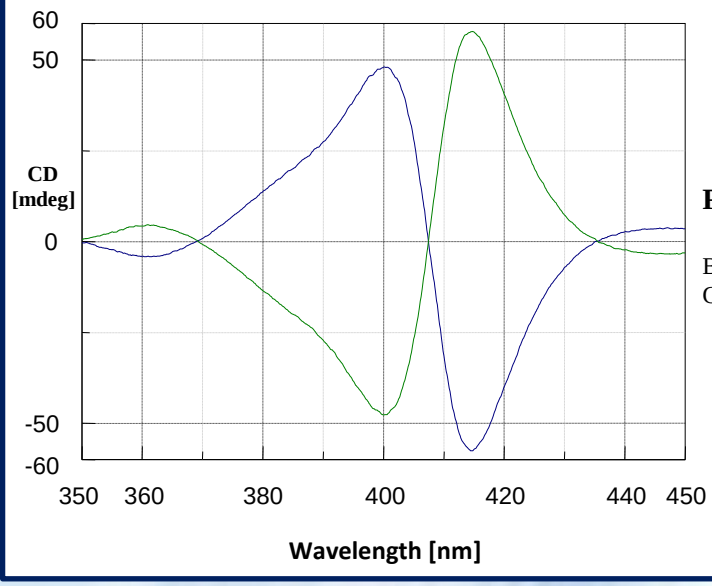


Fig. 4 MCD spectra of the Fe(III) cytochrome C (after subtraction of the CD spectrum)

Blue line: Magnetic field of forward direction
Green line: Magnetic field of inverse direction

MCD of cobalt chloride (II) 6 hydrate

An aqueous solution of cobalt (II) chloride 6 hydrate exists in a 6-coordinate structure of the octahedron type in which the cobalt ion is coordinated with 6 water molecules (Figure 5). By contrast, the cobalt (II) chloride 6 hydrate in a concentrated hydrochloric acid solution is a 4-coordinate structure of a tetrahedron type in which it is coordinated with 4 chloride ions (Figure 6). The MCD spectra demonstrate drastic changes based on the change in the electronic state of the cobalt ion with high-sensitivity[9].

$$[Co(H_2O)_6]^{2+} + 4Cl^- \rightleftarrows [CoCl_4]^{2-} + 6H_2O$$

Measurement of Vibrational Circular Dichroism spectra using the FVS-6000

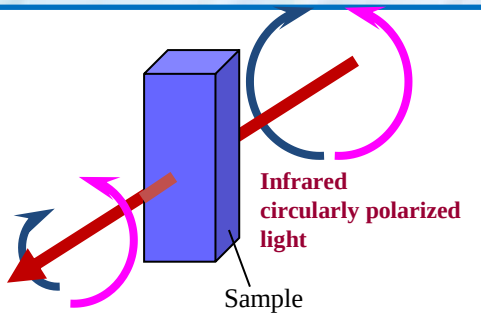
< Introduction >

It is generally understood that chiral compounds have different bioactivities depending upon the absolute configuration of each compound. Some familiar examples include glutamic acid and thalidomide. L-glutamic acid demonstrates the "Umami" taste*[1], while D-glutamic acid has a bitter taste, similarly, the *R* form of thalidomide is a sedative, but the S form has teratogenic activity. Thus, the separation and study of chiral compounds is critical for many reasons.

The functionalities of chiral compounds have been studied for the development of advanced molecules for many applications. The study of chiral compounds has spread to several fields such as natural products, pharmaceuticals and other functional molecules, and it can be pointed out that among those studies, the structural analysis of chiral compounds is a very important topic. X-ray Diffraction (XRD), Nuclear Magnetic Resonance (NMR) and Electronic Circular Dichroism (ECD) using UV/Vis light are employed as primary methods for the structural analysis of chiral compounds. In this paper, the measurement of chiral compounds by Vibrational Circular Dichroism (VCD) using infrared light will be outlined.

VCD is a method to measure the difference of absorbance intensity between left-hand and right-hand circularly polarized light as shown in Figure 1. It is an advantage of VCD that this method can be applied to almost all organic compounds in the same way as infrared (IR) spectroscopy. In addition, by comparing the measurement results with calculated results by *ab-initio* molecular orbital calculations, the absolute configuration of the sample can be determined. However, since the peak intensity of VCD spectra are 1,000 – 10,000 times weaker than that of standard IR spectra, spectroscopic instruments with high sensitivity and stability with very small baseline fluctuations are required. The FVS-6000 VCD system has a high sensitivity detector, suitable optical filter technology and a thermostatted Photoelastic Modulator (PEM) to accurately measure the weak VCD peaks. The measurement results of typical chiral compounds and hemoglobin as a model protein using the FVS-6000 are reported.

*[1] Umami taste is the fifth taste sensation in addition to sweet, acid, salty and bitter taste.



$DAbs = A_L - A_R$

$(De = e_L - e_R)$

Fig. 1. Principles of VCD spectroscopy

Fig. 2. External view of the FVS-6000

< Measurement results >

Figures 3 τηρουγη 6 illustrate the measurement results for α-pinene; 1,1-Bi-2-naphthol; proline and hemoglobin, respectively. Both IR and VCD spectra can be obtained by the FVS-6000. The identification of the absolute configuration of chiral compounds can be determined from both the IR and the VCD spectra as well as the analysis of the molecular structure.

Figure 3 demonstrates the IR and VCD spectra of alpha-pinene which is a typical standard sample to validate a VCD instrument system. IR spectra of the R- and S- form of α-pinene are completely overlapped, while their VCD spectra are symmetric, clearly identifying each alpha-pinene enantiomer. Since the peak shapes of the VCD spectra obtained illustrate typical alpha-pinene spectra, it is confirmed that high quality VCD spectra can be measured by the FVS-6000.

Figure 4 outlines measurement results for 1,1-Bi-2-naphthol which is used as a ligand for transition-metal catalyzed asymmetric synthesis and is a precursor for chiral ligands such as BINAP. The small peaks due to the anisotropy factor 'g value' (VCD peak/IR peak) around 1600 and 1500 cm^{-1} attributed to the benzene ring are clearly shown. These results demonstrate that the FVS-6000 is a very effective system for evaluation of chiral compounds which have similar structures.

Figure 5 illustrates the measurement results of proline which is one of the amino acids. Good, symmetrical VCD spectra were obtained for the D- and L- forms. Since amino acids demonstrate different bioactivities between the D- and L- forms, studies regarding structure and bioactivity are increasingly popular. Liquid samples can be easily measured by VCD instrumentation so the structural analysis of amino acids can be performed under similar to physiological conditions.

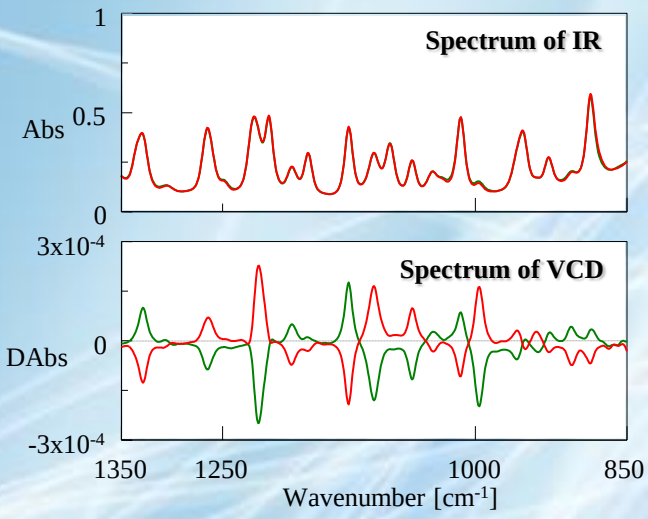
Figure 6 contains the measurement results for hemoglobin. Hemoglobin is known as a spherical model protein which contains rich alpha-helix structures, and in addition, its VCD spectrum shape is very different from that of concanavalin A, which contains a rich beta-sheet structure.

The addition of VCD spectral results to information obtained from ECD and IR spectra can provide much more accurate secondary structure analysis of proteins in solvents. We also believe that VCD can also be a powerful tool for the analysis of DNA and chiral polymers other than proteins.

In this paper, the standard performance and measurement results of typical chiral compounds using the FVS-6000 were reported and we believe the FVS-6000 can be an essential and indispensable tool for analysis of chiral compounds.

Measurement condition

Model name:	FVS-6000
Resolution:	4 cm^{-1}
Detector:	MCT-V
Accumulation:	1000 (α-pinene and 1,1'-Bi-2-naphthol), 2500 (proline), 2000 (hemoglobin)



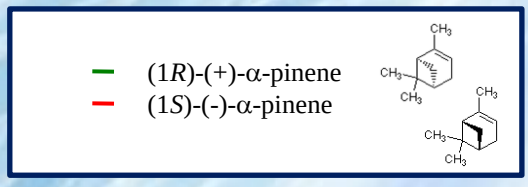
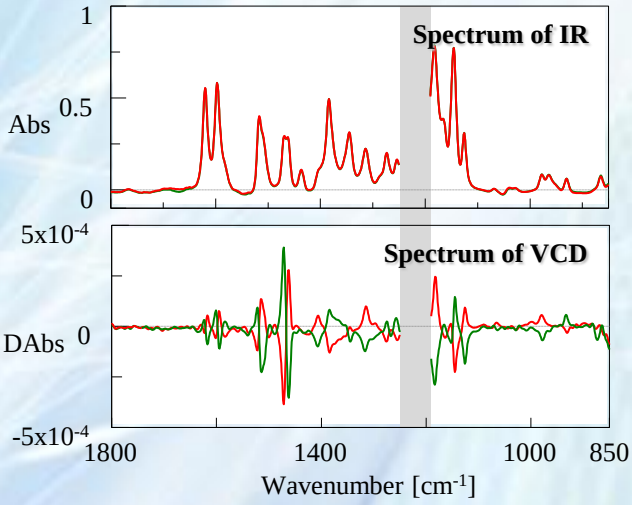
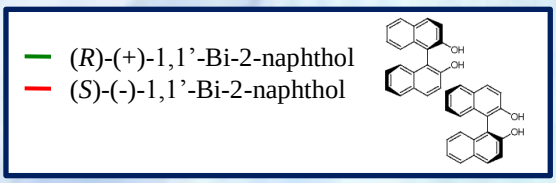


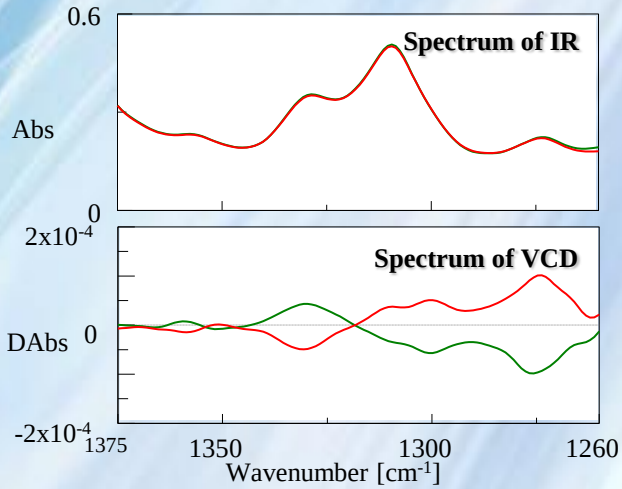
Fig. 3. Spectra of α-pinene
(neat, 50 μm pathlength BaF_2 liquid cell)





* Since the noise in the range of 1250 - 1190 cm^{-1} is excessive due to solvent absorption, the data in this range are cut from the spectral figures.

Fig. 4. Spectra of 1,1'-Bi-2-naphthol
solvent: $CHCl_3$, concentration: 0.162 M, 50 μm pathlength BaF_2 liquid cell)



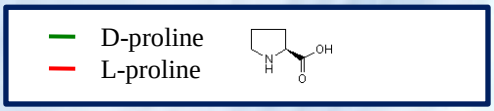
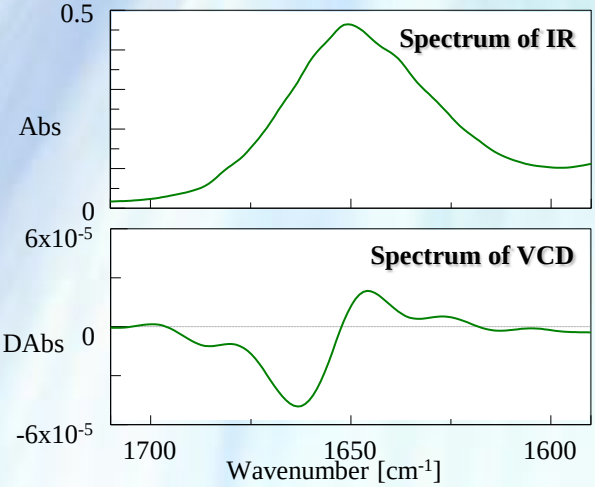


Fig. 5. Spectra of proline
(solvent: D_2O, concentration: 0.9 M,
25 μm pathlength CaF_2 liquid cell)



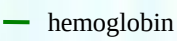


Fig. 6. Spectra of hemoglobin
(solvent: D_2O, concentration: 50 mg/mL,
25 μm pathlength CaF_2 liquid cell)