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Analysis of proteins by high-performance liquid chromatography with circular dichroism spectrophotometric detection

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ABSTRACT

High-performance liquid chromatography combined with circular dichroism spectrophotometry (HPLC–CD) for the separation and conformational analysis of proteins is described. The HPLC–CD measurement of proteins was performed easily and reproducibly with a gradient elution method by using a micro-flow cell unit. Reversed-phase chromatography and hydroxyapatite chromatography of proteins with this system were demonstrated. In addition, the influence of salts, surfactants and the interaction of packing materials on the secondary structure of proteins during HPLC was investigated and the α-helix content of protein was calculated by using a secondary structure estimation program.

INTRODUCTION

High-performance liquid chromatography (HPLC) is now routinely applied to the separation of proteins owing to its high resolution and rapid separation capability. However, the biological activity of a protein is often reduced or lost during the HPLC separation process.

The biological activity of a protein is related to its conformational structure. Therefore, it has been desirable to have a detector which can sensitively monitor the conformational structure. NMR, X-ray diffraction and circular dichroism (CD) are well known methods for the conformational analysis of proteins. Although NMR and X-ray diffraction offer a high resolution capability, NMR analysis requires too much sample and it is very difficult to use as an on-line monitor for an HPLC system. With X-ray diffraction analysis, it is impossible to measure a protein in solution. Of the three methods, CD is the most useful for the conformational analysis of proteins in combination with a separation method. A CD spectrophotometer allows the determination of the secondary structure, which is a conformational structure, of a protein in solution with higher sensitivity than NMR. Therefore, if a CD spectrophotometer can be used as an on-line monitor with an HPLC system, such a system (HPLC–CD)

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would be a powerful tool for the separation and conformational analysis of proteins.

In the early 1980s, preliminary studies on HPLC–CD were reported[1–3]. However, they examined the applicability of HPLC–CD systems only to low-molecular-weight chiral substances. More recently, we reported[4,5] an HPLC–CD system for protein analysis based on gel filtration chromatography (GFC) and a highly sensitive CD system which was equipped with a micro-flow cell. We have now extended the HPLC section of the system to reversed-phase (RP) HPLC and hydroxyapatite chromatography (HAC) with gradient elution, and have investigated the influences of salts, surfactants and stationary phase materials on the secondary structure in protein separation.

EXPERIMENTAL

Materials

All proteins and tyrosine were purchased from Sigma (St. Louis, MO, U.S.A.). Surfactants {sodium dodecyl sulphate (SDS), Tween 20 and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate, (CHAPS)} were purchased from Wako (Osaka, Japan). For GFC, Biofine GFC SI-150K [particle size 7 μm, exclusion size $1 \cdot 10^5$ dalton (protein level), column dimensions 300 mm × 7.5 mm I.D., packing material silica), for HAC, Biofine HAC-5CP (particle size 5 μm, column dimensions 100 mm × 7.5 mm I.D.), RP-HPLC, Biofine RPC-SC18 (particle size 7 μm, column dimensions 250 mm × 4.6 mm I.D.), were used. All column were obtained from JASCO (Tokyo, Japan). Other reagents were purchased from Wako.

Apparatus

HPLC was carried out on a JASCO 800 Series HPLC system with a Multi-320 multi-wavelength UV detector (195–350 nm) (JASCO) and a Model J-600 CD spectrophotometer with a micro-flow cell device attached. Both detectors were connected in series to give an HPLC–UV–CD system.

Preparation of protein solution

Each protein solution was prepared by dissolution in the same solvent as the eluent (concentration 0.01–1%, w/v) except for RP-HPLC, where water was used. In the gradient elution method, the first eluent was used. The solution was stored for about 1 h at 0°C and then HPLC was performed.

Chromatographic procedure and CD measurement

The effluent was first monitored at 195–350 nm by using the multi-wavelength UV detector, and subsequently monitored at 220 nm by using the CD spectrophotometer [spectral band-width (SBW) 2 nm; time constant (TC) 2 s]. The on-line CD spectrum was measured with the following procedures. The pump delivery was stopped at each chromatographic peak top monitored by CD and at the same time the injector valve was set to the intermediate position to stop the eluent flow completely (stopped-flow method). Then each CD spectrum under the operating conditions of SBW = 2 nm, TC = 0.5 s, scan speed = 50 nm/min and a computer of average transient (CAT) = 5 was measured within 10 min. After completion of one CD measurement, the pump delivery was restarted to continue the elution. The CD chro-

matogram and on-line CD spectra were obtained by performing measurements separately. As a reference for the CD spectrum, the effluent at the same retention time as each eluted protein was used.

HPLC was performed under the following conditions. For RP-HPLC, the column was Biofine RPC-SC18; eluent, linear gradient of acetonitrile (10–60%) in 0.1% trifluoroacetic acid (TFA) for 20 min; flow-rate, 1.0 ml/min; and column temperature, room temperature. For HAC, the column was Biofine HAC-5CP; eluent, linear gradient of sodium phosphate (10–300 m*M*) in the presence or absence of 0.1% Tween 20 for 20 min; flow-rate, 1.0 ml/min; and column temperature, room temperature. For GFC, the column was Biofine GFC SI-150K; eluent, (A) 50 m*M* Tris–HCl (pH 7.2) or (B) 50 m*M* Tris–HCl + 300 m*M* sodium chloride (pH 7.2) in the presence or absence of surfactant; flow-rate, 1.0 ml/min; and column temperature, room temperature. For flow-injection analysis (FIA), the same conditions as in GFC were adopted, except for the column.

Secondary structure estimation (SSE) of proteins

After smoothing by fast Fourier transformation (FFT), secondary structure estimation of proteins was performed by an SSE program (JASCO protein SSE program). This program provides the four fractions of the secondary structure of α-helix, β-form, β-turn and unordered form of protein conformation.

RESULTS AND DISCUSSION

HPLC–CD for RP-HPLC and HAC using the gradient elution method

HPLC–CD measurements of some proteins were performed using RP-HPLC with a linear gradient of acetonitrile (10–60%) for 20 min (Fig. 1) and HAC with a linear gradient of sodium phosphate (10–300 m*M*) for 20 min (Fig. 2). The amount of each protein charged was 50 μg [total injection volume, 150 μl (RP-HPLC) and 100 μl (HAC)], which was suitable for CD detection. As a reference for the CD spectrum, the effluent at the same retention time as each eluted protein was used. Although the reference is not completely identical, no difference was observed between CD spectra which were repeatedly measured three times.

Fig. 1 shows (A) the CD chromatogram at 220 nm, (B) the UV chromatogram at 280 and 350 nm and (C) the CD spectra (192–250 nm) of proteins on RP-HPLC. This mode allows proteins to be substantially denatured because of the high concentration of the organic solvent. For example, myoglobin was changed into an apoprotein part (peak 3, first) and a haeme part (peak 3, second). The haeme peak, which did not have a secondary structure such as proteins, was not monitored by CD. The apoprotein peak was determined from both the UV and CD spectra and amino acid analysis (data not shown).

Fig. 2 shows (A) the CD chromatogram at 220 nm, (B) the UV chromatogram at 280 and 350 nm in the absence of Tween 20, and the CD spectra (192–250 nm) of (C-1) myoglobin and (C-2) cytochrome *c* in the absence (solid lines) and presence (dashed and dotted lines) of Tween 20 on HAC. The separation and the CD spectra of cytochrome *c* were greatly influenced by Tween 20. Two peaks appeared in the presence of Tween 20 on HAC. As shown Fig. 2(C-2), each CD spectrum was very different from that in the absence of Tween 20. In contrast, the CD spectra of myoglo-

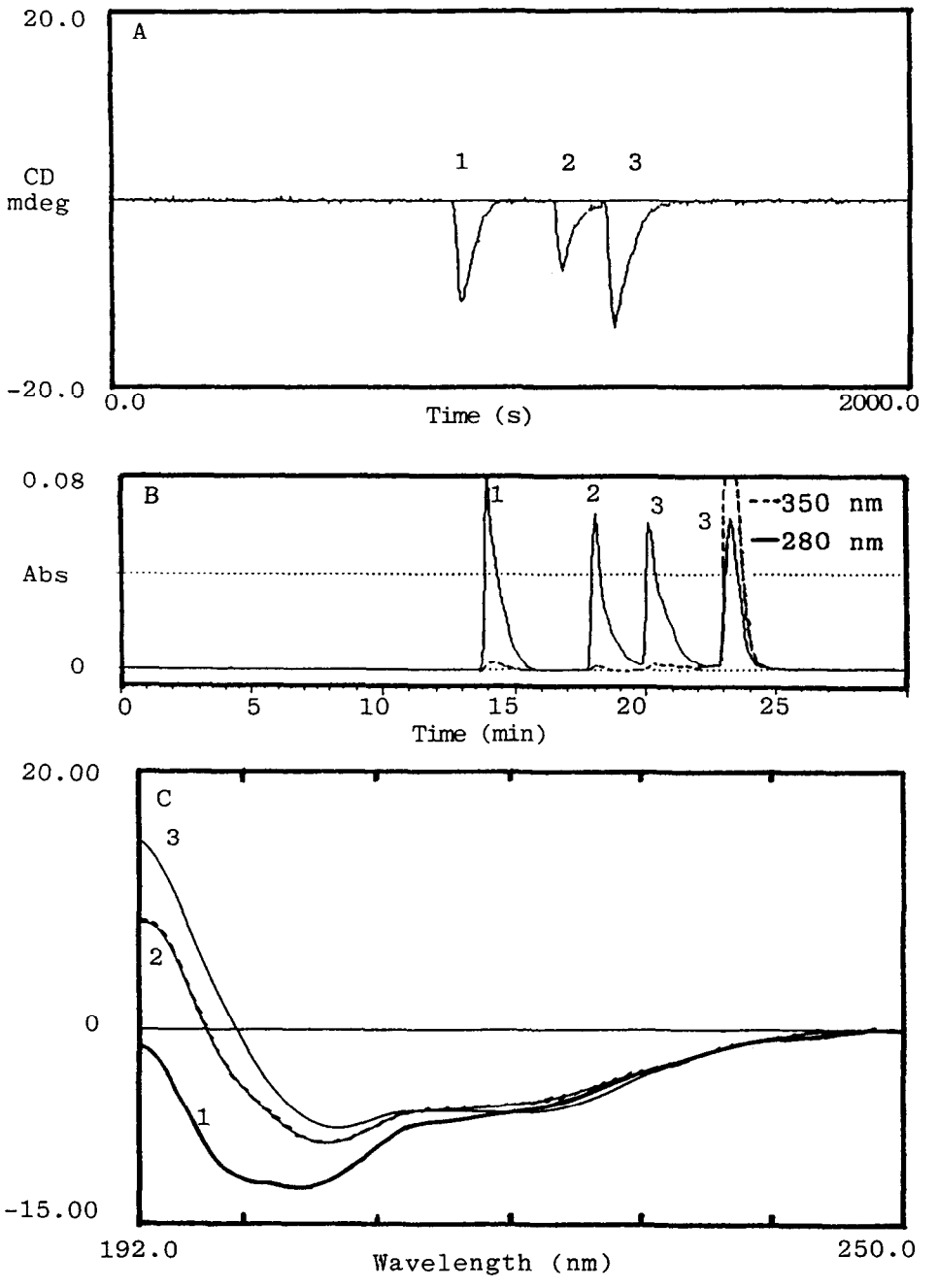
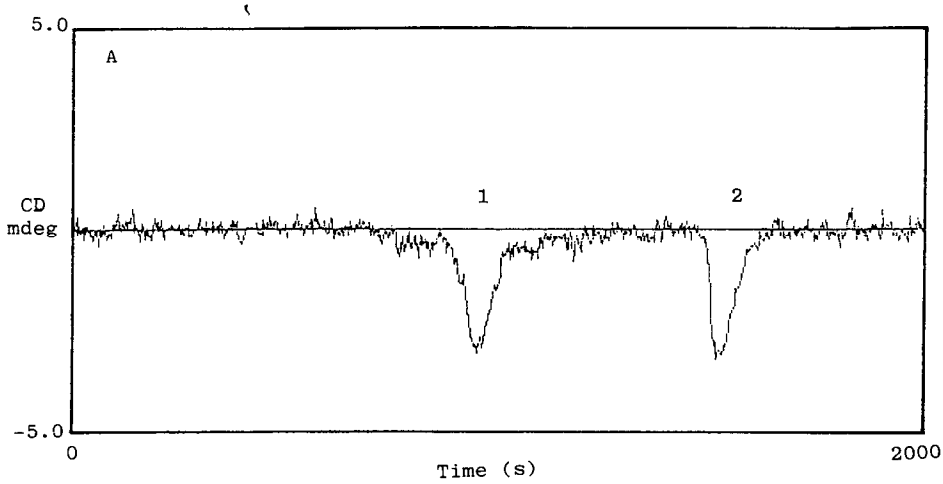
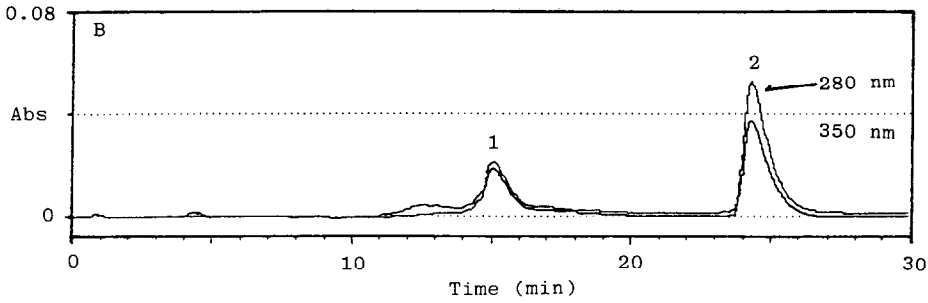


Fig. 1. RP-HPLC of proteins by using the described HPLC–CD system. (A) CD chromatogram; (B) UV chromatogram; (C) CD spectra with the stopped-flow method. Sample: 1 = ribonuclease A; 2 = transferrin; 3 = myoglobin. Chromatographic conditions as described under Experimental.





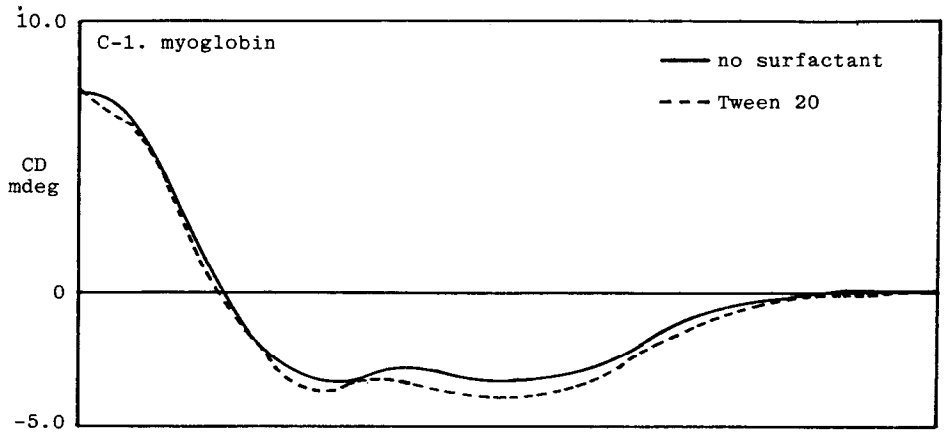


Fig. 2.

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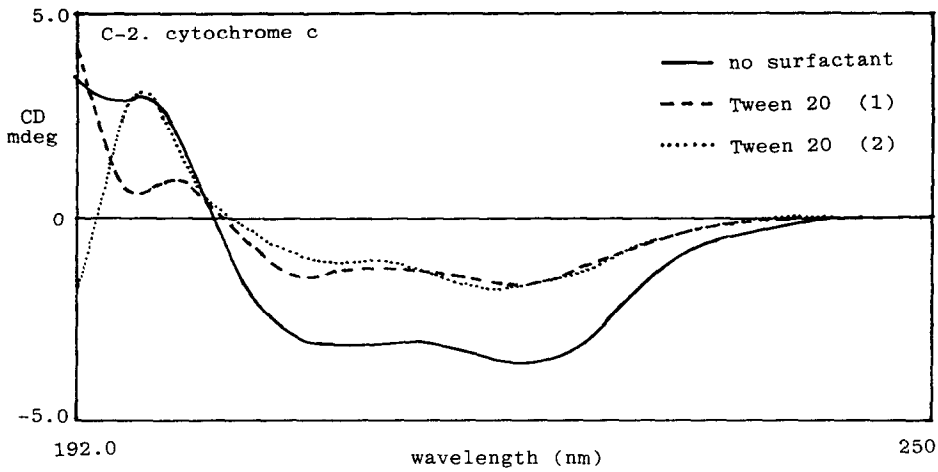

Fig. 2. HAC of proteins by using the described HPLC–CD system. (A) CD chromatogram; (B) UV chromatogram; (C) CD spectra of (C-1) myoglobin and (C-2) cytochrome *c* with the stopped-flow method. Sample: 1 = myoglobin; 2 = cytochrome *c*. Chromatographic conditions as described under Experimental. The CD and UV chromatograms and CD spectra (solid lines) were obtained in the absence of Tween 20. Dashed and dotted lines represent the CD spectra obtained during HAC in the presence of 0.1% Tween 20 (C-1 and C-2). With cytochrome *c*, two peaks appeared on HAC in the presence of 0.1% Tween 20.

bin in the absence and presence of Tween 20 were only slightly different. The HPLC–CD measurement could be performed by the gradient elution method with not only sodium phosphate (10–300 m*M*) but also sodium chloride (0–500 m*M*), which was confirmed by ion-exchange chromatography (IEC) (data not shown). Hence it was demonstrated that HPLC–CD could be performed with the gradient elution method using several separation modes.

Effect of packing materials on α-helix content of proteins

The biological activity of a protein is often reduced or lost during the HPLC separation process. Although the nature of the eluent is known to cause denaturation, we presumed that the packing materials may also be related to denaturation. As FIA can be considered to represent HPLC without an analytical column, in FIA proteins could not be influenced by interactions with packing materials. Therefore, a comparison of GFC with FIA will demonstrate the influence of the packing materials on the conformational structure of proteins under the same chromatographic conditions.

We adopted the α-helix content, which is one factor of secondary structure, to determine the conformational structure, because it was expected to be the most stable conformation in comparison with the β-form, β-turn, etc. The estimation was performed with the SSE program.

As shown Table I, lysozyme was not eluted by 50 m*M* Tris–HCl (pH 7.2) (solvent A) in GFC. However, It was eluted by 50 m*M* Tris–HCl + 300 m*M* sodium chloride (pH 7.2) (solvent B) in GFC. This result indicates a strong interaction between the packing materials and lysozyme in a buffer of weak ionic strength. In myoglobin, although it was eluted by both solvents A and B, the α-helix content with

TABLE I

EFFECT OF PACKING MATERIALS ON THE α-HELIX CONTENT OF PROTEINS

Eluent: A, 50 m*M* Tris–HCl (pH 7.2); B, 50 m*M* Tris + 300 m*M* NaCl (pH 7.2). Other conditions as described under Experimental. The α-helix content was calculated with the SSE program.

Protein	*Eluent*	*α-Helix content (%)*	
		FIA	*GFC*
Lysozyme	A	35.4	Not eluted
	B	38.4	34.9
Myoglobin	A	62.0	57.4
	B	63.1	62.9
Transferrin	A	48.9	40.1
	B	48.2	42.3
Haemoglobin	A	56.1	57.6
	B	56.4	57.7

solvent A in GFC was slightly decreased in comparison with that with solvent A on FIA. In transferrin, when GFC was performed, the α-helix contents with both solvents A and B were decreased in comparison with FIA. In haemoglobin, the α-helix content was not influenced by the nature of the solvent and packing materials. In general, ionic strength is not related to α-helix content in FIA, but it is related to it in GFC, which causes a slight interaction. The results showed no influence of the concentration of sodium chloride (at least in the range 0–300 m*M*) on denaturation. However, the influence of the packing materials on denaturation was suggested during HPLC in the HPLC–CD analysis.

Effect of surfactants on α-helix content of proteins

As shown in Fig. 2, the presence of a surfactant affected HAC. Generally the chromatographic resolution and reproducibility is reduced by the presence of surfactants. In addition, surfactants cause denaturation of active proteins. However, surfactants are very useful for the solubilization of insoluble proteins, *e.g.*, membrane proteins. SDS (strong for solubilization, ionic), Tween 20 (medium, non-ionic) and

TABLE II

EFFECT OF SURFACTANTS ON THE α-HELIX CONTENT OF PROTEINS

Eluent: A, 50 m*M* Tris–HCl (pH 7.2). Other conditions as described under Experimental. The α-helix content was calculated with the SSE program.

Eluent	*α-Helix content (%)*			
	Lysozyme	*Myoglobin*	*Transferrin*	*Haemoglobin*
A	26.2	63.1	48.2	56.4
A–0.1% SDS	23.4	55.6	42.7	52.8
A–0.1% Tween 20	23.9	64.5	47.0	58.9
A–0.1% CHAPS	23.7	63.5	51.9	57.2

CHAPS (weak, amphoteric) were used as surfactants. This investigation was performed by FIA and the use of the SSE program.

As shown Table II, the α-helix content of lysozyme was reduced by all the surfactants used. However, those of myoglobin, transferrin and haemoglobin were hardly changed by Tween 20 and CHAPS. CHAPS in fact caused the α-helix contents of myoglobin, transferrin and haemoglobin to increase slightly.

In addition, the influence of concentration of Tween 20 and SDS on the α-helix content was investigated (data not shown). The results observed were as expected: in myoglobin, the α-helix content was influenced by the concentration of SDS (strong surfactant) and not influenced by that of Tween 20 (medium surfactant). Similar results were obtained on GFC by the presence of SDS. However, the degree of denaturation was enhanced as compared with that on FIA. The α-helix contents of transferrin, myoglobin and lysozyme, with those obtained by FIA under the same conditions in parentheses were 36.6 (42.7), 43.9 (55.6) and 24.5% (23.4%), respectively. The results suggested that both SDS and the packing materials caused denaturation during HPLC. In lysozyme, although the values were almost unchanged, it may represent the limit of denaturation because of the rigid protein structure (lysozyme has four disulphide bonds).

CONCLUSION

As demonstrated, the HPLC–CD system could be easily applied to the analysis of the conformation of proteins during HPLC using a gradient elution method, *e.g.*, RP-HPLC with an organic solvent or HAC with a buffer containing salts.

In addition, the influence of surfactants and packing materials on the secondary structure was observed by using this system during HPLC. Salts such as sodium chloride in the concentration range 0–300 m*M* did not cause denaturation of some standard proteins sodium chloride.

This method will permit theoretical studies of the behaviour of active proteins during HPLC, and in the future, the relationship between the structure of proteins and their activity during HPLC will be studied using this system.

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