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Size-exclusion chromatography combined with small-angle X-ray scattering optics

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ABSTRACT

Size-exclusion chromatography with on-line synchrotron radiation solution small-angle X-ray scattering optics, absorbance and/or refractive index detectors was evaluated by protein characterizations. The radius of gyration value and zero-angle scattering intensity of protein molecules eluted from the chromatography column were estimated using this measurement system. In addition, the characterization of the conformation of the eluted proteins was demonstrated for hen egg lysozyme and bovine submaxillary mucin. The present technique will be useful for not only the determination of the radius of gyration value and molecular weight of proteins with dimensions of 1–10 nm, but also for the structural characterization of the macromolecules during the chromatography.

1. Introduction

Size-exclusion chromatography (SEC) is a technique for separating proteins and other biological macromolecules on the basis of molecular size. Originally developed in the 1950s, the technique was developed using cross-linked dextran [1]. SEC has been used for many purposes, including buffer exchange (desalting), the removal of non-protein contaminants, protein aggregation separation [2], the study of biological interactions, and protein folding [3]. It also has the important advantage of being compatible with physiological conditions.

Protein molecules eluted from the SEC column are most often monitored by absorbance in the ultraviolet range, either at 280 nm, which is suitable for proteins with aromatic amino acids, or at 206 nm, which detects the peptide bond. Fluorescence detection either by the direct detection of fluorescent tryptophan and tyrosine residues or after fluorescence derivatization have been used for protein chromatography. Other detections, such as refractive index, radiochemical, electrochemical, and light scattering [2,3], are also available.

Although the weights of small macromolecules can be determined from the scattering of visible light, the method fails if we seek to use the angular dependence to measure the dimensions of particles with a radius of gyration (R_g) less than about 10 nm [4]. On the other hand, small-angle X-ray scattering (SAXS) is a widely used diffraction method for studying the structure of proteins with dimensions in the neighborhood of 10 nm in solution [5]. Furthermore, the scattering measurements for low protein concentration solutions (~0.05%) are possible using synchrotron X-ray sources [5].

Recently Mathew et al. reported liquid-chromatography-coupled SAXS for sizing of aggregating proteins [6]. Thünemann et al. showed more recently the online coupling of field-flow fractionation with SAXS for the detection of maghemite nanoparticles [7,8]. In this study, since only a few studies have dealt with the separation techniques combined with SAXS, we focused our attention on the evaluation of the SEC–SAXS measurement system by further protein structural characterizations. The size and zero-angle scattering intensity of protein molecules eluted from the SEC column were estimated using the present SEC–SAXS measurement system. In addition, the characterization of the conformation of the eluted protein molecules, such as hen egg lysozyme and bovine submaxillary mucin, was demonstrated by the analyses of the X-ray scattering patterns obtained in this SEC–SAXS system.

2. Materials and methods

The purified bovine submaxillary mucin was purchased as type I-S from Sigma–Aldrich, St. Louis, MO, USA. The bovine serum albumin and hen egg lysozyme were purchased from Nacalai Tesque Inc., Kyoto, Japan. All other chemicals were of analytical grade.

The high-performance liquid-chromatography system was equipped with a sample loop having an internal volume of 300–700 μL, SEC columns and the following sequential detectors, a differential refractometer (RI-2031, Japan spectroscopic Co. (JASCO), Tokyo, Japan) and/or a ultraviolet spectrophotometer (AC-5100S, ATTO, Tokyo, Japan). The differential refractometer was used

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for the protein with a low absorbance at 280 nm, such as the bovine submaxillary mucin. The SEC columns were a main column (GF-7M HQ, 300 mm × 7.5 mm, Shodex, Tokyo, Japan) equipped with a guard column (GF-1G 7B, 50 mm × 7.5 mm, Shodex) for the analysis of bovine submaxillary mucin, or a main column (Superdex200 prep grade, 600 mm × 16 mm, GE Healthcare, UK, Ltd., Buckinghamshire, England) for the analyses of other proteins. The flow rates for the GF columns and Superdex column were 0.35 and 0.7 mL/min, respectively. The applied protein concentration varied from 1.5 to 3% (w/v). The eluent was a 50 mM sodium phosphate buffer, pH 6.9. The protein concentrations were spectrophotometrically determined using the following proteins with known extinction coefficients at 280 nm (mL/mg, for a 1-cm light-path): bovine serum albumin (0.678), ovalbumin (0.735), soybean trypsin inhibitor (0.95) and hen egg lysozyme (2.69) [9].

The synchrotron radiation SAXS measurements were performed with an optics system at the beamline BL-10C station in the Photon Factory of the High Energy Accelerator Research Organization as previously described [10–13]. A wavelength (λ) of 0.1488 nm was used. The temperature of the flow cell with a 1-mm light path and a pair of 20-μm quartz windows was kept constant at 24 °C using a metallic cell holder through which constant temperature water was circulated. The cell volume varied from 15 (3 mm × 5 mm) or 30 μL (3 mm × 10 mm). The scattering data of the elution were collected for 5 min with a position sensitive proportional counter at a sample to detector distance of 980 or 1980 mm. The obtained signals were corrected for solvent scattering and normalized to the beam intensity to yield the net scattering intensity $I(q)$, where q (=$(4\pi/\lambda)\sin\theta$, 2θ is the scattering angle) is the scattering vector. The observed q-range was between ~0.06 and ~3.0 nm^{-1}. The characteristic of the collagen was evaluated using a laboratory-type small-angle X-ray scattering measurement apparatus (M18X, MAC Science, Yokohama, Japan). The theoretical scattering pattern of the hen egg lysozyme was estimated using the Debye formula [14,15] and the atomic coordinates obtained from the Protein Data Bank (code: 6LYS).

3. Results and discussion

Fig. 1 shows the schematic drawing of the SEC–SAXS measurement system. As shown in the figure, the usual high performance liquid-chromatography system was combined with synchrotron radiation solution small-angle X-ray scattering measurement equipment, absorbance and/or refractive index detectors. Fig. 2 shows the typical X-ray scattering patterns of bovine serum albumin used in this system. The sample was equilibrated with the

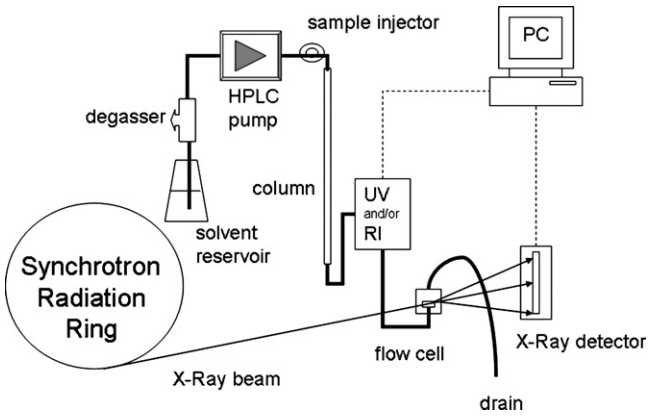

Fig. 1. Schematic drawing of SEC–SAXS measurement system.

solvent buffer solution using SEC columns. Therefore, the patterns show the net scattering of the protein after correcting for the solvent scattering. The scattering intensity in the small-angle regions ($q < 0.5\ nm^{-1}$) increases from 60 min. The scattering curves were analyzed using the Guinier approach [5]. The low q-region of the scattering curves was approximated by

$$I(q) = I(0)\exp\left(\frac{-q^2R_g^2}{3}\right),$$

where $I(0)$ is the scattering intensity at the zero scattering angle and R_g is the radius of gyration. The plot of $\ln(I(q))$ *versus* q^2 yields a straight line, the slope of which gives the R_g value. Fig. 3 shows the Guinier plots (q^2 *vs.* $\ln I$) of the scattering data of the eluted proteins. All the curves in the region of $q^2 < 0.15\ nm^{-2}$ were well approximated by a straight line, while the low-q limit of the Guinier rule ($qR_g < 1$) was $q^2 < 0.02$–$0.11\ nm^{-2}$. No clear sign of significant aggregation, as would be indicated by an upward deviation from the straight line fit in the small-angle region, is observed. The R_g values were estimated using the data in the region of the Guinier rule. Fig. 4 shows the typical chromatograms of the SEC–SAXS system for bovine serum albumin. The aggregate peaks were observed at 58–68 min in the tracing of the ultraviolet absorbance and scattering intensities. Protein species were eluted between 71 and 101 min. Since the zero-angle scattering intensity of the SAXS data is related to the molecular weight of the solutes in solution, the molecular weights of the eluted proteins were estimated using another static measurement data of the bovine serum albumin monomer purified as a standard sample. The molecular weights

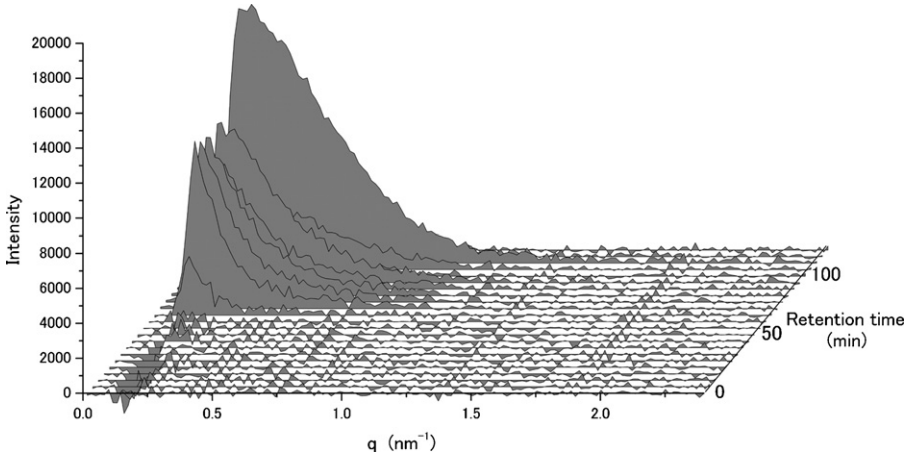

Fig. 2. Typical scattering patterns of bovine serum albumin using SEC–SAXS measurement system.

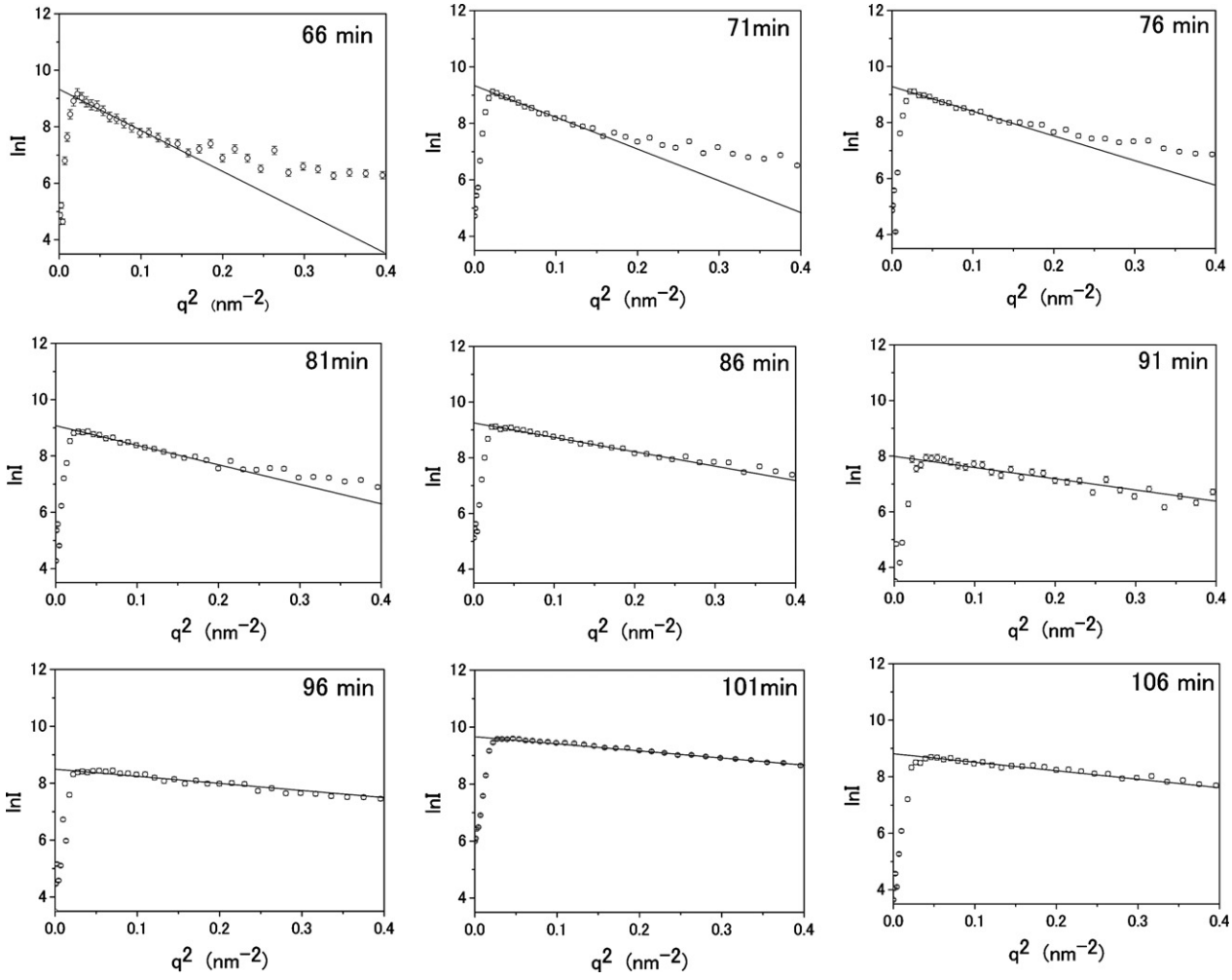

Fig. 3. Guinier plots of the scattering data of the eluted protein for bovine serum albumin. The elution time is shown in each figure. Some error bars are not visible within the symbols.

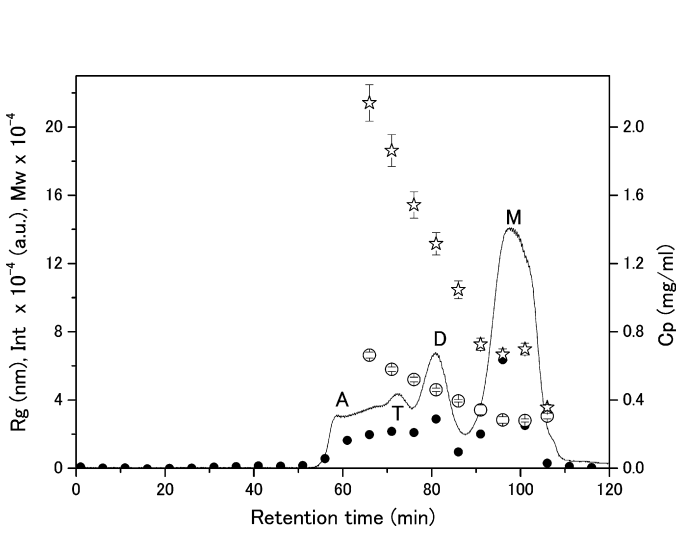

Fig. 4. Typical chromatograms of SEC–SAXS measurement system for bovine serum albumin. The solid chromatogram was obtained using an ultraviolet spectrometer (280 nm). The absorbance was converted to the protein concentration. The filled circles, open circles, and star symbols are the total X-ray scattering intensities, R_g values and molecular weights, respectively. A, T, D, and M are the position of the aggregates, trimer, dimer, and monomer of the bovine serum albumin.

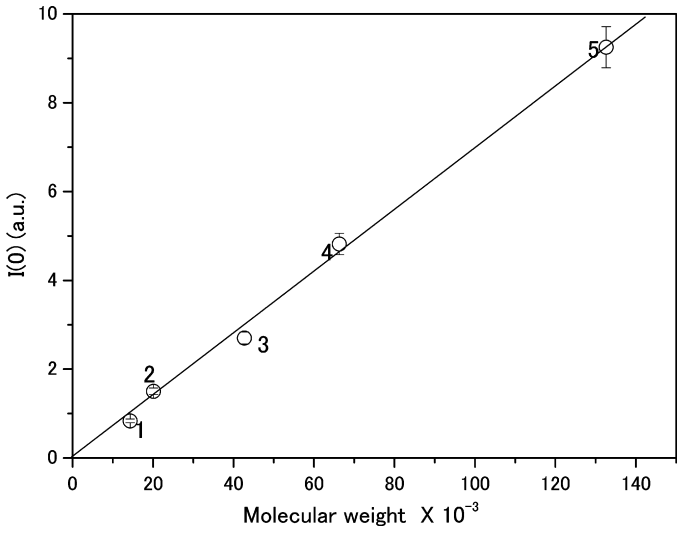

Fig. 5. Relationship between the value of the scattering intensity at the zero scattering angle ($I(0)$) and the molecular weight of the proteins. The number next to each symbol corresponds to (1) the hen egg lysozyme (14 300), (2) trypsin inhibitor (20 100), (3) ovalbumin (42 700), (4) bovine serum albumin (66 300), and (5) bovine serum albumin dimer (132 600). The molecular weight values were obtained from the literature [9].

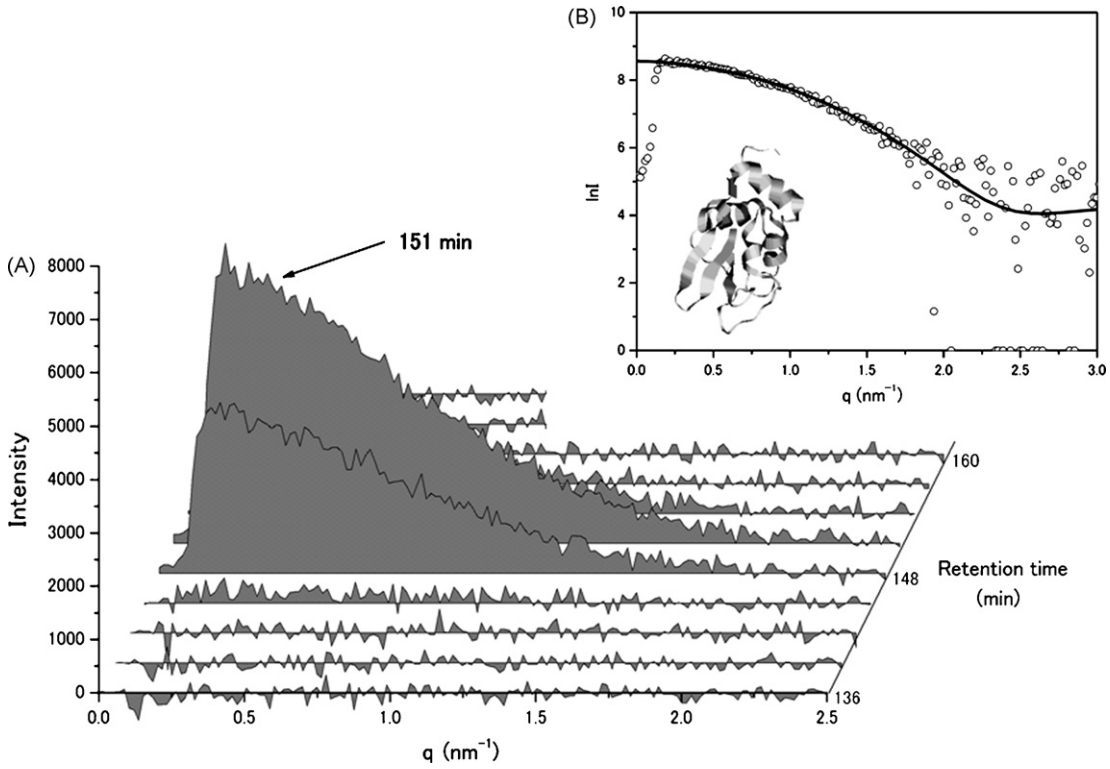

Fig. 6. Characterization of the conformation of hen egg lysozyme. (A) X-ray scattering patterns, (B) relationship between the theoretical scattering curve (the solid line) and experimental curve (open circles) of the hen egg lysozyme. The crystal structure of the protein is shown in the figure. The error bars are not visible within the symbols.

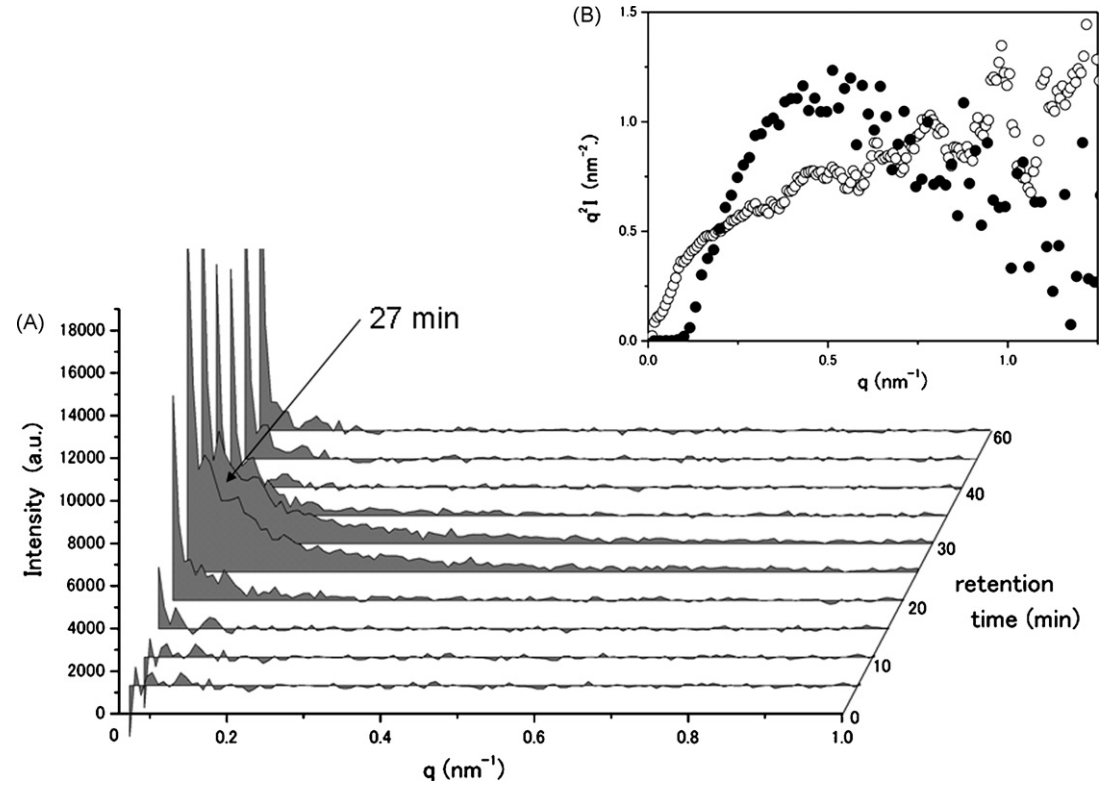

Fig. 7. Characterization of the conformation of bovine submaxillary mucin. (A) X-ray scattering patterns, (B) Kratky plots (q *vs.* q^2I) of bovine serum albumin monomer (filled circles) and the mucin eluted at 27 min (open circles). The error bars are not visible within the symbols.

profile in the peak region of the species shows the trimer, dimer, and monomer of the bovine serum albumin. The molecular weights and R_g values at the peak apex at 101 min for three independent experiments averaged 67 000 ± 2000, and 2.9 ± 0.1 nm that correspond to that of the bovine serum albumin monomer [9] when the protein concentration was about 1 mg/mL. In the previous study on the liquid-chromatography-coupled SAXS for sizing of aggregating proteins [6], the R_g value of bovine serum albumin was also estimated to be 3.0 nm, which is comparable to the present result of the bovine serum albumin monomer. Fig. 5 shows the relationship between the value of the scattering intensity at a zero scattering angle and the molecular weight of some proteins at about 1 mg/mL protein concentrations. The linear plot confirms the reliability of this technique for the molecular weight determination of proteins in solution. It is also advantage of this measurement system that the flow of the sample solution prevents radiation damage to the sample.

In addition, as a preliminary demonstration, the conformation of the eluted protein molecules, such as hen egg lysozyme and bovine submaxillary mucin, was characterized by analysis of the X-ray scattering patterns obtained in this system. Fig. 6A shows the X-ray scattering patterns when the hen egg lysozyme was applied to the SEC–SAXS system. The main fraction, that is the monomeric lysozyme, was eluted at 151 min. Fig. 6B shows the relationship between the theoretical scattering curve (the solid line), which was estimated using the Debye formula [14,15] and the atomic coordinates obtained from the Protein Data Bank (code: 6LYS), and experimental scattering curve (open circles) at 151 min. The overall character of the experimental scattering patterns was confirmed by the scattering patterns calculated for the crystal structure of the protein, while the detailed analysis of the large q-region requires for further experiments in the future. Moreover, Fig. 7A shows the X-ray scattering patterns for application of the bovine submaxillary mucin. The mucin contains a large number of oligosaccharide side chains covalently bound to a protein backbone. The macromolecule typically contains 60–90% (w/w) carbohydrate. The oligosaccharides may contain about 20 monosaccharides. The molecular weight of the bovine submaxillary mucin is about 2 million [12]. Fig. 7B shows the Kratky plots (q *vs.* q^2I) [5] of the bovine serum albumin monomer (filled circles) and the mucin (open circles) at the peak apex for each chromatogram. The Kratky plots are related to the protein configuration [5]. The peak (around $q = 0.4\ \mathrm{nm}^{-1}$) for the bovine serum albumin monomer, which the scattering pattern at 101 min in Fig. 2, is typical of globular molecules. On the other hand, the Kratky plot of the bovine submaxillary mucin shows the Gaussian (random coil) chain conformation.

In conclusion, the SEC–SAXS technique will be useful for the determination of the R_g value [6] and molecular weights of the separated protein molecules with dimensions of 1–10 nm in solution. Furthermore, this measurement system will be also useful for the structural characterization of the proteins and other macromolecules, such as polysaccharides and synthetic polymers, during chromatography. Although the potentiality of the present method depends on the performance of the SAXS instrument, the further development of this method will be important for the future biological and material science.

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