Applications of Size Exclusion Chromatography with Low-Angle Laser Light Scattering Detection to Proteins, Including Somatotropins†

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Size exclusion chromatography with low-angle laser light scattering detection (SEC/LALLS) has been applied to the separation and characterization of a number of proteins as a function of molecular weight using different SEC columns and elution buffers. The use of a LALLS detector in conjunction with a concentration detector eliminated the need for calibration of the SEC column. Data acquisition and analysis were under computer control. The technique has been demonstrated to determine molecular weights for a variety of proteins, including natural bovine and natural porcine somatotropins. The LALLS detector combined with an SEC column was effective in determining an accurate molecular weight even under conditions when the SEC columns' separation was not strictly size-dependent. Applications of the technique to protein samples that contain dimers and higher order aggregates, i.e., samples which may arise in protein stability and formulation studies, will be discussed.

INTRODUCTION

Size exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), is widely employed in the characterization and separation of polymers as a function of their molecular weight or hydrodynamic volume (Provder, 1984; Chuang and Johnson, 1974; Ouano, 1981). Molecular weight determination using SEC generally requires calibration of the column by obtaining elution volumes of polymers of well-defined molecular weight. This calibration procedure may be eliminated by using a low-angle laser light scattering (LALLS) detector to determine molecular weight directly. Studies in the literature have documented the use of SEC/LALLS to characterize molecular weight distribution of polymer systems in organic solvents. Recent advances in SEC/LALLS have led to the development of computer programs that can control data acquisition as well as simplify data reduction (Provder, 1984).

The weight-average molecular weight of a macromolecule, which is the value determined by a light scattering detector, has been defined as

$$M_w = \sum(M_i^2 c_i)/\sum(M_i c_i) \quad (1)$$

where M_i is the molecular weight of each of the fractions of a mixture, as may be encountered in synthetic polymers (Meyer, 1950). For proteins, absenting fragmentation, one would sum over fractions of monomer, dimer, etc. Thus, without SEC separation, the highest molecular weight fraction would dominate, as in eq 1, and LALLS would yield only an overall weight-average molecular weight.

Following the advent of SEC columns capable of separations in aqueous media, studies of polymers and proteins (Fukano et al., 1978; Rollings et al., 1983; Muller et al., 1984; Maezawa and Takagi, 1983; Kopaciewicz and Regnier, 1983; Frigon et al., 1983; Leypoldt et al., 1984; Flapper et al., 1986) including bovine somatotropin (bST) (Stodola et al., 1986) by SEC have been reported in the literature. LALLS detectors were employed in some of the studies (Maezawa and Takagi, 1983; Miklautz et al., 1986; Mhatre et al., 1990). The results obtained from investigations of synthetic polymers in organic solvents suggest that the use of a computer-controlled SEC/LALLS system ought to yield information on absolute molecular weights of proteins and, more importantly, the molecular weight distributions in cases of aggregate formation in somatotropins (Stodola et al., 1986).

The present paper discusses the results obtained from an SEC/LALLS study with computer-controlled data acquisition and analysis of various proteins including natural bovine somatotropin (nbST) and natural porcine somatotropin (npST). Subsequent to the original presentation of this material, SEC/LALLS studies of natural and human somatotropin were reported (Stuting and Krull, 1991). The implications of the SEC/LALLS results obtained for the ST samples to the monitoring of stability studies and to formulation testing are explored.

THEORY OF LALLS

The light scattering detector utilizes the principle that the intensity of light scattered elastically by a molecule (Rayleigh scattering) is directly proportional to the molecular weight. Additionally, data on macromolecular size and shape may be extracted from the Rayleigh scattering (Chu, 1974; Kratochvil, 1987). A detailed description of the theory of light scattering as determined by the model of LALLS instrument employed in this investigation has been given elsewhere (MacRury and McConnell, 1979; Mhatre et al., 1990). A brief summary of the theory follows.

The mathematics which relate the signal of the LALLS detector, the excess Rayleigh factor, to a macromolecule's molecular weight is given by

$$M_w = (Kc/R_\theta - 2A_2c)^{-1} \quad (2)$$

where M_w is the weight-average molecular weight, c is the solute concentration (milligrams/liter), K is the polymer optical constant (see eq 3), R_θ = (Rayleigh scattering of the solution − Rayleigh scattering of the solvent), and A_2 is the polymer second virial coefficient. The polymer optical constant K is defined by

$$K = Bn^2(dn/dc)^2/\lambda^4 N \quad (3)$$

$$B = 2\pi^2(1 + \cos^2\theta')$$

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where $\cos\theta'$ is the cosine of the scattering angle corrected for solvent refractive index, λ is the laser wavelength (632.8 nm), N is Avogadro's number (6.023×10^{23}), n is the refractive index of the solvent, and dn/dc is the differential refractive index increment.

The quantities defined in eqs 2 and 3 are such that they may be obtained from the LALLS instrument, i.e., Rayleigh scattering, or they may be measured independently of the experiment, i.e., A_2, n, dn/dc. The instrumental parameters influencing the Rayleigh factor are only dependent upon scattering angle and cell type and are a function only of the LALLS geometry, which remains unchanged during the course of an experiment. When the LALLS detector is used in conjunction with a UV detector, the injection mass of the protein and the flow rate of the solvent are the only parameters needed for the calculation of molecular weight at each point on the elution curve. The calculation is performed by the computer program MOLWT3 (Chromatix/LDC Milton Roy Co., 1985) using eq 2, where c, the concentration term, is derived from a normalized UV detector response.

EXPERIMENTAL PROCEDURES

In this investigation the GPC or SEC chromatograph employed dual Toya-Soda TSK gel TSK G3000SW;G2000SW columns or a Pharmacia Superose 12 column. In the TSK column system, a TSK SW guard column preceded the separating columns.

The solvent system for the TSK gel columns was 0.14 M NaCl/0.010 M phosphate buffer, pH 6.9. Studies have shown the TSK gel column to be effective in the SEC separation of proteins (Fukano et al., 1978; Maezawa and Takagi, 1983; Stuting and Krull, 1991). One of the limitations of the TSK gel columns is the upper pH limit of ~7.5. For this reason, the Pharmacia column, which has a wider pH range, was also employed. The Pharmacia Superose 12 column used a solvent system of 0.025 M carbonate/bicarbonate buffer (CB), pH 9.85, or 0.1 M tris(hydroxymethyl)aminomethane, pH 7.5.

The columns were connected to a Spectra Physics 8800 ternary gradient HPLC pump. The Spectra Physics 8800 pump was equipped such that wetted parts contacted only inert Teflon or titanium, minimizing metal contamination of proteins. Proteins were manually injected onto the column(s) through an inert Rheodyne injector equipped with titanium injection loops. Flow rates of solvent were 0.5 mL/min.

The solution eluting from the column(s) passed first through a Chromatix KMX-6 LALLS equipped with an HPLC flow cell and then to an Hitachi Model 100-40 spectrometer equipped with a low-volume flow-through cell. A scattering angle of 6–7° and a field stop of 2 mm were used for all light scattering determinations. Quantitation of protein was accomplished by obtaining UV absorbance at 280 nm. Ease of use and availability were the reasons for employment of the UV detector. At other times, detection of protein was also accomplished by a McPherson FL-750 HPLC Plus spectrofluorescence detector. Fluorescence of protein was excited at 280 nm and detected at 314 nm.

The analog signals from the LALLS and spectrometer were simultaneously fed to a Kipp & Zonen two-pen chart recorder and to a Chromatix CMX-10 A analog-to-digital converter. The digital signal from the CMX-10A was sent to a Digital Equipment Corp. Micro PDP-11/23 computer running MOLWT3.

Samples of proteins were dissolved in filtered buffer. Dissolution was hastened by use of a magnetic stirrer. Protein solutions were filtered through either Millex GV 0.2-μm or Millex VV 0.1-μm filters prior to injection. If long-term storage of protein solutions were necessary, solutions were stored at 277 K. The concentrations of the protein solutions varied from 1 to 2 mg/mL.

Proteins other than somatotropins were obtained from Sigma Chemical Co. (bovine serum albumin, ovalbumin, soybean trypsin inhibitor). The nbST and npST were obtained from A. F. Parlow, UCLA Medical Center, Torrance, CA. The solid somatotropin samples had been stored at temperatures less than or equal to 277 K for times ranging from 1 to 5 years.

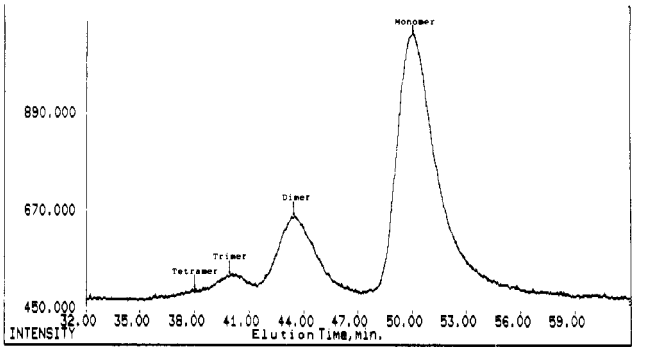

Figure 1. LALLS detector trace for SEC of BSA on dual TSK gel columns. Peaks are marked for monomer, dimer, trimer, and tetramer.

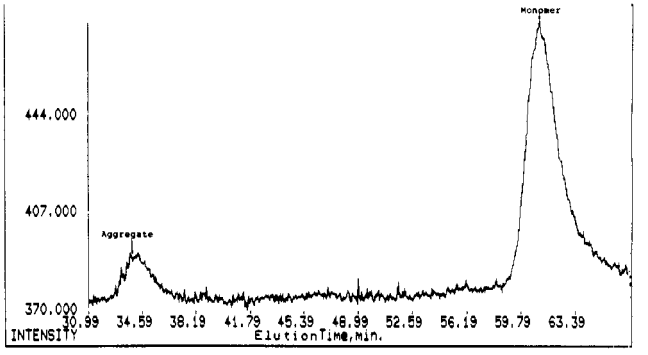

Figure 2. LALLS detector trace for SEC of soybean trypsin inhibitor. Peaks are marked for monomer and oligomer.

EXPERIMENTAL RESULTS

SEC runs on various proteins using dual TSK gel columns resulted in excellent separations. For instance, the LALLS trace for an injection of bovine serum albumin (BSA) is shown in Figure 1. Note that four peaks may be discerned. These four peaks, in order of increasing elution time, correspond to tetramer, trimer, dimer, and monomer. As part of the combined LALLS and UV data analysis by MOLWT3, a running value of M_w at each point of the elution profile of the chromatogram may be generated. Examination of the M_w data for the BSA run shown in Figure 1 yielded values of M_w at the apices of the four peaks of 250 000, 198 000, 133 000, and 66 000 for tetramer, trimer, dimer, and monomer, respectively. The units of all M_w values are daltons. For the entire chromatogram, M_w was 73 000. Data for ovalbumin and soybean trypsin inhibitor (STI) were also obtained.

The LALLS signal trace for STI, displayed in Figure 2, shows a strong peak excursion near 31 min, 15.5 mL, the columns' exclusion volume. The peak was absent from the UV scan. The peak position would correspond to a M_w greater than, or equal to, 500 000, which is the exclusion limit of the TSK 3000 SW column. The peak near 15.5 mL was observed with numerous samples of STI and always at the same position. No attempts were made to further characterize the 15.5-mL peak.

Dissolution of the ST samples in saline/phosphate buffer, pH 6.9, was difficult. Useful chromatograms were only obtained for nbST.

A comparison of the elution times of STI and nbST from the dual TSK gel columns operating at pH 6.9 was quite interesting. The UV traces for each chromatogram are displayed in Figure 3. Note that STI elutes before nbST under the conditions employed here. Thus, the assumption that the SEC separation is strictly a function of molecular size would be incorrect under these circum-

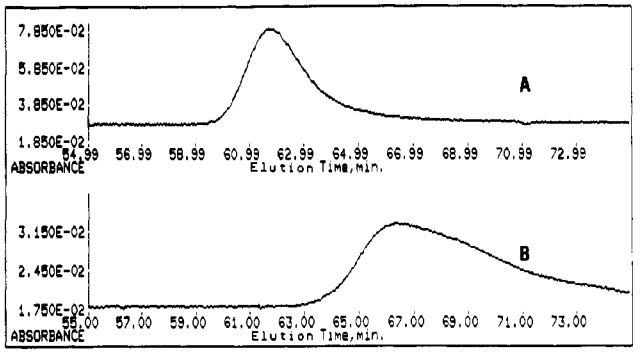

Figure 3. UV traces for SEC of soybean trypsin inhibitor (A) and nbST (B) on dual TSK gel columns.

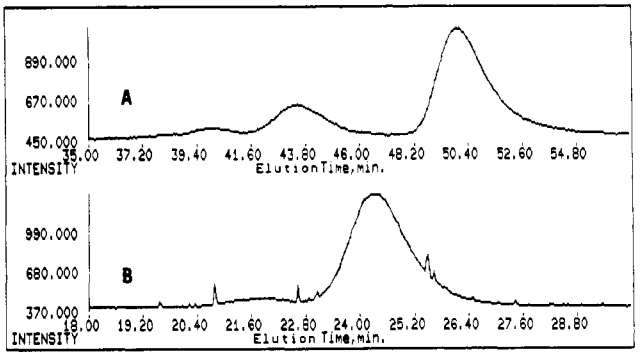

Figure 4. LALLS detector trace for SEC of BSA on dual TSK gel columns (A) and on a Superose 12 column (B).

Table I. Molecular Weights of Proteins in Saline/ Phosphate Buffer, pH 6.9, on Dual TSK Gel Columns or in Tris Buffer, pH 7.5, on a Superose 12 Column

molecule	M_w(literature)	M_w(LALLS)[a]
native bST,	21 600	22 600
soybean trypsin inhibitor	20 000	20 200
ovalbumin	44 500	42 800
bovine serum albumin, monomer	66 300	65 500
bovine serum albumin, dimer	132 600	133 000

[a] Standard deviation of triplicate runs was less than 10%. Units are daltons.

stances. However, the value of M_w obtained from the LALLS detector for each of the proteins was correct, i.e., STI ~20 000 nbST ~21 000, removing any uncertainties from the SEC separations. Overall values of M_w for the various proteins are summarized in Table I.

Excellent separation of proteins was obtained from the dual TSK gel columns, but the time involved to obtain a complete chromatogram approached 90 min. Also, no pertinent information on ST dimers was forthcoming from the dual TSK gel columns. The upper pH limit of 7.5 would preclude the use of basic buffers, and the tendency of ST to aggregate at basic pH has some relevance to stability and formulations (Stodola et al., 1986). For these reasons, a Pharmacia Superose 12 column was employed to examine the several proteins. Most proteins were eluted from the Superose 12 column within 45 min. The resolution of the Superose 12 column was less than that of the dual TSK gel columns, as can be seen from the comparison traces of BSA in Figure 4. However, the LALLS detector sensitivity to M_w served to overcome any decrease in resolution resulting from changing columns.

It is worthwhile to note that at pH 9.85 STI and ST elute at the same time (Figure 5); nonetheless, the M_w for each protein as obtained from the LALLS signal is correct, i.e., 20 000 for STI and 21 500 for ST. The values of M_w for the several proteins of interest are listed in Table II.

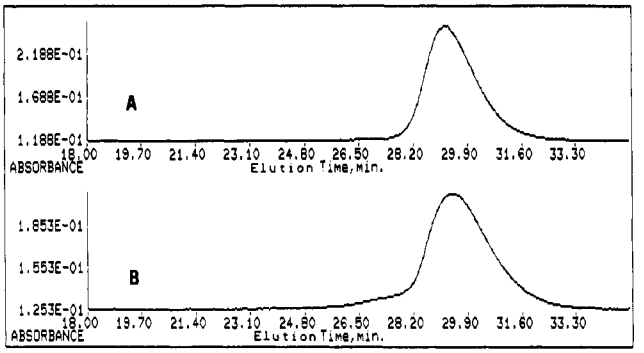

Figure 5. UV traces for SEC of soybean trypsin inhibitor (A) and nbST (B) on a Superose 12 column, 25 mM CB, pH 9.85.

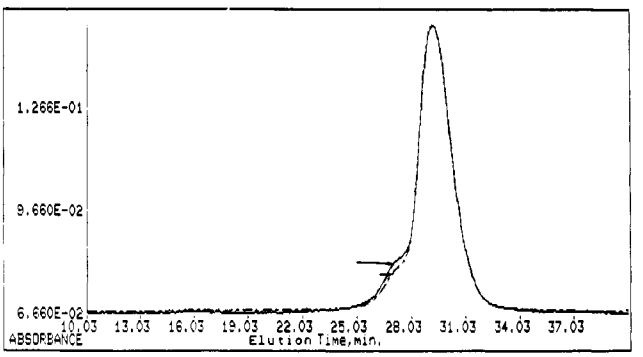

Figure 6. Overlapped UV traces for SEC on a Superose 12 column of nbST solutions in 25 mM CB, pH 9.85. Solid line represents solution after 7 months at 277 K. Dotted line is same solution after a further 9 days at 295 K.

Table II. Molecular Weights of Proteins in Carbonate/ Bicarbonate Buffer, pH 9.85, on a Superose 12 Column

molecule	M_w(literature)	M_w(LALLS)[a]
native bST, AFP 7500	21 600	22 600
native pST, AFP 5324C	21 600	22 000
soybean trypsin inhibitor	20 000	20 200
ovalbumin	44 500	42 800
bovine serum albumin, monomer	66 300	65 500
bovine serum albumin, dimer	132 600	133 000

[a] Standard deviation of triplicate runs was less than 10%. Units are daltons.

The LALLS trace for npST and nbST showed a strong peak at the column exclusion volume. Recall that such a peak was noted for STI in PBS (Figure 2). The molecular weight of the material eluting at the exclusion volume would be 300 000 or greater. A corresponding peak at the column exclusion volume was absent from the UV traces of the ST samples, again, as was the case for STI in saline.

Of interest as well is the increase in M_w of ST under basic conditions. Figures 6 and 7 show the UV and LALLS traces of chromatograms of a solution of nbST in 25 mM CB, pH 9.85. The ST solutions had been prepared in CB and stored at 277 K for 7 months. The two chromatograms were obtained before and after the solution was allowed to remain at 295 K for 9 days.

Note the differences in the appearances of the LALLS and UV traces—there are three peaks in the LALLS traces, while the UV trace shows only a peak with a shoulder. The prominent peak in the UV trace, near 29 min, correspond to elution time for monomer, while the shoulder near 27 min corresponds to dimer. Note that the overlapped UV trace (Figure 6) shows a small increase in the dimer peak over the 9-day time period. The LALLS trace has more clearly defined monomer and dimer peaks near corresponding elution times, and in addition, there is a strong peak near the 15-min elution time. Note the obvious growth

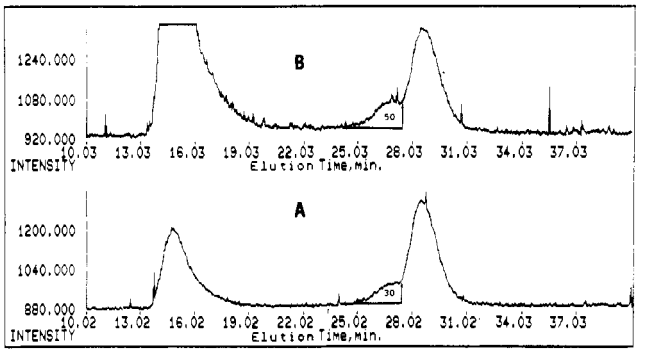

Figure 7. LALLS traces for SEC on a Superose 12 column of nbST solutions in 25 mM CB, pH 9.85. These traces are from the same SEC as the UV traces in Figure 6. The trace marked B corresponds to the solution after 7 months at 277 K. The trace marked A corresponds to the solution in (A) after being held a further 9 days at 295 K. The numbers under the curves are values of LALLS intensity.

in the 15-min peak, and note that the increase in dimer is more evident from the LALLS trace in Figure 7 than it is from the small increase in the UV trace.

The 15-min peak would correspond to a low concentration of material with M_w greater than 300 000. From the intensity differences in the two traces, the amount of this aggregate has approximately doubled. This aggregation tendency is greater for bST and pST than for human somatotropin (Seely et al., 1991). Analysis of the data using MOLWT3 yielded M_w for the 25-min peak of ~42 000 and M_w for the 29-min peak of ~21 000. The data analysis required each peak to be analyzed separately, with the protein injection amount weighted by the height of the UV peak. Protein elution time did not enter into the calculations.

DISCUSSION

The preceding results have shown that the use of LALLS with SEC can quickly and accurately separate proteins and determine their molecular weight without the necessity of column calibration. The use of LALLS detection with computer-controlled data acquisition and analysis is particularly applicable to problems of aggregation in proteins, whether one is employing high-performance liquid chromatographic separations in a gradient (Mhatre et al., 1990) or isocratic SEC, as in the present investigation or that of Stuting and Krull (1991).

The SEC/LALLS employed in the present study required no precharacterization of the protein(s) being studied. The only parameter involved in data processing directly related to the protein was the polymer constant, K, which has been defined in eq 3. For most globular proteins, dn/dc is nearly constant (Chu, 1974; Kratochvil, 1987), absenting strong salt effects (that is, high salt concentrations or high concentrations of denaturants). The value of dn/dc for most biological macromolecules is 0.19 mL/g at 632.8 nm (Chu, 1974). Thus, one may analyze the experimental data without recourse to the physical properties of the protein of interest.

In the present investigation the dn/dc value for nbST at 632.8 nm in saline/phosphate buffer was determined as 0.188 mL/g. The value was determined on a Wood's differential refractometer. A value of dn/dc close to this, 0.166–0.175 mL/g, was reported for natural and recombinant human somatotropins in saline/phosphate buffer by Stuting and Krull (1990). For all calculations in the present work a value of 0.19 mL/g was assumed for dn/dc.

Another prime consideration for the use of the LALLS detector is that its signal is directly proportional to the mass of protein (see eq 2), unlike the signal from the typical UV detector near 280 nm, which is sensitive to the number of aromatic residues.

A second point deriving from the fact that the response of the LALLS detector is proportional to protein mass is that a reasonable estimate of protein concentration may be derived from the M_w value calculated from a chromatogram. An assumed injection mass higher or lower than that actually injected will yield a value of M_w lower or higher than expected. As a check of purity, the LALLS signal response is as reliable as the traditional response factor method employed in most non-LALLS SEC.

This paper has demonstrated that direct determination of molecular weight of proteins by SEC/LALLS may be accomplished without the calibration of columns and without detailed knowledge of protein physical constants. The use of a sensitive UV detector with the KMX-6 LALLS detector directly yields the molecular weight and removes the uncertainties that may arise from column effects involved in non-LALLS SEC or GPC. SEC/LALLS can be a valuable technique in the study of the aggregation properties of porcine and bovine somatotropins.

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