Journal of Chromatography, 591 (1992) 139–148
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 794

Interfacing gradient elution ion-exchange chromatography and low-angle laser light-scattering photometry for analysis of proteins

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(First received July 10th, 1991; revised manuscript received October 8th, 1991)

ABSTRACT

Molecular weights (MWs) of different proteins were determined by interfacing gradient elution ion-exchange chromatography and low-angle laser light-scattering photometry (IEC–LALLS). A high-performance strong cation-exchange column was used to elute proteins using fast (5 min) and conventional (15–30 min) gradients. The eluted proteins were characterized on-line by determining their MWs using LALLS. The specific refractive index (RI) increment (dn/dc) and the RI of the solvent used over the gradient range were determined off-line and used to calculate the absolute weight-average MWs. Four proteins, ribonuclease A, α-chymotrypsinogen A, trypsinogen and β-lactoglobulin A (β-LACT) were studied. Accurate MWs were obtained for all the proteins using fast and conventional gradients, except for β-LACT, which aggregated as a function of the gradient employed. The degree of aggregation of β-LACT increased as the rapidity of the gradient was increased over a fixed gradient range. This study indicated that it is possible to separate and characterize proteins rapidly using IEC–LALLS.

INTRODUCTION

The separation of biopolymers, especially proteins, has gained significant advantage in the past decade or so [1–6]. The development of high-performance liquid chromatography (HPLC) for biopolymers has been due to the development of supports specially designed for biopolymer separation, and understanding the chromatographic behavior of different biopolymers. In spite of the advances in column chemistry, no great importance has been given to characterizing or identifying proteins separated by HPLC. Most of the detection approaches, such as UV, fluorescence and refractive index, provide insufficient information for characterizing biopolymers. Mass spectrometry is the only detection technique that has been able to provide qualitative information in terms of molecular weight (MW), but it is not currently practical for the routine MW determination of high-MW biopolymers.

We have been interested in interfacing low-angle laser light-scattering photometry (LALLS) for on-line detection for HPLC to characterize proteins by determining their absolute MW [7–11]. LALLS had been used for this purpose in the past few years, but only under isocratic conditions, and mainly for size-exclusion chromatography (SEC) [12–16]. Over the past few years, we have succesfully interfaced gradient reversed-phase (RP) and hydrophobic interaction chromatography (HIC) with LALLS. This encouraged us to look at interfacing gradient elution ion-exchange chromatography (IEC).

High-performance IEC has not been used as extensively as RP chromatography or SEC for the separation of proteins. However, as the mass recovery is very high and the biological activity of the protein is maintained in IEC, it has become an important tool for the biotechnology industry. Many research groups have, over the past few years, developed various high-efficiency IEC columns and

0021-9673/92/$05.00

have also concentrated on understanding the retention mechanisms in IEC [17–21]. Fast IEC separations of proteins have been accomplished using non-porous packings [22,23]. Recently, Lloyd and Warner [24] demonstrated the use of wide-pore polymer matrix columns, which have been able to separate proteins very rapidly with high resolution. The columns have also been shown to have a higher loading capacity and rigidity than non-porous columns. The introduction of rigid polymer-based columns has allowed the use of high flow-rates and steep gradients for rapid separations of proteins.

In this paper, we demonstrate the coupling of a wide-pore, polymer-based, strong cation-exchange column for on-line LALLS–UV detection. Our previous attempts at interfacing RP-chromatography and LALLS had indicated that aggregates of proteins were formed on-column as a function of the rapidity of the gradient employed for elution. We have tried to investigate whether similar aggregates would be formed under rapid IEC elution conditions. Detailed discussions of the equations and theory of LALLS can be found elsewhere [8,25–28].

EXPERIMENTAL

Apparatus

The HPLC–LALLS–UV system was obtained from LDC Analytical (Riviera Beach, FL, USA), and consisted of a Model CM4000 ternary gradient pump, a Model SM4000 variable-wavelength UV detector and a Model KMX-6 LALLS detector. The injector was a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading type, fitted with a 20-μl loop. The ion-exchange column was a 10-μm HP-SCX strong cation-exchange column (100 × 7.8 mm I.D.) (Interaction Chemicals, CA, USA).

Three different instrumental set-ups used in this work were an IEC–HPLC system with LALLS–UV detection, an SEC system with LALLS–UV detection and a flow-injection analysis (FIA) system connected to a LALLS detctor.

System I, used for the gradient IEC–HPLC, consisted of a CM4000 gradient system, KMX-6 LALLS detector and an SM4000 UV detector, in series. The detector signals were collected on a Soltec (Sun Valley, CA, USA) Model 1242 strip-chart recorder and an IBM compatible computer using PCLALLS data collection and processing software from LDC Analytical.

System II, used for SEC studies, consisted of an LDC ConstaMetric III metering pump and an injector fitted with a 100-μl injection loop, a TSKgel G4000 SW column (30 cm × 8 mm I.D.) (Phenomenex, Ranchos Palos Verdes, CA, U.S.A.) and an LDC SpectroMonitor-D variable wavelength UV–VIS detector. The remaining experimental set-up was similar to system I.

System III, used for FIA, consisted of a 2-ml injection loop and 1/8-in. narrow-bore tubing of 12 in. length connected between the injector and the LALLS instrument. The LALLS output was collected on a Soltec recorder.

Refractive index (RI) increment (dn/dc) measurements were made in an off-line mode using a KMX-16 633-nm laser-based differential refractometer. RIs of the mobile phases were measured using a Bausch and Lomb (Rochester, NY, USA) Model 3L Abbé refractometer with a 632.8-nm narrow bandpass filter (Melles Griot, Rochester, NY, USA).

Mobile phases

Three mobile phases were used. The first was 5 m*M* Bis-Tris, (pH 5.8) (A) and 5 m*M* Bis-Tris + 0.5 *M* NaCl (pH 5.8) (B). This mobile phase was used to elute α-chymotrypsinogen A (CHY), trypsinogen (TRP) and ribonuclease A (RNase). The second was 10 m*M* sodium phosphate (monobasic) (pH 4.0) (A) and 10 m*M* sodium phosphate (monobasic) + 0.5 *M* NaCl (pH 4.0) (B), and was used to elute β-lactoglobulin (β-LACT). The third was a modified protein buffer (MPB) and was prepared as described [9]. All mobile phases were filtered through a 0.2-μm filter (Millipore, Bedford, MA, USA) and degased before use. The column was equilibrated with mobile phase A (protein dependent) overnight at a flow-rate of 0.2 ml/min.

Chemicals and supplies

HPLC-grade water was purchased from EM Science (Gibbstown, NJ, USA) and Bis-Tris, dibasic sodium phosphate and sodium chloride from Aldrich (Milwaukee, WI, USA). All protein standards were purchased from Sigma (St. Louis, MO, USA). Proteins were used as received.

Procedures

IEC–LALLS–UV studies. Fresh mobiles phases were prepared for each study. Protein standards in

the concentration range 8–10 mg/ml were prepared in 5 m*M* Bis-Tris, except β-LACT, which was prepared in 10 m*M* monobasic sodium phosphate, and refrigerated for about 1 h prior to use. Samples were kept refrigerated between each injection. The mobile phase flow-rate used throughout was 1 ml/min. The UV detector was set at 280 nm and 0.2 a.u.f.s. To study the effect of gradient times (over a fixed gradient range) on MWs of proteins, three gradients of 5, 10, and 15 min were run. Gradient ranges for the elution of individual proteins were as follows: TRP was eluted using a gradient from 5 to 75% B, α-CHY was eluted using a gradient from 15 to 100% B, RNase was eluted using a gradient from 0 to 100% B and a gradient from 0 to 80% B was used for β-LACT.

FIA–LALLS. FIA–LALLS studies were done using protein concentrations in the range 0.25–5.0 mg/ml. Proteins were dissolved in mobile phase A, which was also the mobile phase used for elution. The plots generated from the FIA–LALLS studies were used to calculate the second virial coefficient (A_2), which was eventually used to calculate the MW by IEC–LALLS.

SEC–LALLS–UV studies. System II described under *Apparatus* was used for this study. Protein concentrations ranging from 5 to 10 mg/ml prepared in buffer A were injected. Buffer A was also the mobile phase. Proteins were eluted at a flow-rate of 0.7 ml/min. LALLS and UV peak areas were integrated for calculating the MW of individual proteins [8,25–28].

Calculation of refractive index (RI), specific refractive index increment (dn/dc) and percentage recovery. RI of the mobile phase was measured over the entire gradient range (0–100% B) using an Abbé refractometer. The RI was measured to determine changes in RI over the gradient range used for elution of proteins. For gradient elution to be coupled successfully with LALLS, the two solvents (A and B) have to be "isorefractive"; solvents are considered to be isorefractive if the difference in their RI is $\leqslant 0.025$ units [27]. The differences in the RI of the two buffers was found to be 0.0045, which is much below the limit for isorefractivity. To measure the RI, different concentrations of B and A were prepared with that of B increasing by 10% over the 0–100% B range. The solutions were prepared in volumes of 10 ml. RI measurements were made by following standard operating procedures outlined in the operator's manual.

The d*n*/d*c* measurements were made using a KMX-16 differential laser-based (633-nm) refractometer. The refractometer was connected to a water-bath to maintain a cell temperature of 25°C. Protein solutions were prepared in buffer A and the concentrations ranged from 1 to 5 mg/ml. In previous studies we had determined d*n*/d*c* values at the point of elution of the protein [7,9]. In this particular study, as the *RI*s of the solvents were very close, d*n*/d*c* measurements were determined in buffer A. This assumption was based on calculation of d*n*/d*c* values at the point for β-LACT, which was about 30% B. The d*n*/d*c* values calculated by dissolving the protein in 30% B matched very well those using 100% A. Also, as most of the proteins analyzed seemed to elute at about or below 30–35% B buffer composition, it was safe to assume that the d*n*/d*c* values calculated in buffer A were accurate. Protein solutions were prepared and refrigerated for about 1 h prior to use. The solutions were then placed in the refractometer cell and 6–8 readings were determined for each solution; d*n*/d*c* values were calculated for 4–5 different concentrations of the protein.

The molar absorptivity of β-LACT was calculated on-line by FIA, using a B concentration at the point of elution in IEC, and was used to determine the percentage recovery as described in detail previously [10].

RESULTS AND DISCUSSION

The purpose of this study was primarily to determine the compatibility of interfacing gradient IEC with LALLS. To the best of our knowledge, there are no published reports on interfacing gradient IEC with on-line LALLS detection for determining the MWs of proteins. In our previous attempts, we were successful at interfacing gradient RP chromatography with LALLS for determining MWs on-line [9]. However, we noticed that all the proteins studied seemed to aggregate as a function of the gradient time over a fixed gradient range. At gradient times below 15 min, higher MWs were obtained, indicating the formation of aggregates. When longer gradient times were used (>15 min), the MWs obtained matched literatures values.

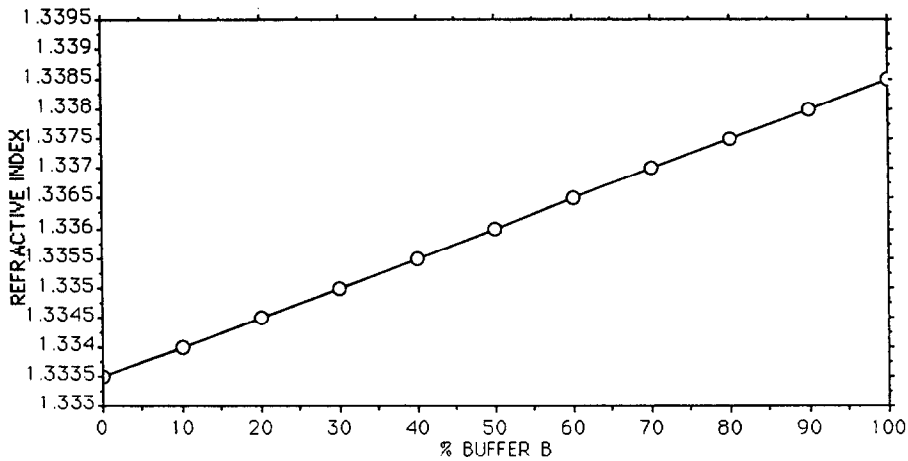

Fig. 1. Plot of refractive index *versus* percentage of buffer B. Mobile phase: (A) 5 m*M* Bis-Tris; (B) A + 0.5 *M* sodium chloride (pH 5.8).

Those results had indicated that RP separations using conventional means of detection may have led to the formation of higher MW species of proteins. However, this could not be determined as the precise nature of the eluting species was not known.

Based on the above results, we tried to study the effects of changes in gradient times in ion-exchange chromatography on the MWs of some commercially available proteins. In most of these studies, the gradient times used were 5, 10 and 15 min over a fixed gradient range. We report several studies done to determine the MWs of proteins, including (1) n and dn/dc calculations; (2) IEC–LALLS–UV for four commercially available proteins with MWs obtained at different gradient times; (3) SEC–LALLS–UV studies using MPB and phosphate buffer to calculate the MW of β-LACT; and (4) FIA–LALLS–UV studies to calculate A_2.

n and dn/dc determinations

Fig. 1 shows a plot of the change in RI over the gradient range 0–100% B (5 m*M* Bis-Tris + 0.5 *M* NaCl). The change in RI was minimal (0.0045), which satisfied the condition of isorefractivity. The change in RI for the buffer system used to separate β-LACT was also well below the isorefractivity limit of 0.025. These results indicated that gradient IEC could be interfaced with LALLS without any further corrections for RI changes.

Table I is a list of the calculated dn/dc values for the different proteins. As seen, all the dn/dc values fell between 0.16 and 0.17. These values were typical of what we have determined for various proteins in related studies [7–11].

IEC–LALLS–UV analysis of proteins

The purpose of this study was to determine if the rapidity of the gradient caused any alterations in the biopolymer, such as the formation of aggregates, as observed with RP gradients. RNase aggregated as a function of the rapidity of the gradient (percentage change in B per minute) when eluted using RP-HPLC–LALLS [9]. Mixtures of aggregates were obtained for RNase when a 10-min RP gradient was used, and the degree of aggregation

TABLE I

dn/dc VALUES OF DIFFERENT PROTEINS

Relative standard deviations (%) (n = 3) are given in parentheses.

Protein	dn/dc[a]
Trypsinogen	0.171 (1.75)
α-Chymotrypsinogen A	0.168 (0.8)
Ribonuclease A	0.162 (0.6)
β-Lactoglobulin A	0.166 (100% A) (1.3)
	0.164 (30% B–70% A) (0.7)

[a] dn/dc measurements were made in buffer A (5 m*M* Bis-Tris) for all proteins except β-LACT, where buffer A was 10 m*M* sodium phosphate (monobasic) and B was A + 0.5 *M* NaCl.

TABLE II

MOLECULAR WEIGHT DATA FOR PROTEINS OBTAINED BY IEC–LALLS

Mobile phase: A = 5 m*M* Bis-Tris, B = A + 0.5 *M* NaCl (pH 5.8). Column: HP-SCX, 10 μm, 100 × 7.8 mm I.D. Detection: UV at 280 nm, 0.2 a.u.f.s. LALLS: 0.2 mm field stop, 6–7° annuli, G_o = 400–425 mV [2–4]. Relative standard deviations (%) (n = 3) are given in parentheses.

Gradient time (min)	CHY	TRP	RNase
5	25 500 (4.7)	25 100 (4.2)	15 200 (1.3)
10	26 150 (2.7)	25 200 (6.0)	14 900 (3.5)
15	25 300 (6.3)	24 900 (4.0)	14 400 (1.9)
Theoretical MW	26 000	24 000	13 800

increased with a 5-min RP gradient. The first IEC results that were obtained for gradient times of 5–15 min are shown in Table II. As seen from the results, the MW for CHY, TRP and RNase agreed well with literature values of monomer MWs. This suggested that some proteins could be eluted under fast gradient conditions without aggregate formation. These results seemed very encouraging, and indicated that fast IEC separations were compatible with LALLS. Figs. 2, 3 and 4 illustrate the LALLS and UV results for TRP, CHY and RNAse, respectively. At least three injections were made to determine the relative standard deviations (R.S.D.) of the MWs. In general, there was excellent reproducibility and precision (low R.S.D.).

β-LACT was studied next. Previous reports indicated that β-LACT aggregated very easily, as a function of pH [29–32]. Based on the above, we studied β-LACT to investigate the effect of fast gra-

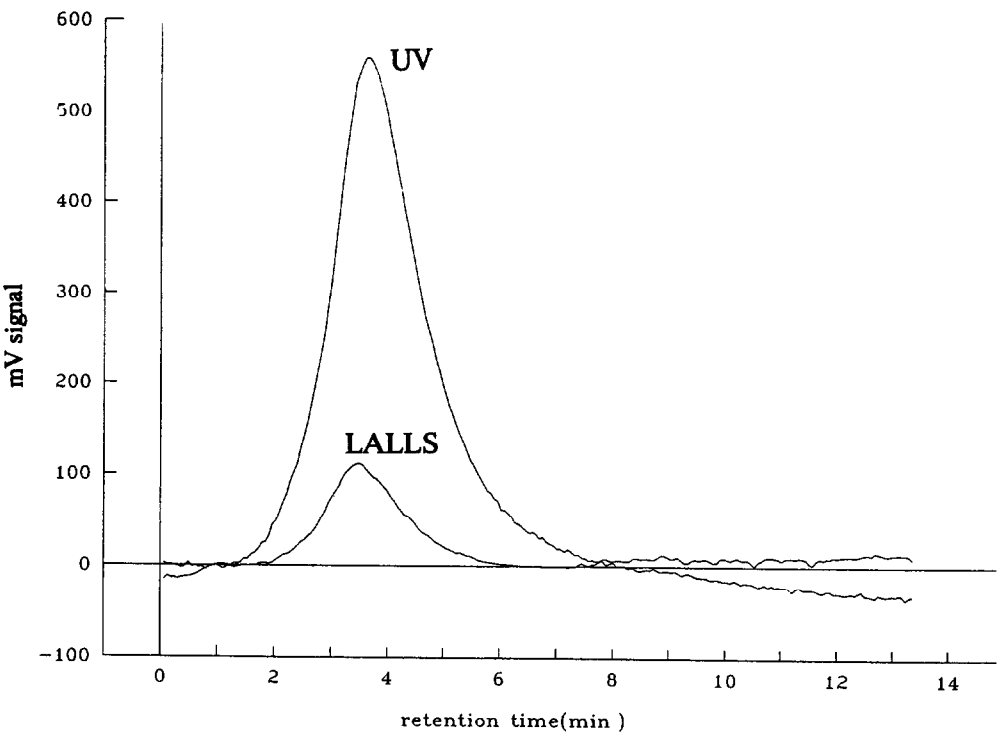

Fig. 2. LALLS–UV signals of trypsinogen 10-min gradient. Column, HP-SCX; mobile phase, (A) 5 m*M* Bis-Tris, (B) A + 0.5 *M* sodium chloride (pH 5.8), 15–75% B in 10 min at 1 ml/min; UV-detection, 280 nm, 0.2 a.u.f.s.; LALLS, 0.2 mm field stop, 6–7° annuli, G_o = 400 mV; concentration 10 mg/ml through a 20-μl loop.

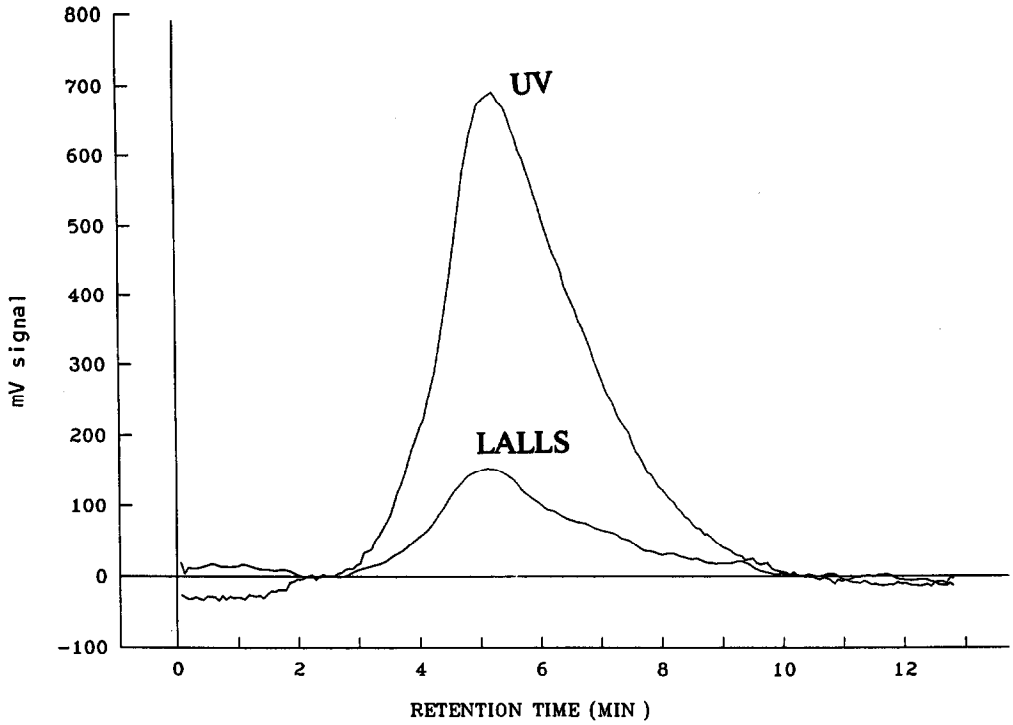

Fig. 3. LALLS–UV signals of α-chymotrypsinogen 5-min gradient. Conditions as in Fig. 2 except gradient 15–100% B in 5 min.

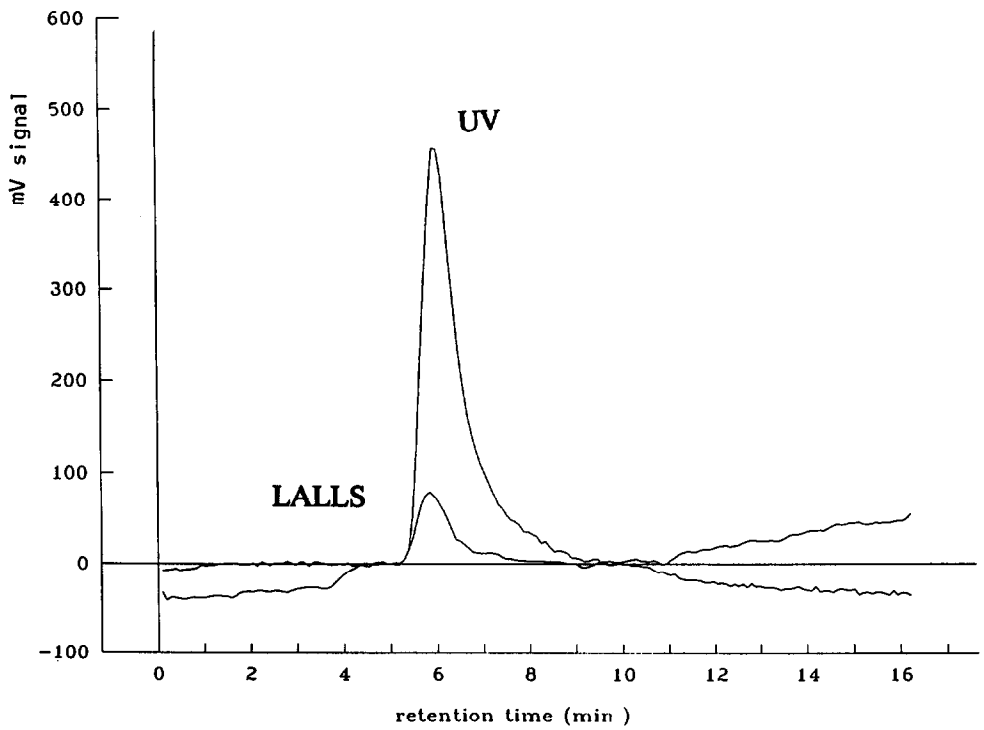

Fig. 4. LALLS–UV signals of ribonuclease A 15-min gradient. Conditions as in Fig. 2 except gradient 0–100% B in 15 min.

TABLE III

MW OF β-LACTOGLOBULIN A

Mobile phase: A = 10 m*M* NAH_2PO_4; B = A + 0.5 *M* NaCl. Gradient 0–80% B. Relative standard deviations in % (n = 3) are given in parentheses.

Gradient time (min)	MW
5	71 000 (5.1)
15	63 300 (2)
30	46 000 (2.1)
Theoretical MW	18 700

dients (5 min) on MW. Table III shows the MW of β-LACT obtained for the 30-, 15- and 5-min gradients. The MW increased from that obtained in the 30-min gradient (46 000 dalton) to the 15-min (63 000 dalton) and the 5-min gradients (71 000 dalton). This was the first protein studied that exhibited such behaviour. β-LACT was always present as a dimer or higher aggregate under the elution conditions. What remained to be determined was whether the protein was present as a dimer when supplied, aggregated in the injection solution, or aggregated on-column. The above results suggested that β-LACT was probably aggregating on-column, from a dimeric to higher aggregate states. Figs. 5 and 6 illustrate the LALLS and UV signals of β-LACT at 30 and 5 min, respectively. As seen, the LALLS and UV peaks were fairly symmetrical. In the RP studies, peaks for the 5-min gradients were unsymmetrical as mixed aggregates were formed and were partially resolved [9]. The symmetry of the LALLS peak indicated that mixed aggregates were probably not formed for the 5-min gradient. The MW from the 30-min gradient was slightly higher than that for a dimer, indicating that a dimer and some other aggregate form were present. The 5-min gradient indicated the formation of a tetramer. The MW from the 15-min gradient again indicated the formation of mixed aggregates which were not being separated owing to the low resolution of IEC.

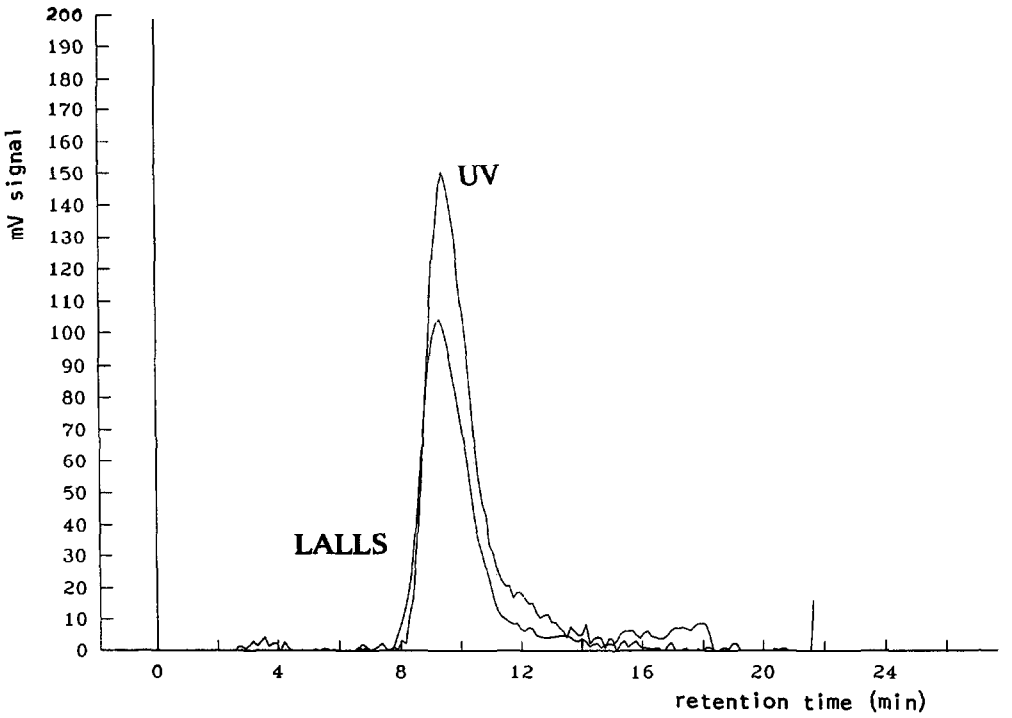

Fig. 5. LALLS–UV signals of β-lactoglobulin A 30-min gradient. Mobile phase, (A) 10 m*M* NaH_2PO_4, (B) A + 0.5 *M* sodium chloride (pH 4.0), 0–80% B in 30 min; other conditions as in Fig. 2.

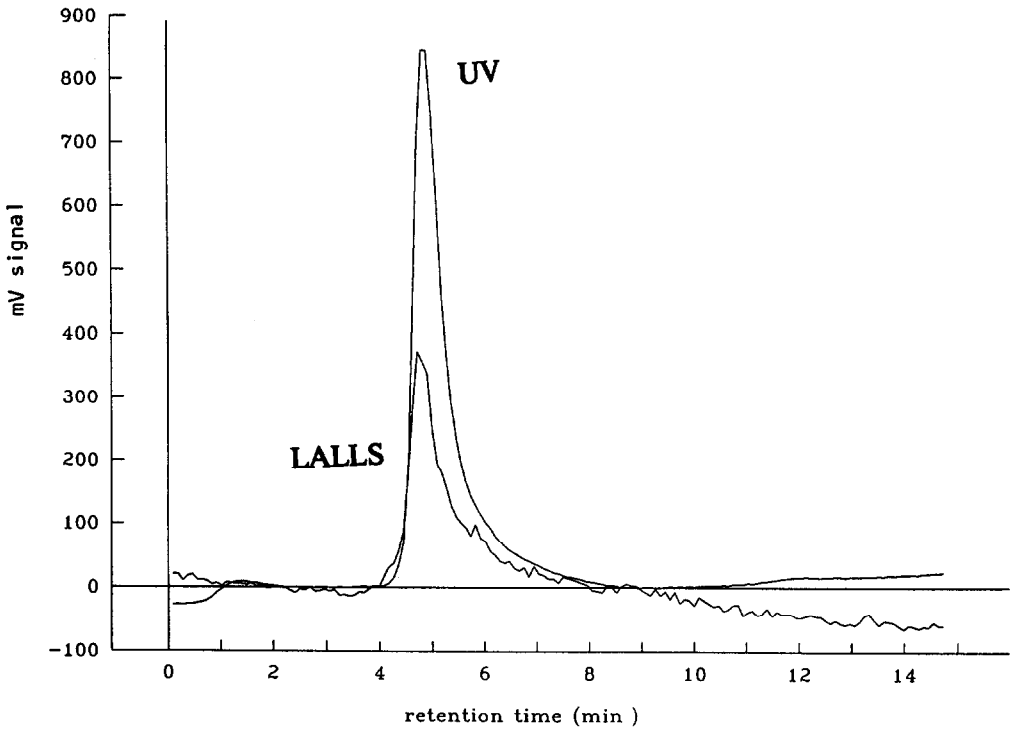

Fig. 6. LALLS–UV signals of β-lactoglobulin A 5-min gradient. Conditions as in Fig. 5 except gradient 0–80% B in 5 min.

The recoveries of β-LACT were above 95% for all the gradient times. This suggested that there was little loss of sample on-column. Also, the decrease in MW from the 5- to 30-min gradient time was due to a lower degree of aggregation with the latter gradient, and not to lower recovery of sample. Recoveries for the other proteins were assumed to be 100%. As the MWs for these proteins matched literature values, our assumptions were indeed correct. In a separate study, the recoveries for some other proteins eluted from a column similar to that used in this work were above 95% [33]. The increase in MW of β-LACT could not be an artifact of the system as none of the other proteins aggregated under any gradient conditions.

TABLE IV

SEC-LALLS OF β-LACTOGLOBULIN A

Column: TSK-GEL G4000SW. Flow-rate: 0.7 ml/min. Injection volume: 100 μl. Relative standard deviations (%) (n = 3) are given in parentheses.

Mobile phase	Concentration injected (mg/ml)	MW
MPB	4.2	19 200 (4.4)
10 mM Na_2HPO_4	10.68	37 900 (2.6)

TABLE V

MOLECULAR WEIGHTS DETERMINED BY FIA–LALLS

FIA conditions: 5 mM Bis-Tris at 0.2 ml/min; 2-ml injection loop, except for β-LACT, where 10 mM NaH_2PO_4 was used for elution. LALLS: 0.2 mm field stop, 6–7° annuli, G_o = 200 mV. Relative standard deviations (%) (n = 3) are given in parentheses.

Protein	MW	Literature value[a]
Trypsinogen	25 600 (3.5)	24 000
α-Chymotrypsinogen A	26 968 (1.6)	26 000
Ribonuclease A	15 000 (1.2)	13 800
β-Lactoglobulin A	37 700 (2.6)	18 700

[a] Taken from Sigma Chemical Co. catalog (1991).

SEC–LALLS–UV of β-LACT

SEC–LALLS studies were performed to determine whether β-LACT was present as an aggregate as received from the supplier and aggregated further on-column, or if it aggregated in the injection solution prior to injection. To determine the state of β-LACT in the sample, it was dissolved in a non-aggregating buffer, MPB. MPB has been reported not to aggregate proteins [7–11]. Table IV shows that the MW obtained using SEC–LALLS and MPB matched that of the monomer (19 200 dalton).

β-LACT was then dissolved in buffer A, the injection solvent, and eluted using the same solvent. The MW was 37 900 dalton (97% recovery). This was close to the MW obtained using the 30-min gradient. As *ca.* 10 mg/ml of β-LACT were used per injection, these results suggested that the protein was present as a dimer prior to injection and aggregated further on-column as a function of the gradient. Fig. 7 shows the LALLS–UV signals for a 10 mg/ml concentration of β-LACT.

FIA–LALLS

Table V shows the MWs of RNase, CHY, TRP and β-LACT obtained by FIA–LALLS. The MWs matched well with literature values, except for β-LACT (MW 37 700 dalton), which formed a dimer.

CONCLUSIONS

This study has helped us to understand further the significance of LALLS as a qualitative detection technique for biopolymer analysis. UV peaks that seem to be single/monomeric species may in fact be aggregates or mixtures of aggregates, as with β-LACT. It is possible to interface gradient elution IEC and LALLS for the rapid separation and characterization of biopolymers on-line. Of the four proteins studied, it was possible to interface very steep gradients (5-min 0–80% B) and deduce accurate MW information. β-LACT was the only protein studied that aggregated as a function of the gradient. We have also been able to interface suc-

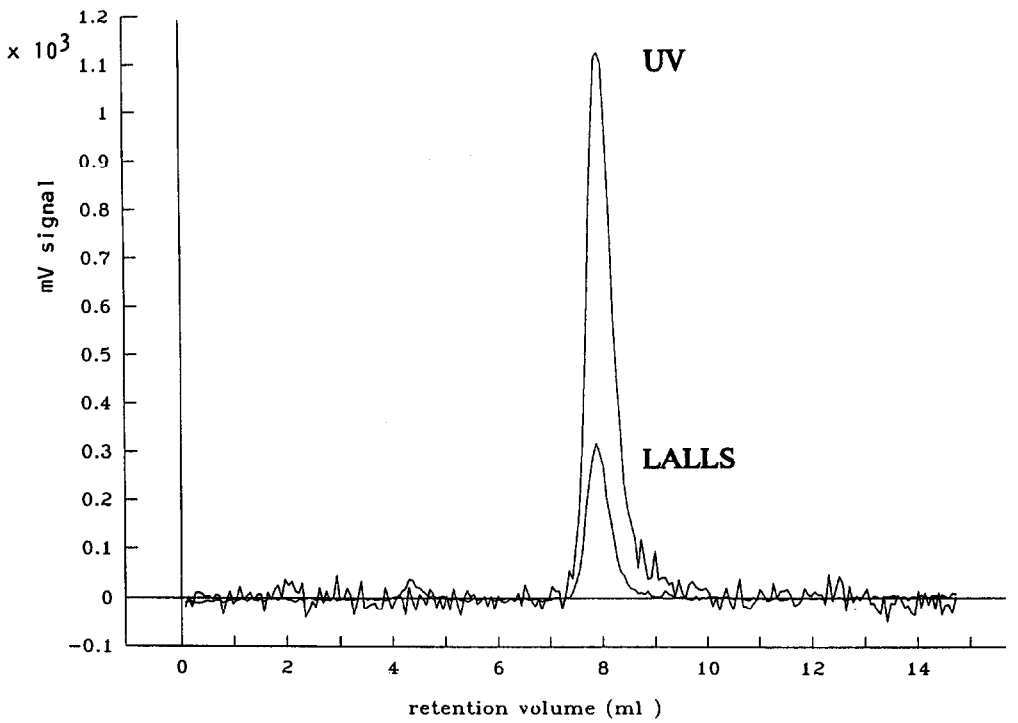

Fig. 7. LALLS–UV signals of β-lactoglobulin A SEC. Column, TSKgel G4000 SW; mobile phase, 10 m*M* NaH_2PO_4 at 0.7 ml/min; concentration, 10 mg/ml; LALLS conditions as in Fig. 2.

cessfully fast-gradient HIC with LALLS and the results will be published in the near future.

ACKNOWLEDGEMENTS

Gratitude is expressed to Laboratory Data Control (LDC) Analytical, Division of Thermo Instruments, (Riviera Beach, FL, USA), for their instrumentation donations, funding and technical support. This work would not have been possible without the continuing support of certain individuals within LDC, especially H. Kenny, B. Freeman, W. Mahn, M. Munk and B. Howe. We also acknowledge Interaction Chemicals, Division of E. Merck (D. Hometchko), for their donation of the ion-exchange column used. Additional funding for this work was provided, in part, by Merck Sharp & Dohme Research Laboratories (H. Perpall and M. Brooks), Pfizer (G. Forcier and K. Bratin) and the NIH-Biomedical Research Support Grant to NU, NO. RR07143 (DHHS) (S. Fine). Various individuals within Northeastern University have shown considerable interest in the work described, especially B. Karger, D. Magiera and A. Quazi. We appreciate their interest and continued encouragement. This is contribution No. 486 from the Barnett Institue at Northeastern University.

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