Journal of Chromatography, 606 (1992) 264–271
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 378

Short Communication

Determination of alkaline phosphatase aggregation by size exclusion high-performance liquid chromatography with low-angle laser light scattering detection

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(First received January 23rd, 1992; revised manuscript received May 26th, 1992)

ABSTRACT

The formation of aggregates of alkaline phosphatase (AP) is examined in two different buffer systems: phosphate-buffered saline and modified protein buffer, using isocratic size-exclusion chromatography combined with low-angle laser-light scattering, ultraviolet spectroscopy, and a modified differential refractive index detector, all in series. The effects of buffer conditions, concentrations, and different batch preparations of alkaline phosphatase, as obtained from a manufacturer, were examined.

INTRODUCTION

Low-angle laser-light scattering (LALLS) has been interfaced with an ultraviolet (UV) and a differential refractive index (DRI) detector to collect on-line weight-average molecular mass (M_w) information of protein molecules separated by a prior high-performance liquid chromatography (HPLC) separation technique, the most popular technique being size-exclusion chromatography (SEC) [1–5]. Over the past twenty years, LALLS has been used to measure the molecular masses of various polymers. Static or traditional LALLS was used during the past 15 years for measuring synthetic polymer characteristics, such as M_w, second virial coefficients (A_2) and the degree of copolymer branching. Recently, our group and others [6–8] have focused on using LALLS to measure the M_w of biopolymers, as long as the refractive index (n) of the solvent and dn/dc (where c is concentration) of the biopolymer in solution are known [9–11]. An on-line system consisting of HPLC technique followed by LALLS, UV and DRI detectors, connected in series, was developed in our laboratory and used to calculate on-line M_w and dn/dc, with excellent reproducibility [12]. A detailed description of LALLS theory can be found in approaches to SEC–LALLS [13].

The tri-detection system described above is very useful for detecting protein aggregation in salt containing buffer solutions. We have compared the extent of aggregation of alkaline phosphatase (AP) in two different types of salt buffer systems, phosphate-buffered saline (PBS) and a surfactant-en-

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hanced modified protein buffer (MPB). SEC was used to separate the aggregates formed from the monomer unit of AP. Because LALLS provides absolute M_w values, no recalibration of the SEC columns was required. The purity of AP from three different batch preparations was analyzed and compared, in order to observe different degrees of aggregation as a function of protein concentration and batch-to-batch preparation.

EXPERIMENTAL

Apparatus

The SEC–LALLS–UV–DRI system consisted of a ConstaMeteric II analytical metering pump (LDC Analytical/Thermo Instruments, Riviera Beach, FL, USA) operated at 0.7 ml/min, a Rheodyne (Cotati, CA, USA) Model 7125 20-μl injection loop, a medium TSK-PW 6000 size-exclusion column (30 cm × 8 mm I.D.) or a TSK-PWXL 3000 size-exclusion column (30 cm × 8 mm I.D., from Supelco/ Division of Rohm & Haas, Bellefonte, PA, USA), a 0.22-μm (GV membrane) on-line filter apparatus (LDC), a Chromatrix KMX-6 (HeNe equipped laser operating at a wavelength 632.8 nm) LALLS photometer (LDC) equipped with a 10-μl flow through cell, a UV–visible (VIS) spectroMonitor-D (LDC) variable-wavelength absorbance detector operating at a 280 nm wavelength (0.2 AUFS at 10 mV full scale) and a modified refractoMonitor IV DRI detector (LDC), operated at $0.2 \cdot 10^{-3}$ RI units (10 mV full scale). The modifications to the DRI enabled d*n*/d*c* measurements to be made online at *ca.* 650 nm, as discussed elsewhere [12]. A Soltec (Sun Valley, CA, USA) Model 1242 strip chart recorder operating at 10 mV full-scale for UV or DRI outputs and 1 V full scale for LALLS output, at a recording speed of 15 cm/h was used to record raw chromatograms from LALLS and UV or DRI detectors. All detector outputs were connected to a PCI 2000 analog-to-digital converter (Burr Brown, Palo Alto, CA, USA) for computer data acquisition (CompuAdd 810, CompuAdd, Austin, TX, USA) and graphics/data manipulation. Software packages for simultaneously collecting data from the three detectors for calculating molecular weights were obtained from LDC, version PCLALLS. In-house software was developed [12] on LOTUS-123 to calculate on-line d*n*/d*c* measurements and percent recoveries. RI measurements were made as described previously [9].

Mobile phases

All water used to make the PBS and MPB buffers was deionized by a Nanopure System (Millipore, Bedford, MA, USA) and filtered with a 0.22-μm nylon membrane (Millipore) and degassed under vacuum before use as HPLC solvent. PBS was made with 150 m*M* sodium chloride, 25 m*M* monobasic sodium phosphate and 25 m*M* dibasic sodium phosphate pH 6.8 at room temperature, $n = 1.33$ at 633 nm. MPB was composed of 18 m*M* N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 7 m*M* imidazole, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 3 m*M* sodium azide, 200 m*M* sodium acetate, 0.5 m*M* non-ionic surfactant, octaethylene glycol-mono-N-dodecyl ether (Sigma, St. Louis, MO, USA), $n = 1.335$ at 633 nm, pH 7.0 at room temperature.

Chemicals and supplies

Bovine human AP was received from Sigma without further purification and stored immediately in the freezer (−20°C). Three different batch preparations of AP were analyzed as provided by the supplier. All solutions were made daily and stored in the refrigerator (5°C) in-between analyses or injections. All refrigerated samples were allowed to equilibrate to room temperature for 30 min before chromatographic analysis.

Ammonium acetate was reagent grade and obtained from EM Science (Gibbstown, NJ, USA). Imidazole, 99% pure, was used without further purification as obtained from Aldrich (Milwaukee, WI, USA). ACS reagent-grade chemicals EDTA, monobasic sodium phosphate, dibasic sodium phosphate and HEPES were obtained from Sigma. Sodium acetate, HPLC reagent grade, was obtained from Fisher. Sodium azide, used for a bacterial group repressor, was obtained from Eastman Kodak (Rochester, NY, USA).

Procedures

SEC–LALLS–UV–DRI studies. The columns for SEC analysis were equilibrated with buffer for 1 h before analysis at a flow-rate of 0.5 ml/min. The column was washed out at the end of each day's analysis with deionized water at a flow-rate of 0.5

ml/min for 1 h and then the flow-rate was lowered to 0.2 ml/min overnight.

Medium-resolution SEC study. A batch of AP, corresponding to lot 3 in Table I, was analyzed by medium-resolution SEC in the two different buffer systems, by weighing out the sample and adding it to the buffer solution to provide concentrations ranging from 1–3 mg/ml. The theoretical M_w of the monomer species was 68 000. A second batch of AP, lot 1, was analyzed by medium-resolution SEC; but only in the MPB buffer system to compare the purity of different batches of AP. An off-line d*n*/d*c* value of 0.146 was used for AP M_w calculations, as measured previously [9] for both MPB and PBS buffer systems. The percent recoveries (calculated by comparing computer collected UV areas at the same concentrations injected) were in the ranges of 60% for PBS and 50% for MPB.

High-resolution SEC study. For this study two different batches of AP, lots 1 and 2, were chromatographed on a high-resolution system. The first batch of AP, lot 3, was not used because of sample limitations for this study and was also unobtainable from the supplier, as it was out of stock. The same procedures described above were used for sample storage and HPLC analysis, except for the column, which was replaced with a high-resolution TSK-PWXL gel SEC column. Injections were made in both buffer systems, MPB and PBS, at concentrations ranging from 1.0–14.0 mg/ml (n = 3). All samples were prepared for injection by adding 1 ml of buffer to a weighed mass of lyophilized protein, always avoiding foaming.

The percent recoveries were calculated by measuring the molar absorptivity off-line of AP, dissolved in MPB and PBS buffers, by flow injection analysis. The procedure involved plotting the peak height versus concentration for three different dilutions of the injection solution (n = 3). A least-squares fit of this line provided the molar absorptivity (ε) of the protein, as the slope. The final percent recovery determined on-line was then calculated by using the in-house developed Lotus software [13]. The average percent recovery on the high-resolution

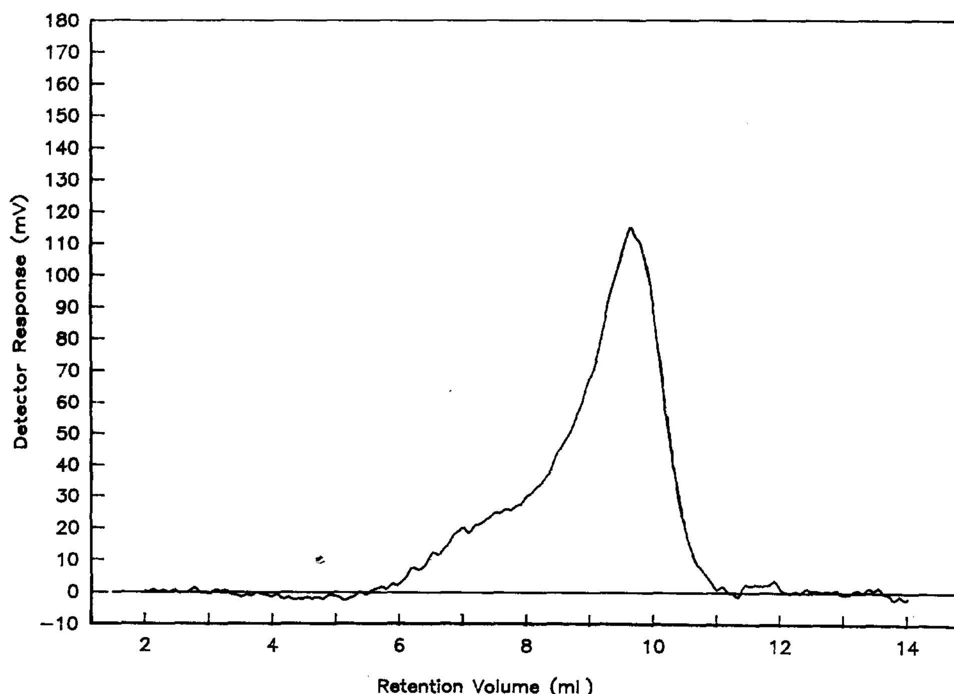

Fig. 1. Medium-resolution SEC–LALLS chromatogram of AP lot 3 in MPB. Conditions: concentration, 2.6 mg/ml; 100-μl loop; flow-rate, 0.7 ml/min.

column was *ca.* 90.0% in both buffer systems. An on-line calculated d*n*/d*c* for AP in MPB was 0.174, and in PBS 0.175 at *ca.* 5.0 mg/ml concentrations.

RESULTS AND DISCUSSION

PBS buffer was orginally thought to support aggregation of AP in solution. That is, as one increases the concentration of AP in an aggregating buffer solution, one would expect to see increased aggregation. The buffer components for PBS buffer, such as relatively high salt concentration, make it a suitable buffer to support the formation of AP aggregates. The aggregation of AP itself in PBS can be attributed to the weak dipole–dipole and hydrophobic forces of attraction, causing incomplete dissociation to the monomer species in solution. The next question that had to be answered was whether or not AP could be totally dissociated in any salt containing buffer solution.

A non-aggregating buffer solution was critical to this type of study because it served as the control. We have chosen to use an MPB buffer, which has not caused any aggregation of proteins upon dissolution [9–14].

The M_w results are listed in Table 1 for AP lot 3 in PBS and MPB buffers. The M_w calculated for lot 3 of AP dissolved in PBS buffer, approximately 78 400, was greater than the M_w corresponding to that calculated from the MPB buffer and suggested the existence of increased aggregation.

The existence of an aggregate was shown in the LALLS chromatograms (Figs. 1 and 2). Even though complete resolution of the species was not obtained, a reproducible M_w could still be obtained. The higher M_w calculated in the PBS buffer for lot 3 was compared to the M_w calculated for the same species at a similar eluate concentration dissolved in MPB.

High-resolution SEC–LALLS–UV–DRI was next used to obtain better resolution between the AP aggregate and monomer, which could discriminate between the different degrees of aggregation already present in the lyophilized samples. The AP aggregate was separated from the monomer species on the high-resolution SEC column (Figs. 3 and 4).

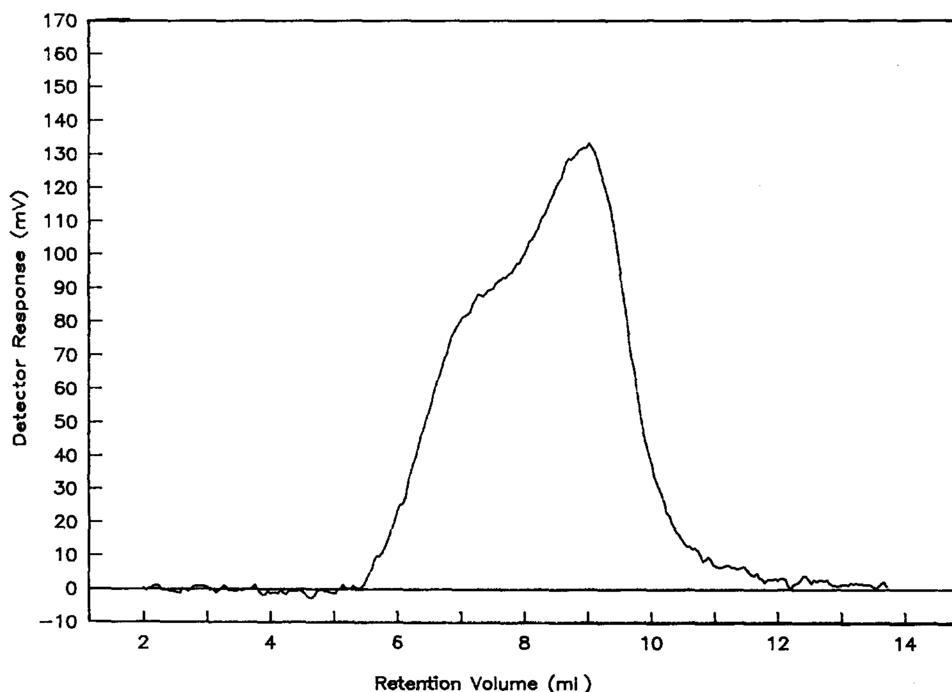

Fig. 2. Medium-resolution SEC–LALLS chromatogram of AP lot 3 in PBS. Conditions as in Fig. 1.

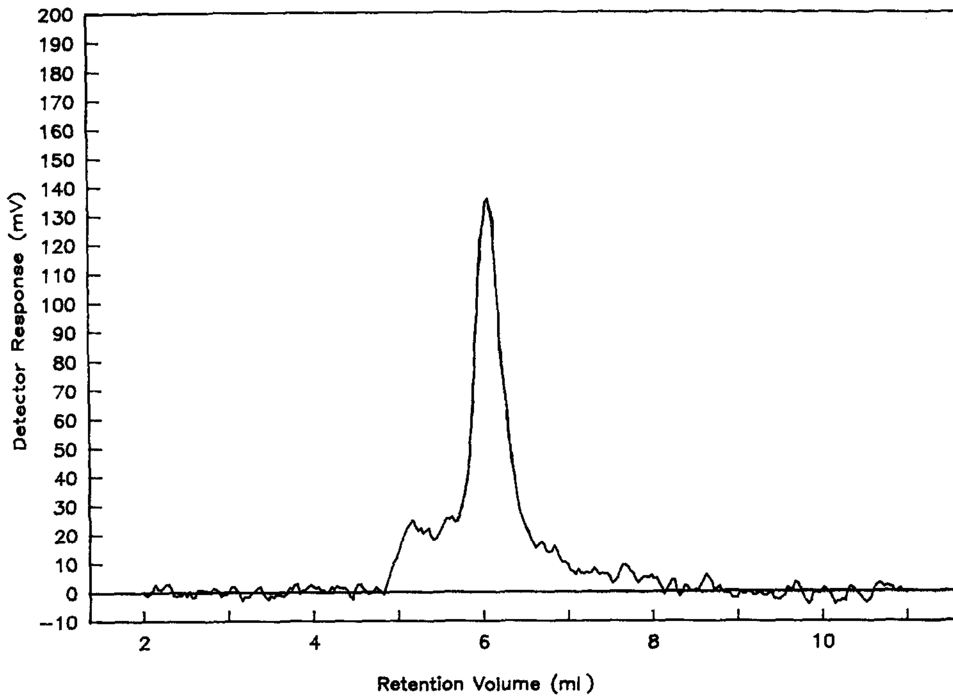

Fig 3. High-resolution SEC–UV chromatogram of AP lot 1 in PBS. Conditions: concentration, 1.33 mg/ml; 20-μl loop; flow-rate, 0.7 ml/min.

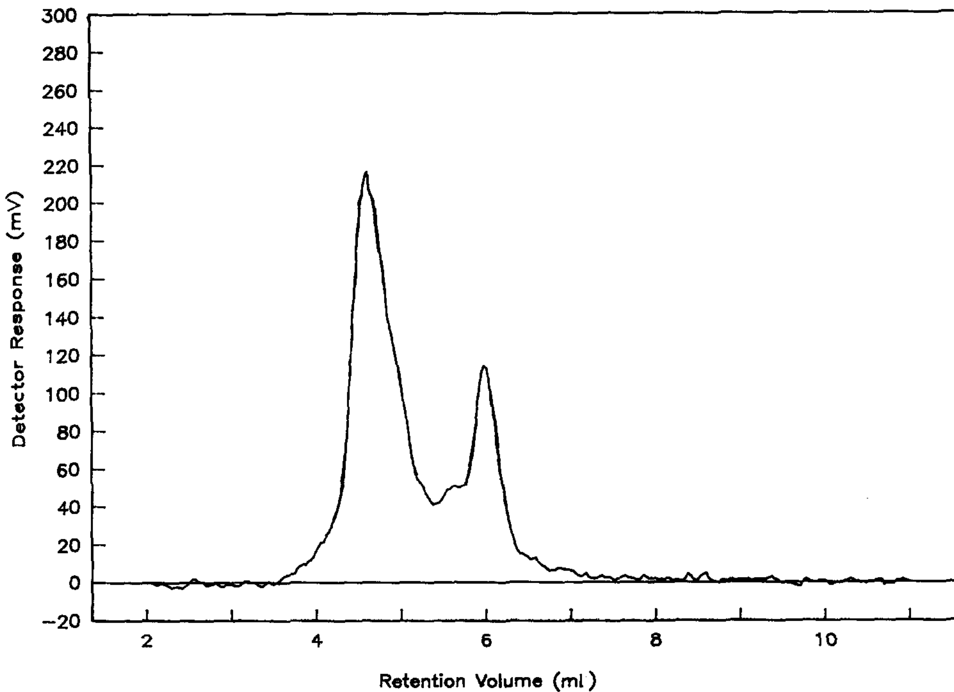

Fig. 4. High-resolution SEC–LALLS chromatogram of AP lot 1 in PBS. Conditions as in Fig. 3.

TABLE I

M_w OF ALKALINE PHOSPHATASE SAMPLES

A = Medium-resolution SEC–LALLS (n = 2); B = high-resolution SEC–LALLS (n = 3). SEC–LALLS–UV–DRI conditions: HPLC 20-μl loop for high-resolution and 100-μl loop for medium-resolution SEC; columns: TSK-PWXL 3000 for high resolution and TSK-PW 6000 for medium resolution; mobile phase PBS or MPB buffer, flow-rate 0.7 ml/min; UV = 280 nm; DRI = $1.0 \cdot 10^{-4}$ refractive index units; LALLS: LALLS attenuator constant (D) = $3.9223 \cdot 10^{-9}$, 6–7 degree annulus, 0.2 mm field stop, LALLS flow through cell system.

SEC–LALLS	AP sample (Lot No.)	Concentration (mg/ml)	Buffer	M_w	Average M_w	Relative S.D. (%)
A	3	2.8	PBS	82 500 – 80 000	81 300	
	3	2.6	PBS	77 800 – 73 200	75 500	
	3	1.50	MPB	70 700 – 70 500	70 600	
	3	2.7	MPB	73 400 – 67 900	70 700	
	1	1.24	MPB	76 400 – 78 600	77 500	
B	2	1.1	PBS	87 200		5.8
	2	5.5	PBS	87 600		5.8
	2	11.0	PBS	89 700		9.4
	1	1.3	PBS	76 600		5.8
	1	5.8	PBS	76 700		5.2
	1	11.7	PBS	79 100		11.2
	2	1.17	MPB	89 600		5.3
	2	5.1	MPB	89 600		2.5
	2	13.4	MPB	90 200		0.6
	1	1.7	MPB	81 800		8.2
	1	5.1	MPB	81 900		7.5
	1	10.0	MPB	81 200		2.8

On-line M_w analyses along with % recoveries and on-line dn/dc measurements are reported in Table I. The aggregate impurity was determined to be the dimeric species of AP, having a M_w of 136 000. The high-resolution SEC results are shown in Tables II and III for aggregate formation as the concentration of protein was increased in each buffer system, MPB and PBS, for lots 1 and 2. The graphs of % aggregate /% monomer *versus* concentration of AP in solution are shown in Figs. 5 and 6. The

TABLE II

HIGH-RESOLUTION SEC–LALLS–UV–DRI AP DEGREE OF AGGREGATION STUDY (MPB)

SEC–LALLS–UV–DRI conditions: HPLC: 20-μl loop for high-resolution SEC; column: TSK-PWXL 3000; mobile phase MPB buffer; flow-rate 0.7 ml/min; UV = 280 nm, DRI = $1.0 \cdot 10^{-4}$ refractive index units; LALLS: D = $3.9223 \cdot 10^{-9}$, 6–7 degree annulus, 0.2 mm field stop, LALLS flow through cell system.

AP Lot No. (n = 3)	Concentration (mg/l)	% Monomer	% Aggregate
1	1.7	79.69	20.30
	5.1	79.59	20.41
	10.0	80.23	19.56
2	1.17	68.12	31.87
	5.1	68.30	31.70
	13.4	67.34	32.66

TABLE III

HIGH-RESOLUTION SEC–LALLS–UV–DRI AP DEGREE OF AGGREGATION STUDY (PBS)

SEC–LALLS–UV–DRI conditions: HPLC: 20-μl loop for high-resolution SEC; column: TSK-PWXL 3000; mobile phase PBS buffer; flow-rate 0.7 ml/min; UV = 280 nm, DRI = $1.0 \cdot 10^{-4}$ refractive index units; LALLS: D = $3.9223 \cdot 10^{-9}$, 6–7 degree annulus, 0.2 mm field stop, LALLS flow through cell system.

AP Lot No. (n = 3)	Concentration (mg/l)	% Monomer	% Aggregate
1	1.3	87.42	12.58
	5.8	87.20	12.80
	11.7	83.70	16.30
2	1.1	71.85	28.15
	5.5	71.11	28.89
	11.0	68.16	31.84

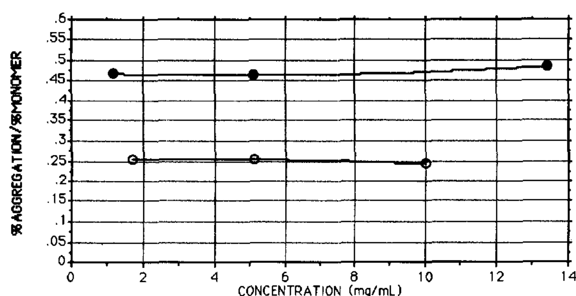

Fig. 5. Graph of % aggregate / % monomer *vs.* concentration for MPB. ○ = AP lot 1; ● = AP lot 2. SEC–LALLS–UV–DRI conditions as in Table II.

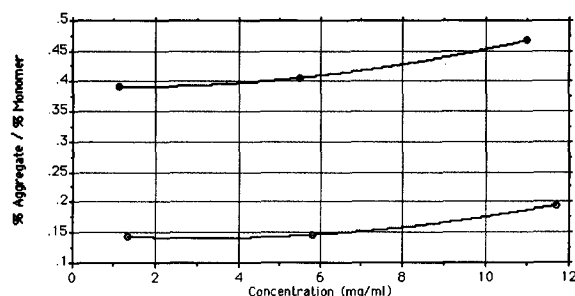

Fig. 6. Graph of % aggregate / % monomer *vs.* concentration for PBS. ○ = AP lot 1; ● = AP lot 2. SEC–LALLS–UV–DRI conditions as in Table III.

% monomer and % aggregate were determined by the ratio of their integrated UV areas to the total UV area collected by computer with the PCLALLS software. The degree of aggregation in PBS was shown to increase with increasing protein concentration for both lots 1 and 2.

The degree of aggregation observed in MPB did not increase with increasing protein concentration (Table II). The starting ratios of aggregate-to-monomer were almost the same as in PBS buffer at the 1.0 mg/ml concentration. But, as the concentration of AP was increased in the MPB solution, no increase in the aggregate-to-monomer ratio was seen over a broad concentration range (Fig. 5).

CONCLUSIONS

The degree of aggregation of AP has been studied using the SEC–LALLS–UV–DRI system in two different buffer systems, PBS and MPB. The monomer species was observed at M_w 68 000 and the dimer as an impurity at M_w 136 000. The degree of aggregation of AP in PBS increased with increasing protein concentration, and it may therefore be classified as an aggregating buffer system. The degree of aggregation in MPB remained constant and is probably present in the lyophilized powder before dilution. Therefore, it is concluded that since the ratios of dimer-to-monomer were constant over a broad range of increasing concentrations for all AP batches in MPB, the manufacturer had provided us with three differing purities.

ACKNOWLEDGEMENTS

Research funding, instrumentation donations and maintenance, and technical assistance were provided by LDC Analytical, Division of Thermo Instrument Systems, Riviera Beach, FL, USA. Additional funding for the SEC–LALLS program at Northeastern University has been provided by Merck Sharp and Dohme Research Laboratories, Inc., West Point, PA, USA (M. Brooks). We also wish to acknowledge the assistance of H. H. Stuting (Director of Analytical Chemistry, Roche Biomedical Laboratories, Raritan, NJ, USA), Wm. Mahn (LDC), C. Luckas (LDC), H. Kenny (LDC), and B. Howe (LDC) for their technical assistance. Several colleagues within Northeastern University provided stimulating discussions and support throughout this project, especially R. Mhatre, M. Szulc, J. Mazzeo, A. Quazi, A. J. Bourque, F.-X. Zhou and L. Dou.

This is contribution number 540 from The Barnett Institute at Northeastern University.

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