Journal of Chromatography, 442 (1988) 29–52
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 420

CONFORMATIONAL STUDIES OF BOVINE ALKALINE PHOSPHATASE IN HYDROPHOBIC INTERACTION AND SIZE-EXCLUSION CHROMATOGRAPHY WITH LINEAR DIODE ARRAY AND LOW-ANGLE LASER LIGHT SCATTERING DETECTION

IRA S. KRULL* and HANS H. STUTING
Department of Chemistry, The Barnett Institute, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)
and
SUSAN C. KRZYSKO
U.S. Food and Drug Administration, Winchester Engineering and Analytical Chemistry Branch (WEAC), 109 Holton Street, Winchester, MA 01890 (U.S.A.)
(First received October 12th, 1987; revised manuscript received February 17th, 1988)

SUMMARY

Alkaline phosphatase has been studied in hydrophobic interaction chromatography (HIC), using a bonded C_1-ether phase on a silica gel support, together with an aqueous salt gradient. Its behavior under various gradient elution conditions has demonstrated good chromatographic performance and retention of enzymatic activity under aqueous conditions. It has now been studied using linear photodiode array (LDA) spectroscopy in combination with low-angle laser light scattering (LALLS) in gradient elution HIC. HIC–LALLS permitted the use of routine salt gradients for collection of molecular weight information, despite small changes in the baseline, via computerized baseline substraction. Size-exclusion chromatography (SEC)–LALLS measurements, under various isocratic conditions, meant to mimic HIC elution, have indicated the presence of monomer/dimer, dimer/trimer, or mainly trimer. Aggregates of alkaline phosphatase can also be detected under salt gradient HIC conditions, but at lower levels relative to the monomer.

This paper also describes the behavior of alkaline phosphatase when detected using LDA under various chromatographic, temperature, and concentration (injected) conditions. The results indicate a facile equilibrium of at least two monomeric forms of alkaline phosphatase of the same molecular weight, which change relative populations as a function of operational conditions. Most interesting is the suggestion that alkaline phosphatase undergoes rapid conformational interconversions on the chromatographic detection time scales, and that these interconverting conformations, concentration dependent, produce a novel dual wavelength ratioing, *viz.*, a pseudo-Gaussian peak mimicking the chromatographic elution profile at either wavelength. The reasons for these observations and their possible use in future high-performance liquid chromatographic biopolymer studies are discussed and described.

0021-9673/88/$03.50

INTRODUCTION

The separation and determination of biopolymers by modern high-performance liquid chromatography (HPLC) has become an area of interest within the past few years[1–5]. Unfortunately, methods for the detection of natural or synthetic biopolymers have lagged somewhat behind the advances realized in the separation areas[6,7]. Most of the routine detectors for HPLC, such as variable-wavelength ultraviolet–visible (UV–VIS), fixed-wavelength fluorescence (FL), and electrochemistry (EC), offer little in the way of biopolymer characterization. Most biopolymers show little or no response under conventional EC conditions, in the absence of some type of derivatization. Though UV–FL detectors may provide some detection, usually not at trace levels, they rarely provide analyte identification or characterization of biopolymers.

Linear diode array spectrophotometry (LDA) has been used in the past to obtain on-line spectra mainly for non-polymeric materials, but at times for a limited number of biopolymers[8–11]. Unfortunately, many biopolymers, especially proteins and enzymes, have strikingly similar UV–VIS spectra, and thus identification can be difficult. On the other hand, LDA might be utilized to demonstrate the purity of a biopolymer, assuming that it exists in a single, preferred conformational state, and that it does not undergo aggregation on-column or upon injection.

Low-angle laser light scattering (LALLS)[12–15] has been utilized in the past for various biopolymers in HPLC, but it has been restricted to isocratic conditions, mainly via size-exclusion chromatography (SEC). It has rarely been utilized in combination with salt or mobile phase gradient elution conditions[16]. However, much of modern HPLC of proteins involves gradients, for example hydrophobic interaction chromatography (HIC) utilizes some type of salt gradient elution condition for biopolymers[17–20]. It would prove useful to be able to utilize gradient elution HIC conditions together with LALLS for biopolymer identification and characterization.

This paper describes results on the interfacing of salt gradient elution HIC with both LDA and LALLS detection for bovine alkaline phosphatase. Alkaline phosphatase has been chosen in view of its current importance in the detection of pasteurization efficiency in milk and dairy products. Alkaline phosphatase is currently analyzed using a batch, enzyme–substrate reaction with final colorimetric detection, in the absence of HPLC[21].

EXPERIMENTAL

Apparatus

For the HPLC–UV/LDA studies, a modular HPLC system was assembled using, as components for gradient elution HIC, two Waters Chromatography Division (Millipore, Milford, MA, U.S.A.) Model 501 solvent delivery systems, a Waters Model 660 solvent programmer, a Laboratory Data Control (LDC) solvent mixer (Milton Roy/LDC Division, Riviera Beach, FL, U.S.A.), a Rheodyne (Cotati, CA, U.S.A.) Model 7125 syringe loading injector, and a Spectra-Physics (San Jose, CA, U.S.A.) Model SP4270 recording integrator or a Hitachi (EM Science, Cherry Hill, NJ, U.S.A.) Model D2000 Chromatointegrator. Detectors and associated equipment included the Kratos (Ramsey, NJ, U.S.A.) Spectroflow Model 757 variable-wave-

length UV–VIS detector, and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1040A high-speed spectrophotometric linear photodiode array detector, HP-85B work station, DPU multichannel integrator, HP-7470A graphics plotter, HP-9153 disc drives, and HP think jet printer.

For the HPLC–LALLS studies, all performed at 25°C, three modular HPLC systems were assembled using components for gradient elution HIC or isocratic SEC and FIA/STATIC–LALLS (*i.e.*, flow injection analysis coupled directly to LALLS for determination of molecular weight without the HPLC column, but mimicking a mobile phase used, at times, in HPLC–LALLS). System I was composed of an LDC Model CM4000 multiple solvent delivery system, a Rheodyne Model 7125 syringe loading injector, a Chromatix (Milton Roy/LDC Division) Model KMX-6 low-angle laser light scattering detector set-up for SEC–LALLS analyses, an LDC SM4000 programmable UV–VIS detector, an LDC Refractomonitor IV differential refractive index detector linked to a Soltec Model 1242 strip chart recorder (Soltec, Sun Valley, CA, U.S.A.), and an LDC Model CMX-10A(A/D) converter. At times, this system was changed to utilize Waters Model 501 solvent delivery systems with a Waters Model 660 gradient programmer, or Beckman (Beckman Division, Smith, Kline, and Beckman, Fullerton, CA, U.S.A.) Model 114M solvent delivery systems with a Beckman Model 421A gradient controller.

System II consisted of an LDC Constametric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve, a TSK-SW-G3000SW size-exclusion column (Phenomenex, Ranchos Palos Verdes, CA, U.S.A.), a Chromatix KMX-6 LALLS set-up for SEC–LALLS analyses, an LDC Spectromonitor-D variable-wavelength UV–VIS detector, an LDC Refractomonitor III DRI detector linked to both a Soltec Model 1242 strip chart recorder and an LDC CMX-10A(A/D) converter.

System III consisted of an LDC Constametric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve equipped with a 1 ml loop, a zero dead volume in-line filter unit, and a Chromatix KMX-6 LALLS set-up for SEC–LALLS with the analog output sent to a Soltec Model 1242 strip chart recorder. Systems I and II were linked to a DEC (Digital Equipment Corp., Boston, MA, U.S.A.) Micro PDP-11/23+ computer system complete via several Chromatix CMX-10A converters for digitization of instrumental analog outputs, storage of data, and further graphics/data manipulation. Software for calculating LALLS molecular weight information was from Chromatix, version MOLWT3.

The dn/dc (change in refractive index with change in concentration of analyte biopolymer) determinations were performed with an off-line, Chromatix Model KMX-16 laser differential refractometer. These measurements were made against blank buffer with exact amounts of supporting salt equal to that found in lyophilized powder alkaline phosphatase samples. This avoided the need to dialyze the alkaline phosphatase, and true dn/dc values have been measured.

For the constant-temperature HIC studies, a Precision Scientific (Fisher Scientific, Fair Lawn, NJ, U.S.A.) water bath was used, which controlled the temperature ± 0.1°C. Initial UV–VIS spectra of alkaline phosphatase were obtained, off-line, with a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3B UV–VIS spectrophotometer with a PE Model 660 printer and a PE Model 3600 data station. At times, UV–VIS data were also collected with a Spectronic 1201 UV–VIS spectrophotometer [Milton Roy/Analytical Products Division (formerly a Division of Bausch & Lomb)].

The HIC column used was a Beckman Spherogel CAA-HIC hydrophobic interaction (C_1-ether phase), silica-based column, 10 cm × 4.6 mm I.D., 5 μm, 300 Å pore diameter. The SEC column used for the SEC–LALLS studies was a Toyo Soda SW3000, 300 mm × 8 mm I.D., or a Shodex (Alltech Assoc. Deerfield, IL, U.S.A.) Model WS803 GPC column, 300 mm × 8 mm I.D.

Mobile phases

The salt gradient elution mobile phases used for the HIC–UV/LDA studies consisted of different gradient profiles of salts, starting with 2.5 *M* ammonium sulfate alone. Two salt solutions were mixed according to the particular Waters gradient profile selected. The first (A) consisted of a solution of 2.5 *M* ammonium sulfate in 0.5 *M* ammonium acetate (pH = 6.0), and the second (B) consisted of 0.5 *M* ammonium acetate (pH = 6.0). These were mixed at varying gradient elution profile curves, and total flow was held constant at 1.00 ml/min. The gradient isocratic hold and run times for each gradient elution profile are indicated (Results and Discussion).

The above salt gradient HIC conditions were used for the gradient HIC–LALLS–UV studies (10–90% B), closely paralleling the initial HIC–UV conditions, (0–100% B) optimized for alkaline phosphatase. The SEC mobile phase conditions were all isocratic, using varying ratios of mobile phase A and B above, to approximate the actual elution conditions effective in gradient elution HIC. All gradient HIC–LALLS and SEC–LALLS studies were performed at room temperature (25°C), without constant temperature conditions (ambient). Off-line d*n*/d*c* measurements were performed using the same isocratic mobile phase conditions used in SEC–LALLS studies, utilizing exacting quantities of Tris and citrate buffers in the reference side of the cell, according to the manufacturer's specifications, to avoid dialysis and sample deterioration.

Chemicals

Alkaline phosphatases, both bovine intestinal mucosa and bacterial, were obtained from Sigma (St. Louis, MO, U.S.A.), as either solutions in 3.0 *M* sodium chloride containing 1 m*M* magnesium chloride, 0.1 m*M* zinc chloride, and 30 m*M* triethanolamine, pH 7.6, or as the lyophilized powder containing 37.5% protein by a Biuret test (bovine). Bacterial alkaline phosphatase is available only as a suspension. Reagents for the off-line determination of enzymatic activity of alkaline phosphatase included: 4-nitrophenylphosphate disodium salt (Thiokol/Ventron Division, Danvers, MA, U.S.A., Sigma), glycine (Fisher), zinc chloride (Fisher), magnesium chloride (Fisher), and potassium hydroxide (Fisher). Inorganic reagents and mobile phases for the HPLC/SEC and HIC studies included: acetonitrile, HPLC-grade (Omnisolv brand, EM Science, or Fisher), methanol, HPLC-grade (EM Science or Fisher), water, HPLC-grade (EM Science), sodium hydroxide (Fisher), potassium phosphate monobasic (Fisher), ammonium sulfate, grade III, granular (Sigma), and ammonium acetate, anhydrous (Sigma).

Procedures

All solvents, buffers, and samples were filtered through a 0.45-μm hydrophilic polyvinylidenedifluoride membrane filter (low water extractable, low protein binding), either 47 mm, 25 mm, or 13 mm, or disposable cartridges having a Luer lock

syringe adaptor. Alkaline phosphatase activity was determined using the standard, off-line, enzyme–substrate approach[22]. This involved mixing known amounts of the commercial enzyme solution with the substrate, *p*-nitrophenylphosphate, and as a function of time and temperature, monitoring the absorbance of *p*-nitrophenol at 405 nm. The *p*-nitrophenylphosphate substrate contained varying amounts of *p*-nitrophenol, lot dependent, which had to be substracted as a blank from the enzyme activity results. This yielded activity units (*U*) as a function of concentration or amount protein. Off-line denaturation of the originally active enzyme was performed as a function of time and temperature, following the off-line activity before and after.

HIC–UV/LDA studies utilized known concentrations and volumes of the enzyme injected, with on-line, automated data collection of chromatograms as a function of wavelength chosen, wavelength ratioing (280:235 nm) of peak heights at any point in the eluting peak, and individual UV–VIS spectra at three points in the eluting peak. The standard data handling approaches available with the HP 1040A LDA system and software were employed to interpret and plot this data.

HIC/SEC–LALLS–UV–DRI studies were performed so as to best mimic the initial HIC–UV/LDA work, again using known concentrations and volumes injected of the enzyme. Data here were simultaneously collected, interpreted, and plotted using the Chromatix MOLWT3 or PCLALLS software, to provide LALLS and UV chromatograms. These were then reduced, by standard computer approaches, to provide the final weight average molecular weights (M_w^{ave}), verified and validated via FIA/STATIC–LALLS measurements. The d*n*/d*c* values were obtained using standard approaches, and as a function of d*n*/*c* *vs.* *c*. Appropriate d*n*/d*c* values were derived, following manual plotting of the data, for various gradient elution mobile phase compositions. The mean d*n*/d*c* was taken to be the true d*n*/d*c* value.

RESULTS AND DISCUSSION

There were basically two separate, yet related, major spectroscopic studies performed: (1) HIC–LDA chromatograms and dual-wavelength absorbance ratios (280:235) as a function of different mobile phase conditions, temperature, and concentrations of alkaline phosphatase injected; and (2) HIC– or SEC–LALLS–UV–DRI determination of molecular weight moments for alkaline phosphatase, with off-line d*n*/d*c* determinations. The latter data have suggested the precise species responsible for the HIC–LDA results. It is shown that there is a dual-wavelength absorbance ratio produced in HIC–LDA that appears not due to a single species of alkaline phosphatase under all HIC conditions, temperature of separation, and concentrations injected. The nature of the species present can only be deduced after determining the molecular weight of the species present under the same HIC condition first used with LDA. Rather than present the LALLS results first, which would not relate to the HIC–LDA studies, we have chosen to present the dual-wavelength absorbance ratio data first (HIC–LDA).

We present the results of several related studies dealing with bovine alkaline phosphatase, which have been sub-divided as: (1) reproducibility of HIC–LDA results, conditions, and resultant chromatographic data; (2) off-line determinations of alkaline phosphatase enzymatic activity; (3) UV spectra of bovine and bacterial alkaline phosphatase obtained by HIC–LDA; (4) HIC–LDA dual-wavelength absor-

bance ratio plots for bovine alkaline phosphatase; (5) HIC–LDA/UV spectra as a function of changes in dual-wavelength ratio plot; (6) HIC/SEC–LALLS–UV/DRI molecular weight results for bovine alkaline phosphatase; (7) off-line dn/dc determinations via laser differential refractometry; and (8) FIA/STATIC–LALLS determinations of M_w^{ave} and second virial coefficients (A_2).

HIC–LDA interfacing and optimization, reproducibility of HIC–LDA conditions, chromatographic data

To demonstrate the overall reproducibility of retention time and peak height: area values, studies were undertaken using HIC with a fixed-wavelength UV detector, intra-day and between days for alkaline phosphatase (bovine) (Table I). A single, symmetric peak (tailing factor range = 1.00–1.17) was observed under all isocratic and gradient elution conditions. The results indicated high reproducibilities for retention time, peak area, and peak height, under the given HIC conditions. We have performed analogous HIC studies with three other enzymes, *viz.*, ribonuclease A, lysozyme, and α-chymotrypsinogen A, yielding similar reproducibility data[23].

Off-line determinations of alkaline phosphatase enzymatic activity

Alkaline phosphatase solution (bovine) was analyzed for activity using the standard enzyme–substrate, off-line procedure involving *p*-nitrophenylphosphate as the substrate (Experimental). The results indicated an original activity for the enzyme which agreed fairly well with the manufacturer's stated value. The bacterial alkaline phosphatase was also analyzed for activity using the same procedures and was also similar to that reported by the supplier. It too had a single symmetric peak under gradient elution conditions, and a similar retention time as the bovine variety. It has not yet been possible using any HIC conditions to resolve these two enzymes.

These two enzymes are of interest with regard to their presence or absence in milk and dairy products, and it is possible that they may be markers of pasteurization and storage efficiencies[24]. Additional work is needed to demonstrate this hypothesis.

HIC–LDA/UV spectra of bovine and bacterial alkaline phosphatase

Although the two alkaline phosphatase enzymes were eluted at the same re-

TABLE I

REPRODUCIBILITY STUDIES FOR BOVINE ALKALINE PHOSPHATASE

HPLC conditions: 20 min linear gradient, 100% A to 100% B; mobile phase A = 2.5 *M* ammonium sulfate in 0.5 *M* ammonium acetate, mobile phase B = 0.5 *M* ammonium acetate; flow-rate = 1.00 ml/min, injection volume = 20 μl, UV detector at 280 nm (0.1 a.u.f.s.), ambient temperature, alkaline phosphatase concentration, 1 mg/ml.

Day No.	*Retention time (min)*	*Ret. time (R.S.D., %)*	*Peak area (R.S.D., %)*	*Peak height (R.S.D., %)*
1	10.38	1.6 (n=4)	7.1 (n=4)	3.6 (n=4)
2	10.37	1.1 (n=3)	4.0 (n=3)	2.3 (n=3)
3	10.27	0.5 (n=3)	13.7 (n=3)	4.5 (n=3)
4	10.11	3.4 (n=3)	9.6 (n=3)	7.7 (n=3)
5	10.45	0.3 (n=3)	6.1 (n=3)	2.3 (n=3)

tention times, they had substantially different UV spectra (Fig. 1). On-line enzyme–substrate post-column reaction studies, under these HIC conditions, indicated that the peak in question was the active form of bovine alkaline phosphatase[23,25]. The bacterial alkaline phosphatase had a much lower extinction coefficient at 280 nm than did the bovine alkaline phosphatase, though both had about the same absorbances at 225–230 nm. The concentrations injected in Fig. 1 for both enzymes were shown to be within the linear calibration region for both the linear diode array detector and a stand-alone, conventional UV–VIS spectrophotometer. Coefficients of linearity at all temperatures were approximately 0.999. Though the absorbances at 225–230 nm might appear to saturate the detector, this was demonstrated not to be the case. Overlapping of these two spectra highlighted the relative differences in UV extinction coefficients, though their maximum absorbances were similar.

HIC–LDA dual-wavelength absorbance ratio plots

We were interested to determine the chemical and enzymatic purity of the bovine alkaline phosphatase and thus utilized dual wavelength absorbance ratioing. It was expected that these ratio plots would be perfectly linear and horizontal, which is customary for a single, pure species[8–11]. The dual-wavelength ratios were apparently a function not only of the particular alkaline phosphatase present, but also (!) of all chromatographic parameters: (1) concentration injected; (2) temperature of separation; and (3) gradient conditions.

We chose a wavelength ratio of 280:235, mainly because these wavelengths appeared to be the general absorbance maxima for most proteins and enzymes. Our initial LDA work with bovine alkaline phosphatase at low concentrations indicated a wavelength ratio that was pseudo square wave, the usual result for dual wavelength

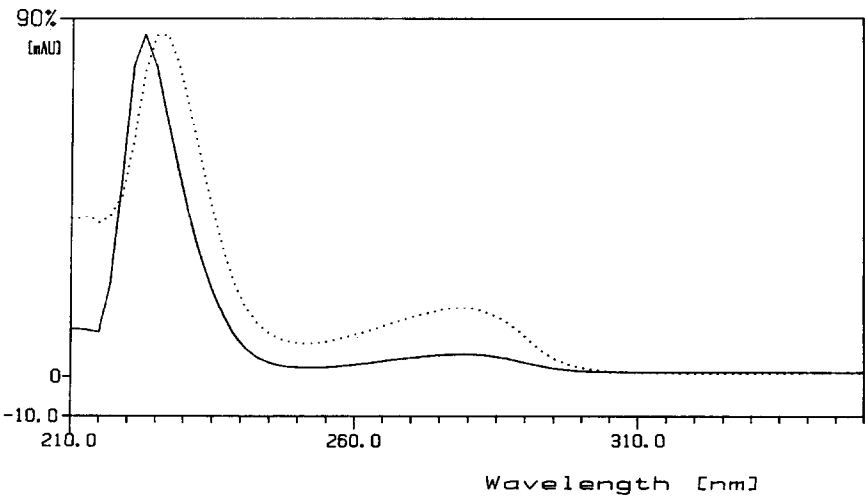

Fig. 1. LDA/UV spectra for bovine (· · · ·) and bacterial (———) alkaline phosphatase under typical gradient HIC conditions: 20 min linear gradient, 100% A to 100% B; A = 2.5 *M* ammonium sulfate and 0.5 *M* ammonium acetate; B = 0.5 *M* ammonium acetate; flow-rate, 1 ml/min, injection volume, 20 μl; ambient temperature; bacterial alkaline phosphatase concentration, 5.5 mg/ml, calf alkaline phosphatase concentration, 7.0 mg/ml.

ratioing in HPLC–LDA, wherein only a single species is present. There have been three main types of wavelength ratios reported in the literature; (1) flat and horizontal, suggesting a single, pure material; (2) linear, but non-horizontal, for at least two materials present with different UV spectra, but similar elution properties; and (3) non-linear and non-horizontal, for at least two materials present with different UV spectra and elution properties. A fourth possibility might be a Gaussian or pseudo-Gaussian peak that paralleled the chromatographic peak elution profile at a given wavelength of detection. This appears not to have been described previously, though it has been suggested as arising from instrumental causes rather than being analyte induced[9,10].

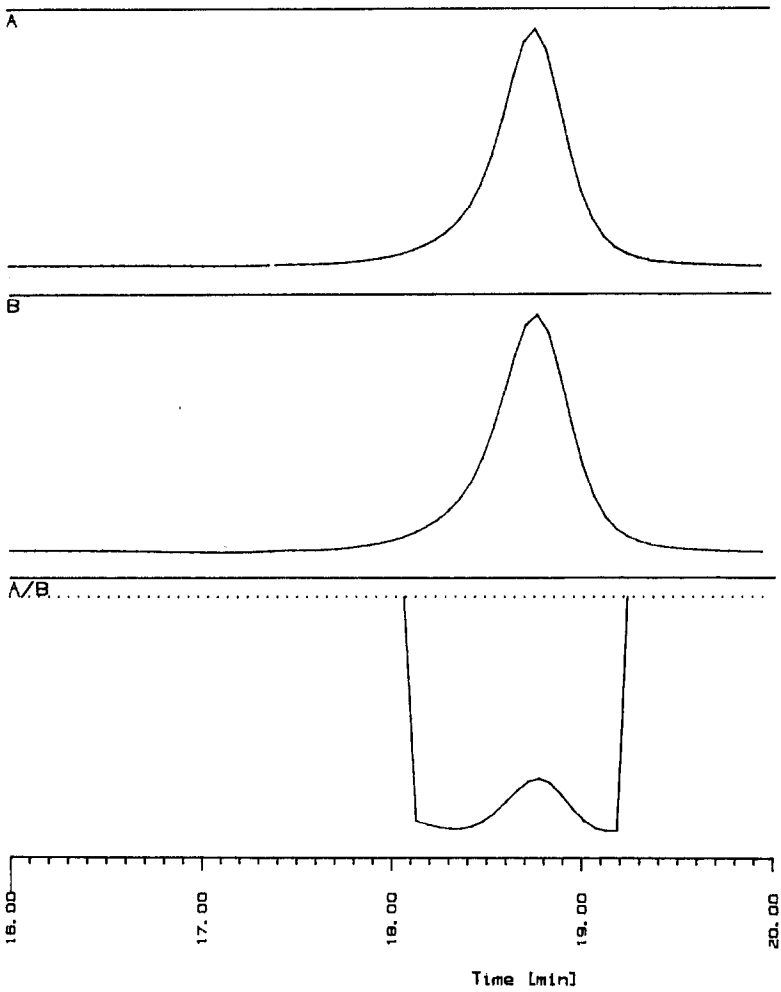

Fig. 2. Typical dual wavelength (A:B = 280:235 nm) ratio plot for bóvine alkaline phosphatase under one particular set of HIC conditions and concentration injected. Gradient elution profile No. 9, 100% A to 100% B in 20 min, 37°C, 7 mg/ml concentration, 20 μl-injections.

Lu *et al.*[18] have described a similar situation where a mixture of two closely eluting proteins produced a pseudo-Gaussian absorbance ratio plot. This type of dual-wavelength ratio peak is unusual. Its presence and intensity may be functions of several HIC parameters, including: (1) concentration injected; (2) temperature of separation; (3) gradient conditions; and (4) others.

Under virtually all HIC conditions and temperatures, at sufficient levels injected, the wavelength ratio peak resembled in shape that of the chromatographic elution peak, (Fig. 2). This particular plot was chosen to illustrate the nature of the wavelength ratio plot, that is, high concentration injected, gradient elution with long initial hold period, and high temperature for the separation (37°C). All of these conditions have been varied, singly, in order to study how they each affected the absorbance ratio peak height. Such studies were done in triplicate, and all of these results are summarized in Table II. The majority of the relative standard deviations (R.S.D.) ($n=3$) are less than 5%, which indicates that these numbers are reproducible. If we attempt to summarize these results, several things become evident.

First, holding all other chromatographic conditions constant, increasing the concentration injected always increased the peak height for the absorbance ratio peak. Second, if the gradient elution profile was changed from No. 6 (linear) to No. 9 (concave), and the concentration was held constant, thereby forcing the enzyme to remain on the HIC column for a longer perod of time before elution started, again the peak height for the absorbance ratio peak became greater, except at 5°C. Holding the original enzyme on-column for a longer gradient time in the initial solvent at the lowest temperature studied, apparently favored a species having a lower 280:235 absorbance ratio. Thirdly, holding concentration and elution conditions constant, but now varying the temperature, showed that higher temperatures led to a higher absorbance ratio peak height. It was also noted that increasing the injection temperature and HIC chromatography temperature (via submersion of both in a constant-temperature bath) increased the retention time. This is opposite to what is normally observed when one increases the temperature in HPLC, but is common for the HIC of proteins[26].

The Gaussian or pseudo-Gaussian dual absorbance ratio plots that mimicked the fixed-wavelength, concentration-dependent UV profiles, suggested concentration dependency. That is, there were changing concentrations and concentration ratios of the species contributing to the final, observed absorbance ratio plots. An increasing concentration of a second species, at the expense of the first species, might have contributed to the constantly increasing absorbance ratio plots. This requires the assumption that increasing concentrations of the initially injected alkaline phosphatase, as the peak eluted from the HPLC, led to the induction/formation of a second species, perhaps aggregate or conformation, at ever-increasing concentrations until the maximum elution concentration was reached.

The trends indicated in Table II, which could be plotted graphically, can be summarized as follows. It is possible that conformational changes and/or aggregate formation could account for these observations. However, in view of the M_w^{ave} LALLS studies described below, we discuss only conformational changes.

(1) Holding the temperature constant, increased concentrations injected led to increases of the ratio peak heights. This phenomenon was enhanced at higher temperatures. If the interconversion of the initial, major conformation was concentration

TABLE II

HIC–LDA DUAL ABSORBANCE RATIOS FOR BOVINE ALKALINE PHOSPHATASE UNDER VARIOUS CONCENTRATION, GRADIENT AND TEMPERATURE CONDITIONS

Concentration (mg/ml)	*Gradient (min)*	*Retention time (min)*			*S.D./R.S.D.(%)*			*Peak height (mm)*			*S.D./R.S.D.(%)*		
		5°C	*20°C*	*37°C*	*5°C*	*20°C*	*37°C*	*5°C*	*20°C*	*37°C*	*5°C*	*20°C*	*37°C*
7	20, linear	8.44	10.72	11.31	0.16/2.0	0.13/1.2	0.11/1.0	3.42	5.08	6.38	0.14/4.2	0.14/2.8	0.38/6.0
3.7	20, linear	8.54	10.39	11.37	0.17/2.0	0.26/2.5	0.10/0.9	1.67	2.33	6.38	0.14/8.6	0.14/6.2	0.38/6.0
1.75	20, linear	8.21	10.51	11.32	0.21/2.5	0.07/0.7	0.11/1.0	−2.92*	0	0	0.14/4.9	0/0	0/0
7	20, linear	8.44	10.72	11.31	0.16/2.0	0.13/1.2	0.11/1.0	3.42	5.08	6.33	0.14/4.2	0.14/2.8	0.38/6.0
7	20, No. 9	17.26	18.37	18.74	0.21/1.2	0.05/0.3	0.07/0.4	2.92	7.42	10.58	0.14/4.9	0.14/1.9	0.52/4.9
7	30, No. 9	24.38	25.99	26.67	0.17/0.7	0.06/0.2	0.12/0.5	1.67	5.5	6.67	0.14/8.6	0.25/4.5	0.14/2.2

* This is apparently real, but actually a function of the method of representing wavelength ratios, which cannot yield a negative value. It really signifies a ratio less than 1.00, but more than 0.00.

dependent, then injecting higher concentrations would lead to a greater percentage of the second conformation. Higher temperatures also appeared to cause a greater conversion of one conformation to the second, along with an increased absorbance ratio peak height. For simplicity's sake, we argue here from only two conformations in equilibrium, but in reality this has not been proven. There might indeed be more than just two conformations responsible for these observations, but it is currently impossible to determine the precise number. The M_w^{ave} results described below suggest that aggregates do not play a significant role in these LDA spectral changes.

It has generally been assumed that protein conformational changes can not be induced just by concentration increases. In the absence of additional experimental results, we are here assuming that in the case of alkaline phosphatase, this assumption may not be valid. All of the data in-hand supports the idea that changing concentrations of the first species injected can indeed lead to conformational equilibrium between more than one species, perhaps several?

(2) At room temperature and 37°C, concentration held constant, increased periods of time for alkaline phosphatase on the column caused increases in ratio peak heights. However, the peak height decreased as one went from 18 to 25 min. At 5°C, however, for increased lengths of time that the compound sat on the column, the ratio peak height decreased. At the first two temperatures, increased column residence times led to a greater interconversion of the first conformation to the second, with the now-expected increased wavelength ratio peak height. It is not apparent why there was no further change in this ratio from 18 to 25 min, unless the maximum possible ratio of conformations was already reached at 18 min. At lower temperatures, apparently the initial conformation is favored, and with longer column residence times, more of the second conformation reverts to the first with a decreased peak height (purer composition).

(3) For any particular time period that the enzyme sat on the column, with the concentration held constant, increasing the temperature caused an increased ratio of peak heights. This increase in peak height was most dramatic when the time spent on the column went from 10 min to 18 min to 25 min. As the column temperature increased, for a fixed residence time, the initial conformation converted to the final, there was a greater mixture of the two, and a higher absorbance ratio peak height.

The explanation for the observed phenomena appear to be somewhat straightforward. What is most important is to understand the cause of the Gaussian or pseudo-Gaussian peak shape for the absorbance ratio. That is, this peak shape was always identical to the chromatographic profile peak, Gaussian produced Gaussian, skewed produced skewed, leading produced leading, and tailing produced tailing. The fact that the absorption ratio was not constant over the elution profile, under most conditions studied, suggested the presence of at least two species or conformations of the same basic enzyme. And, that these two conformational species were easily interconverted as a function of concentration, temperature, and time on the column. The ratio profile peak must have been concentration dependent, and as the concentration of the eluting peak changed, this was directly reflected in the ratio peak height, which then affected its final shape.

The ratio plot represents a mixture of at least two species with different spectra and distributed over the concentration profile with the same concentration distribution of the profile itself, otherwise the so-called "peak maxima" can not coincide.

The conformational change was very fast relative to the elution of the profile.

The UV spectra for at least two conformations involved can only be suggested from the observed changes in UV maxima heights (280:235 nm) as a function of concentration injected, residence time on column, and column temperature. The ratio of 280:235 intensities for the second conformation was obviously greater than for the first, since increased amounts of it led to greater ratio peak heights. This meant that the second conformation had a higher extinction coefficient at 280 nm than the first conformation, and/or a lower extinction coefficient at 235 nm.

It would appear that conformational changes induced by the HIC operating conditions, are slow enough to be detected after elution from the analytical column. Refolding and/or disaggregation (*vide infra*) would appear to be slow kinetic processes on the chromatographic detection time scales. The ability to detect conformational changes, as well as the aggregate formation below, appear to be functions more of the mobile phases or temperatures present, rather than the HIC stationary phase.

HIC–LDA/UV spectra as a function of changes in wavelength ratio plot

If the above observations and explanations for the dual-wavelength ratio plots are valid, then one should observe differences in the summary UV spectra taken under different conditions. That is, as the second conformation built up in relative concentration to the original, the summary UV spectrum for the combination of the two would have been different than either alone. These summary or combination spectra for whatever conformations were present, were taken at the point of maximum peak height (apex) in the normal HPLC elution profile, *e.g.*, Fig. 2 at about 18.75 min. This is illustrated in Fig. 3, which shows the summary spectra under differing HIC operating conditions, temperature, and concentrations injected, as well as the correlative ratio plots (Table II). It is clear that the UV spectra changed sub-

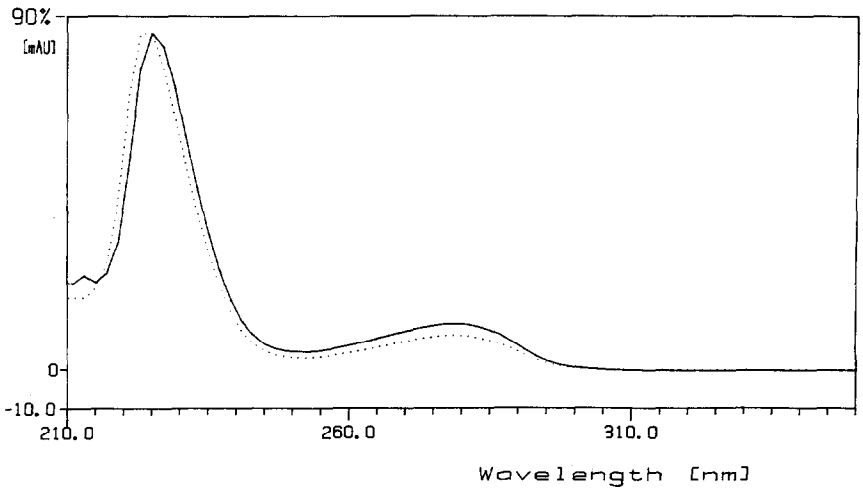

Fig. 3. HIC–LDA/UV spectra for bovine alkaline phosphatase under two different HIC temperature conditions, all other conditions identical. Gradient elution profile No. 9, 100% A to 100% B in 30 min, 5°C (· · ·) and 37°C (———), 7 mg/ml concentration, 20-μl injections.

stantially with changes in wavelength ratio plots. There was a shift in the spectrum taken at 5°C to that at 37°C of the absorbance maxima from about 225 ± 1 nm to 227 ± 1 nm, but that at 280 ± 1 nm remained at the same wavelength. There were increases in absorbances at both 235 nm and 280 nm, again in going from 5°C to 37°C.

The above data could be interpreted on the basis of: (1) interconverting monomeric species having different UV spectra; or (2) interconverting monomer–aggregate(s) formed under HIC conditions, wherein these two species had different UV spectral absorbances at 280:235 nm. Without molecular weight information it would have been impossible to assign a single mechanism to the LDA observations. The demonstration of little aggregate formation under these HIC conditions, using the on-line LALLS measurements below, left interconverting conformers as a reasonable mechanism.

HIC/SEC–LALLS–UV–DRI interfacing and optimization

One of the major goals of this study with biopolymers was to identify individual chromatographic peaks using LALLS. Most of the literature on HPLC detection of biopolymers has monitored analyte peaks without identifying molecular weights. Literature exists related to HPLC/SEC–LALLS, though mostly with SEC conditions for organic or biopolymers. Much less had been done with modern HPLC

TABLE III

SEC–LALLS–UV–DRI MOLECULAR WEIGHT AVERAGES WITH VARYING A–B BUFFER COMBINATIONS

Column, SW 3000

*Eluent/conditions**	M_z^{ave}	M_w^{ave}	M_n^{ave}	M_w^{ave}/M_n^{ave}
A–B (50:50)				
(Low salt, μ = 4.625, 1.25 *M* $(NH_4)_2SO_4$ + 0.5 *M* ammonium acetate, downswing of HIC peak)**				
UV–LALLS	99000	75400	70300	1.07
DRI–LALLS	107800	75200	65200	1.15
Monomer peak at 11.14 min = 68000 daltons from M_ic_i printout				
Dimer peak at 8.75 min = 134000 daltons from M_ic_i printout				
A–B (60:40)				
(Higher salt, μ = 5.450, 1.5 *M* $(NH_4)_2SO_4$ + 0.5 *M* ammonium acetate, upswing of HIC peak)				
UV–LALLS	188400	149000	139000	1.07
DRI–LALLS	165000	142000	133400	1.07
Dimer peak at 13.07 min = 130000 daltons from M_ic_i printout				
Trimer peak at 9.40 min = 195000 daltons from M_ic_i printout				
*A–B (70:30)****				
(Highest salt, μ = 6.275, 1.75 *M* $(NH_4)_2SO_4$ + 0.5 *M* ammonium acetate, before elution of HIC peak)				
UV–LALLS	266000	167000	155000	1.08

* Concentrations injected under SEC conditions were 4.0 mg/ml, 100-μl injection, 400 μg mass, $N - 1 = 4$ (only mean reported).

** μ = ionic strength = $1/2\ \Sigma\ c_i z_i^2$ where c_i = concentration of a given ion and z_i = charge on that ion.

*** Enzyme is barely soluble in this high salt concentration buffer. Peaks eluting suggest presence of both dimer and/or trimer, under these conditions.

approaches, such as HIC. It has generally been felt that only isocratic SEC conditions are fully compatible with LALLS operations.

SEC/HIC–LALLS–UV–DRI results

Molecular weight determinations via LALLS (633 nm) require the determination of the excess Rayleigh scattering factors ($\bar{R}_\theta$) for known concentrations of the biopolymer, at each point in the elution profile. This data is obtained by determining $\bar{R}_\theta$ via the LALLS instrument, and concentration changes via either UV (fixed wavelength) or DRI. Also needed are accurate and precise, off-line determinations of dn/dc, under mobile phase conditions identical to those used for either SEC or HIC of the biopolymer. The dn/dc determinations are made under off-line laser (633 nm) differential refractometer conditions, separate from the HPLC–LALLS–UV–DRI determinations (Experimental).

In the initial chromatograms and calculations of SEC–LALLS–UV–DRI for bovine alkaline phosphatase, we assumed certain values for n (refractive index) and dn/dc (changes in n with changes in concentration). The final calculations (Table III) were done using experimentally determined n and dn/dc values, at different concentrations of alkaline phosphatase. Based on the initial gradient HIC–UV/LDA results, our initial studies with SEC utilized a A–B (50:50, v/v) mobile phase run isocratically with a TSK SW3000 SEC column (Fig. 4a). This figure illustrates the UV wavelength detection at 280 nm (top), as well as a typical LALLS trace (bottom). Both chromatograms indicate the presumed formation *in situ* (mobile phase) of more than a single species/peak, but with incomplete resolution. In view of the FIA/STATIC–LALLS data, without any HPLC column, it was likely that these species were more strongly influenced by the mobile phase make-up. The M_w^{ave} data (Table III), indicated that the major peak was a monomer, since its M_w^{ave} agreed with that reported for this species (69 000 daltons)[24]. The earlier eluting, second major peak, had a M_w^{ave} equal to about 134 000 daltons. Table III was obtained from SEC–LALLS chromatograms having incomplete resolution of the two peaks in question, under

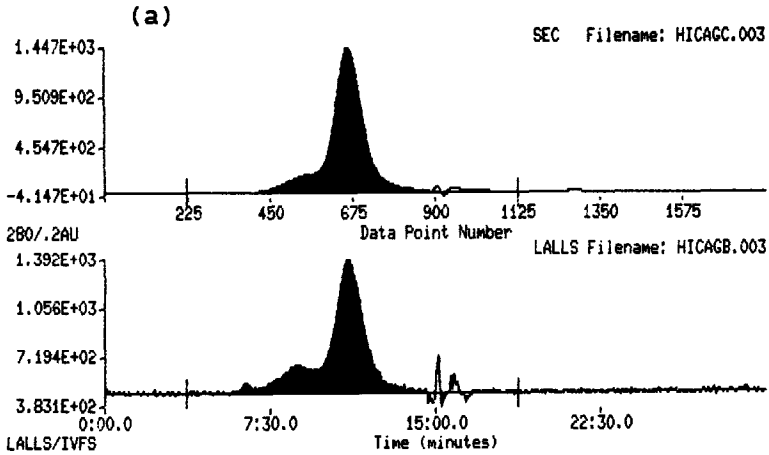

Fig. 4.

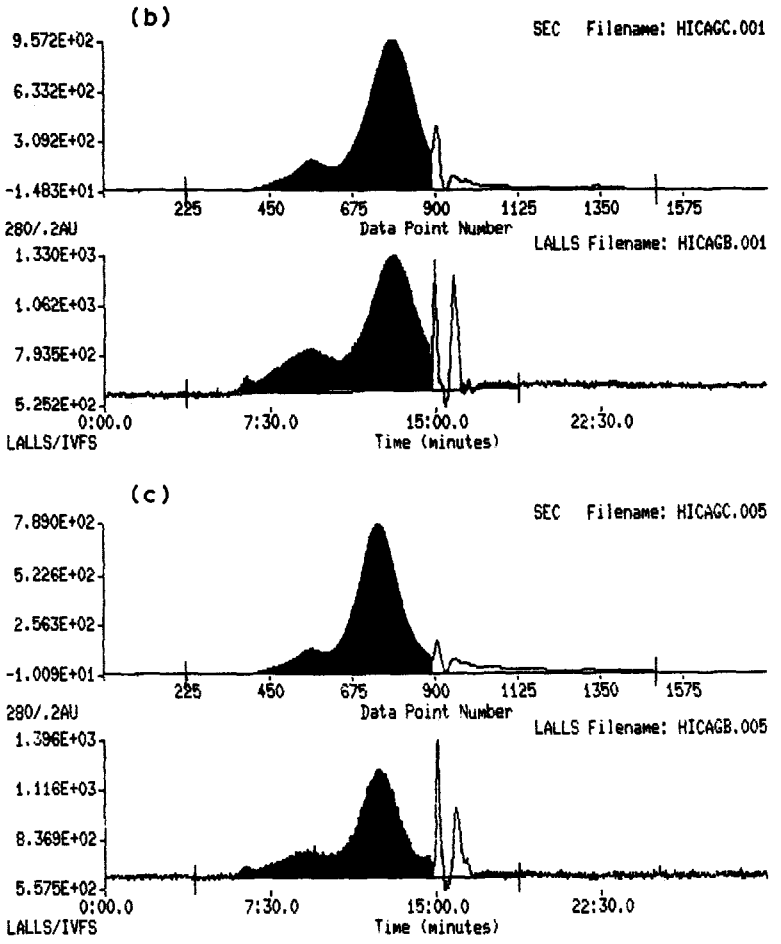

Fig. 4. (a) SEC–LALLS–UV–DRI chromatograms of bovine alkaline phosphatase with A–B (50:50) buffer. Flow-rate, 0.75 ml/min (μ = ionic strength = 4.625), 1.25 *M* ammonium sulfate; injected mass, 100 μl of 4.05 mg/ml = 405 μg; column, TSK-SW-3000 (30 cm); KMX-6 parameters, 6–7 degrees annuli, 0.2 mm field stop, 5 mm flow through cell, G_0 = 400 mV with $D = 4.36982 \cdot 10^{-9}$, solid angle = 651.81, G_θ = 310 mV, $K = 1.5213 \cdot 10^{-7}$ moles cm^2/g^2 with $n = 1.3600$ and $dn/dc = 0.142$ ml/g, detector lag to spectromonitor D UV–VIS detector = 3.5 s monitoring at 280 nm at a sensitivity of 0.2 a.u.f.s., and the detector lag to Refractomonitor IV DRI detector = 12.75 s operating at 1 V.f.s. = $1.00 \cdot 10^{-3}$ RIu.f.s. LALLS, UV, and DRI outputs were linked to a DEC Micro PDP-11/23+ using MOLWT3 and/or an IBM PC-AT using PCLALLS software and also to a strip chart recorder. (b) SEC–LALLS–UV–DRI chromatograms of bovine alkaline phosphatase A–B (60:40) buffer. Conditions same as in a, except μ = 5.450, 1.50 *M* ammonium sulfate; injected mass = 100 μl of 4.055 mg/ml = 405.5 μg; solid angle = 653.84; $K = 1.0714 \cdot 10^{-7}$ moles cm^2/g^2 with $n = 1.3642$ and $dn/dc = 0.1188$ ml/g. (c) SEC–LALLS–UV–DRI chromatograms of bovine alkaline phosphatase with A–B (70:30) buffer. Conditions same as in a except, μ = 6.275, 1.75 *M* ammonium sulfate; injected mass, 100 μl of 4.055 mg/ml = 405.5 μg; solid angle = 655.88; $K = 6.9806 \cdot 10^{-8}$ moles cm^2/g^2 with $n = 1.3684$ and $dn/dc = 0.0956$ ml/g.

isocratic mobile phase conditions meant to mimic the above gradient elution HIC mobile phase compositions.

Thus, the M_w^{ave} values were, depending on the method of calculating, in between those for the monomer and dimer (70 000–140 000). However, when individual M_w values at a given elution concentration, known as M_ic_i data points ($M_i = M_w$ at point i, c_i = concentration at point i), were taken at the apex of each eluting peak [*e.g.*, 11.14 and 8.75 min (Table III), A–B (50:50)], there was excellent agreement between the expected (literature) and experimental results for monomer and dimer.

There is evidence in the literature that alkaline phosphatase exists, at times, as a glycoprotein of 140 000 daltons, which could explain an earlier eluting peak in SEC–LALLS[24]. It is also possible, and perhaps more likely, in the absence of other information, that this peak was/is the dimer, comprising two very similar or identical subunits of approximately 69 000 daltons. We now wonder if alkaline phosphatase ever existed as a species of 140 000 daltons, or whether previous workers have always observed a dimer? In the absence of an appropriate buffer that has been shown to prevent aggregation, previous studies may have already measured the dimer? The somewhat higher value of 75 400 daltons for the monomer, as compared to the expected 69 000, may be due to the presence of some dimer in the monomer peak, or experimental error.

Fig. 4a [A–B (50:50) mobile phase] clearly showed the presence of two separate peaks, in the ratio of about 10:1 monomer–dimer by UV and a bit different by LALLS, perhaps 5:1. LALLS response, being concentration and molecular weight dependent gave an artificially higher, apparent ratio, biased by the higher molecular weight of the smaller, earlier eluting peak (dimer/aggregate). Fig. 4b was similar to this, but now a A–B (60:40) mobile phase mixture of higher salt concentration than before was used, and again showed the presence of two species. Under these HIC conditions (Table III) calculations suggested the presence of two aggregates. In addition to the same dimer as before, there was also an earlier eluting trimer. In Fig. 4c a A–B (70:30) mobile phase mixture was used, and the UV trace again showed two major species. Weight-average molecular weight calculations (Table III), indicated that the major peak here was a dimer, similar to that observed in Fig. 4b.

There appeared to be a shift in retention times for the same species as a function of the SEC mobile phase conditions. This was unusual for SEC, where the same retention times were expected, if purely a size-exclusion mechanism was operative. We believe that these SEC conditions promoted a mixed-mode mechanism, involving both conventional size exclusion, along with some type of hydrophobic interaction or insolubility mechanism/pathway. At very high ionic strengths (high %A compositions), partial insolubility of the aggregates could have led to a longer retention time.

Under the last set of conditions (Fig. 4c), the enzyme was barely soluble in this high-ionic-strength salt mobile phase, and thus these particular molecular weight measurements may not be as useful as the initial ones. In order for LALLS measurements to be truly valid and determinative of the molecular weight of the species, all of the biopolymer must be soluble. Mass balance laws must be adhered to for the chromatography and LALLS measurements to be thermodynamically correct. Calculation of the molecular weight for any species depends on knowing or assuming the concentrations in solution, as well as measuring dn/dc and the excess Rayleigh

scattering factors. Where some of the aggregate is insoluble, then the assumed concentration is actually too high, leading to a lower, apparent molecular weight value. This assumed insolubility at high %A mobile phases, (Fig. 4c), was strongly supported by the off-line, FIA/STATIC–LALLS determinations reported below. The second virial coefficient, A_2, found for this mobile phase mixture of A–B (70:30) was unusually negative in comparison to those determined at lower %A compositions (Table VI). This A_2 value has usually been taken to denote semisoluble polymer systems.

It appears that, as a function of the mobile phase composition, it is possible to force alkaline phosphatase to exist mainly as the monomer, with some dimer present (Fig. 4a), mainly dimer with some trimer present (Fig. 4b), and finally as mainly a dimer, with perhaps higher aggregates present (Fig. 4c). LALLS traces have clearly suggested a third, perhaps higher order aggregate eluting earlier than the monomer–dimer.

The gradient HIC–UV (280 nm) chromatograms at fixed wavelengths (Figs. 2 and 5a), had suggested that the major peak under gradient HIC–LDA conditions was the monomer, rather than any aggregate. However, Fig. 5b is the first chromatographic evidence that under gradient HIC–LALLS conditons, the leading shoulder on the main peak was really another species. Chronologically, the gradient HIC–LALLS studies were done before the isocratic SEC–LALLS ones. Fig. 5b contains a gradient HIC–LALLS trace for bovine AP. Scale expansion and baseline subtraction of Fig. 5b, included here, further supported the presence of a leading shoulder. Fig. 5c illustrates an improved computer subtraction of the baseline drift and noise, under the same chromatographic injection conditions as in Fig. 5b. There appears to be a leading shoulder (peak) eluting just before the main peak. LALLS appeared to be a better chromatographic determinant of other species present under both SEC and HIC conditions than fixed-wavelength UV detection alone. This was because under LALLS determinants, the amount determined and reported is a multiplicative of both concentration and M_w^{ave}, whereas for UV it is just the concentration of each species that appears. However this meant that the UV traces were more closely resembling actual concentrations present (about 10:1, monomer–dimer), assuming equal extinction coefficients on a molar basis. This again suggested that the true ratio of monomer–dimer under any HIC condition was probably reflected by the UV and not LALLS trace.

Table IV summarizes the set of gradient HIC–LALLS data which showed an average M_w^{ave} moment of about 88 500 daltons. This was higher than the above SEC measurements for the monomer, but below the analogous measurements for the dimer, indicating a mixture of both species, with more monomer than dimer present. Evidence for the presence of more than a single species also came from the polydispersity factor, M_w^{ave}/M_n^{ave}, which ranged from 1.15 to 1.36 (Table IV). This term has often been used to denote homogeneity for a polymer–biopolymer sample.

In view of the above, it must have been the monomer that was undergoing conformational changes as a function of HIC parameters, since the absorbance ratio plots (peaks) were all mimicking the concentration-dependent, fixed-wavelength UV plots. If the monomer to dimer (smaller, earlier peak) conversion was the cause of the changing absorbance ratio plots, with the dimer not co-eluting with the main, monomer peak, this system could not have been mimicking the shape of the chro-

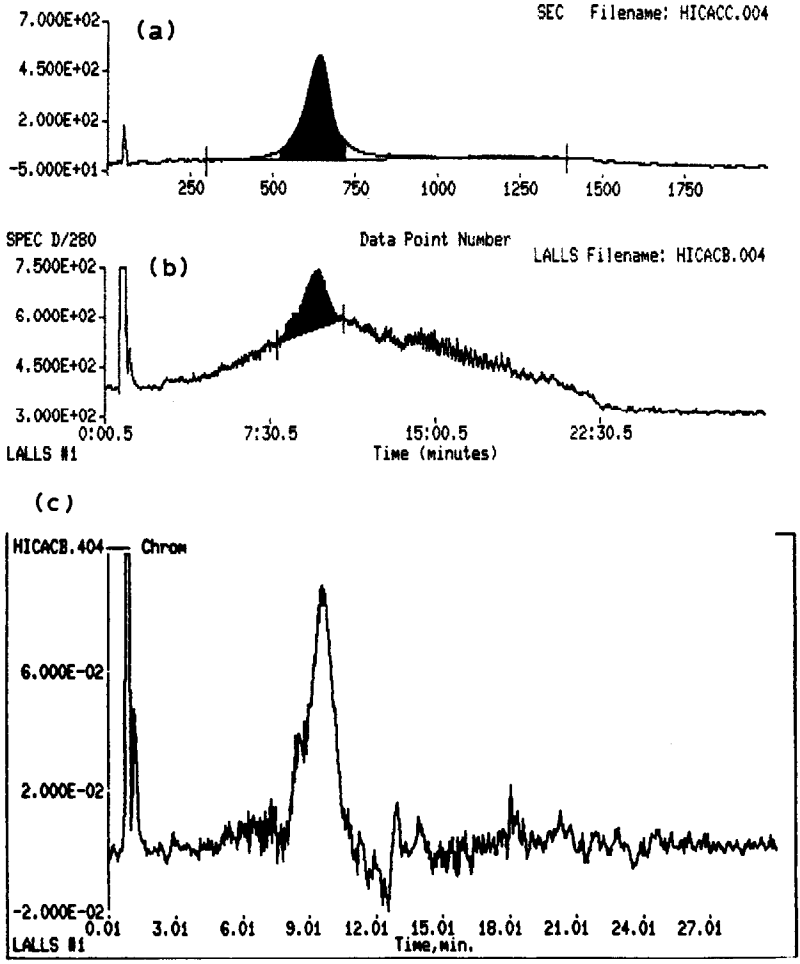

Fig. 5. Gradient HIC–LALLS–UV chromatograms of bovine alkaline phosphatase (quantitating with 45% B parameters, peak apex). Gradient, 10–90% B in 20 min (linear); flow-rate, 1.00 ml/min; injected mass, 20 μl of 7.00 mg/ml = 140 μg; column, Beckman C_1-ether phase HIC; KMX-6 parameters, 6–7 degrees annuli, 0.2 mm field stop, 5 mm flow through cell, G_0 = 200 mV with D = 4.36982 · 10^{-9}, solid angle = 652.83, G_θ = 190 mV, K = 1.2868 · 10^{-7} moles cm^2/g^2 with n = 1.3621 and dn/dc = 0.1304 ml/g, detector lag to Spectromonitor D UV–VIS = 3.5 s monitoring 280 nm at a sensitivity of 0.2 a.u.f.s. LALLS and UV outputs linked to a DEC Micro PDP-11/23+ using MOLWT3 software and/or an IBM PC-AT using PCLALLS software and also to a strip-chart recorder. (a) UV; (b) LALLS; (c) as in b, but with better computer-software baseline subtraction to improve drift and smooth-out baseline noise.

matographic, concentration peak at a fixed wavelength. In addition, since we have concluded that the UV traces were more diagnostic of aggregate–monomer concentrations, under gradient HIC–UV/LDA conditions, by far the major contributor to the dual absorbance ratio plot was the monomer and not the dimer. The dimer, at most, was accounting for less than 10% of the total peak area.

TABLE IV

GRADIENT HIC–LALLS–UV MOLECULAR WEIGHT AVERAGES

Concentrations injected under HIC conditions were 7.0 mg/ml, 20-μl injection, 140 μg mass. Column, Beckman Spherogel CAA-HIC; gradient, linear 10–90% B in 20 min; flow-rate 1.00 ml/min (45% B parameters for quantitation = peak apex).

	M_z^{ave} ± S.D. (% R.S.D.)	M_w^{ave} ± S.D. (% R.S.D.)	M_n^{ave} ± S.D. (% R.S.D.)	M_w^{ave}/M_n^{ave} ± S.D. (% R.S.D.)
	97 700	92 900	80 500	1.15
	90 600	84 100	62 100	1.35
	95 300	86 400	75 400	1.15
	93 100	90 200	69 200	1.30
	94 500	89 100	72 100	1.24
Mean MW moments	94 300 ± 2600 (2.76)	88 500 ± 3400 (3.84)	71 900 ± 6900 (9.60)	1.24 ± 0.09 (7.26)

There is yet another conclusion that may be drawn, tentatively, from all the above observations. If the dimer had a very different UV spectrum from the monomer, and was undergoing significant dual absorbance ratio changes under different HIC elution conditions, then it would have offset or distorted the Gaussian or pseudo-Gaussian absorbance ratio peaks observed, since it was a leading edge to the monomer. This was not observed. One may assume that the dimer of alkaline phosphatase had similar UV properties, though not actually measured, and it did not undergo conformational changes together with UV spectral changes under any of the HIC elution conditions. The dimer appeared to be much more rigid and less fluxional than the monomer. If it was not, this was not evident from changes in dual absorbance ratios. It is not yet possible to pinpoint the exact phenomenon, but all of this suggests that the dimer is more rigid and less prone to conformational changes.

Off-line dn/dc determinations via laser differential refractometry

Crucial to the accuracy of the above M_w^{ave} measurements via both SEC–LALLS and HIC–LALLS was the accuracy of the dn/dc values as a function of mobile phase composition through the HIC gradient of the eluting peaks. These dn/dc values were measured under off-line conditions, as is customary, using isocratic mobile phases meant to mimic compositions present under isocratic SEC– or gradient HIC–LALLS (Table V). Determinations at A–B (50:50) buffer composition provided a value for dn/dc of 0.142 ± 0.002 ml/g, which was close to an assumed or expected value of 0.15 ml/g. Determination at the other most important A–B ratio, 62:38, provided a dn/dc value of 0.114 ± 0.002 ml/g. The refractive index measurements (Table V) at these two mobile phase conditions were almost equal, 1.360 and 1.365, respectively.

Thus, one can not assume equal dn/dc values for typical biopolymers under gradient elution conditions. The dn/dc values must be independently measured for various parts of any elution peak, or measured via on-line techniques. It remains crucial to physically determine dn/dc under HIC or any gradient elution conditions, off-line. Unfortunately, off-line measurements of dn/dc are manpower intensive and biopolymer consuming. In the future, methods should be developed to overcome

TABLE V

EXPERIMENTALLY DETERMINED SPECIFIC RI INCREMENTS AND MOBILE PHASE RI FOR DIFFERENT A–B BUFFER COMBINATIONS

*%B in buffer combination**	*n(RIu.)*	*dn/dc (ml/g)*
38	1.3650	0.1144
50	1.3600	0.1420

* Solvent/buffer contained exacting quantities of equimolar Tris and citrate buffers per manufacturer's (Sigma) specifications to avoid dialysis and sample deterioration.

these problems, such as by providing standard tables of d*n*/d*c* values for typical biopolymers *vs.* eluting solvents. Another approach would be to perform on-line d*n*/d*c* measurements as part of the gradient HIC–LALLS–UV–DRI process. This could circumvent all of the problems with current approaches.

FIA/STATIC–LALLS determinations of the weight average molecular weight (M_w^{ave}) and second virial coefficient (A_2)

The above on-line LALLS determinations were supported by traditional FIA/STATIC–LALLS measurements for molecular weight moments of alkaline phosphatase under four different mobile phase conditions. These conditions (Table VI and Fig. 6a and b) were meant to mimic portions of the gradient HIC elution. At high %B compositions (50, 45%), the lines approached similar intercepts. At lower %B compositions (40, 30%), the intercepts became higher. The final M_w^{ave} data again suggested the presence of higher-order-mers of alkaline phosphatase, as well as the presence of mainly the monomer under at least some buffer/elution conditions. The extremely large negative A_2 values (which is a measure of solute–solvent interaction) at low %B indicated a high degree of biopolymer interacting with biopolymer, rather than with solvent, which then gave rise to insolubility and thus inaccurate values for M_w^{ave}.

FIA/STATIC–LALLS determinations, which have long been the more traditional method for determining weight-average molecular weights of organic and bio-

TABLE VI

FIA/STATIC–LALLS–UV–DRI DATA UNDER VARYING A–B BUFFER COMBINATIONS

A–B (%)	*M_w^{ave} daltons ± S.D. (% R.S.D.)*	*A_2 (ml-mol/g²)*
50:50	135800 ± 500 (0.37)*	$8.539 \cdot 10^{-4}$
55:45	150800 ± 1700 (1.15)	$-4.817 \cdot 10^{-4}$
60:40	53900 ± 100 (0.21)	$-9.813 \cdot 10^{-3}$
70:30**	16200 ± 1000 (6.14)	$-1.634 \cdot 10^{-1}$

* Numbers represent the weight-average MW ± standard deviation of the reciprocal of the *y*-intercept and % relative standard deviation (S.D./average · 100%).

** Enzyme is barely soluble, if at all, in this A–B (70:30) buffer combination.

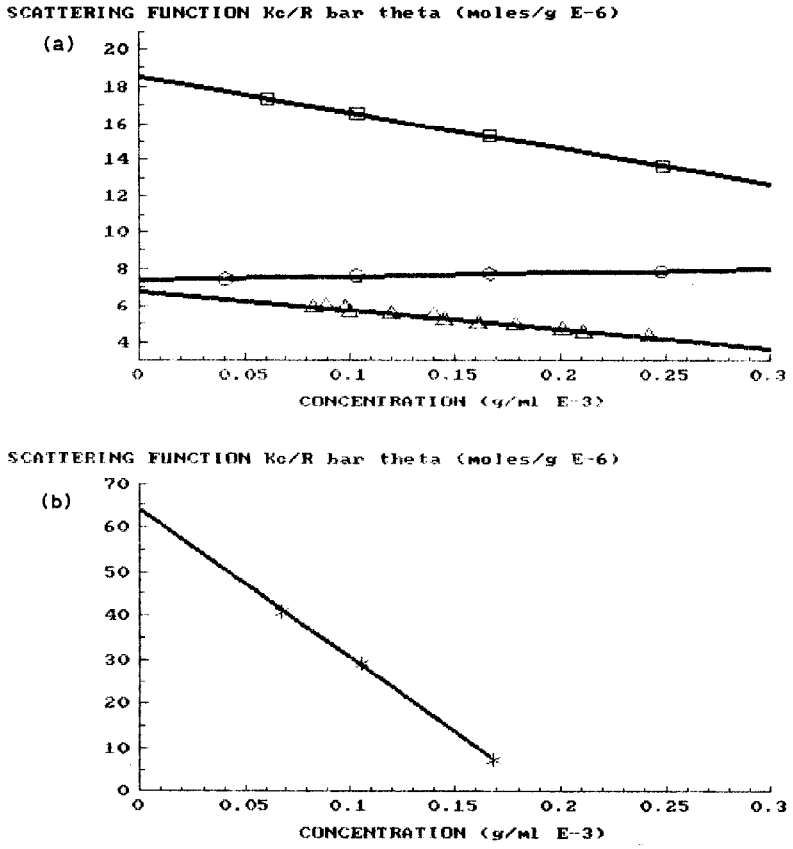

Fig. 6. FIA/STATIC–LALLS–UV–DRI plot for (○) A–B (50:50) (△) A–B (55:45), and (□) A–B (60:40) buffer combinations. Flow-rate, 0.1 ml/min; 1000 μl loop; injected concentration range = 0.04–0.25 mg/ml; KMX-6 parameters, 6–7 degrees annuli, 0.2 m field stop, 5 mm flow through cell, G_0 = 400 mV with D = 4.04249 · 10^{-9}, solid angle = 651.81, G_θ = 725 mV, K = 1.5213 · 10^{-7} moles cm²/g² with n = 1.3600 and dn/dc = 0.142 ml/g; Spectromonitor D UV–VIS detector was set at 280 nm with a sensitivity of 0.2 a.u.f.s.; Refractomonitor IV was set at 1 V.e.s. = 1.0 · 10^{-3} RIu.f.s. LALLS, UV, and DRI outputs were collected on a strip chart recorder. Solvent/buffer contained exacting quantities of equimolar Tris and citrate buffers per manufacturer's specifications to avoid dialysis and sample deterioration. (b) FIA/STATIC–LALLS–UV–DRI plot for A–B (70:30) buffer. Conditions same as in a, except injected concentration range = 0.06–0.17 mg/ml; KMX-6 parameters, G_0 = 400 mV, solid angle = 655.88, G_θ = 690 mV, K = 6.9806 · 10^{-8} moles cm²/g² with n = 1.3684 and dn/dc = 0.0956 ml/g.

polymers in the absence of SEC–HPLC, can at times rely on an inhomogeneous solution, as here. In the case of alkaline phosphatase, we now know that, depending on the specific mobile phase (buffer) conditions, higher order aggregates may be present in varying amounts, with insolubility problems. Thus, the measured scattering function ($Kc/\bar{R}_\theta$), and therefrom derived M_w^{ave} values reported in Table VI, are really representative of mixtures and not of individual species. We also know that at low %B composition, high salt concentrations and high ionic strengths, much of alkaline phosphatase and its aggregates become insoluble and are forced out of solution, leading to incorrect values for M_w^{ave}. This is the basic mechanism of HIC

separations for biopolymers. The fact that as we decrease the %B present, the apparent M_w^{avc} decreases, is only a reflection of the increased insolubility of higher aggregates and lower concentrations of monomeric species.

These results should be a warning to others that FIA/STATIC–LALLS measurements for molecular weight moments of biopolymers may be misleading, depending on the nature of the biopolymer employed and the mobile phase/buffer conditions. Clearly, one could derive just about any final value for M_w^{avc} using FIA/STATIC–LALLS for alkaline phosphatase and possibly other biopolymers, again depending on the nature of the biopolymer employed and the mobile phase/buffer conditions. Determinations of weight average molecular weights for any biopolymer using FIA/STATIC–LALLS must be viewed with extreme caution and reservation, at least until some type of SEC or HPLC separation is combined with on-line LALLS/UV/DRI and possibly dn/dc measurements.

CONCLUSIONS

Linear diode array spectroscopy using wavelength ratioing can now be used to detect conformational equilibria, and changes in ratios of conformations as a function of concentrations injected, temperature of column, and gradient elution parameters. The rates and thermodynamics of these interconversions can also be studied via HIC–LDA approaches, but such information awaits more detailed mathematical treatments. Non-linear, Gausian or pseudo-Gaussian ratio peaks have been observed and explained, but they should be general for any species that interconverts rapidly on the chromatographic-detector time scales, especially proteins/enzymes. It is expected that other biopolymer and organometal fluxional isomers will exhibit this same phenomena, when such isomers elute under the same chromatographic peak.

Low-angle laser light scattering should be used to confirm other spectroscopic data, such as LDA, in order to assign such data to the correct species; monomer or aggregates. Molecular weight information can be obtained using salt gradient elution high-performance HIC conditions, with on-line LALLS–UV–DRI, suggesting its more widespread application to many different biopolymer systems. However, it should be emphasized that gradient HIC–LALLS techniques are not trivial nor simple, and that a large amount of instrumentation, computer hardware/software, experience and expertise in the laboratory, in HPLC and LALLS–UV–DRI, are all required. It is not quite as simple an approach as obtaining HPLC– fixed-wavelength UV chromatograms nor even HPLC–LDA data. But, at the same time, it can be and is a perfectly viable and practical technique. Future developments in our laboratory should be promising.

The heretofore restriction of LALLS to isocratic SEC or isocratic HPLC conditions, in general, has now been lifted, opening the way to a much more general utilization of this most valuable approach for determining molecular weight information of many other natural or man-made biopolymers. We shall shortly report a completely analogous study utilizing β-lactoglobulin A under similar gradient HIC–LALLS conditions, illustrating different degrees of aggregation[27]. The on-line utilization of LALLS with UV and DRI for simultaneous Rayleigh factor and dn/dc values is clearly another possible step forward in the general acceptance of such approaches for biopolymer characterization[28].

ACKNOWLEDGEMENTS

We acknowledge the technical assistance, guidance, and interest of C. Selavka and N. Grinberg at Northeastern University's Chemistry Department and The Barnett Institute. B. L. Karger at Northeastern University provided encouragement and stimulating discussions during the course of these studies, as well as making valuable suggestions on the final manuscript. Certain initial HIC conditions were derived and provided by N. Miller at Cambridge Analytical Assoc. We wish to express our appreciation to the Science Advisor Research Associate Program (SARAP) Advisory Committee for providing partial financial support that enabled the work to be conducted. The HPLC–LALLS (Chromatix "LALLS" Products) instrumentation was donated to Northeastern University by Milton Roy/LDC Division. Special appreciation is indicated to individuals within the LDC organization, H. R. Smith, J. Kelley, G. Cleaver, H. Kenny and B. Freeman. The Spectronic 1201 scanning UV–VIS spectrophotometer was donated by Milton Roy/Analytical Products Division (formerly Division of Bausch & Lomb), via R. Jarrett and R. Flores. We further acknowledge the donation to Northeastern University by Hewlett-Packard and S. George of an HP 1040A LDA spectrophotometer. A Hitachi D2000 Chromatointegrator was donated to Northeastern University by EM Science, via M. Gurkin and G. Desotelle. A Shodex SEC column was donated to Northeastern University by Alltech Assoc. Bovine alkaline phosphatase was donated to Northeastern University by Sigma, via D. Fagan. This is contribution number 345 from The Barnett Institute at Northeastern University.

REFERENCES

1 M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983.
2 S. Stein, in I. W. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development, An Introduction*, Aster Publ. Corp., Springfield, OR, 1985, p. 305.
3 F. E. Regnier and K. M. Gooding, in P. M. Kabra and L. J. Marton (Editors), *Liquid Chromatography in Clinical Analysis*, The Humana Press, Clifton, NJ, 1981, Ch. 14.
4 W. S. Hancock and J. T. Sparrow, *HPLC Analysis of Biological Compounds, A Laboratory Guide*, Marcel Dekker, New York, 1984.
5 *10th International Symposium on Column Liquid Chromatography, San Francisco, CA, May, 1986, J. Chromatogr.*, 371, 384, 385, 386 (1987).
6 T. M. Vickrey (Editor), *Liquid Chromatography Detectors*, Marcel Dekker, New York, 1983.
7 E. S. Yeung (Editor), *Detectors for Liquid Chromatography*, Wiley, New York, 1986.
8 J. F. Izn, A. Braat and J. A. Duine, *Anal. Biochem.*, 162 (1987) 65–73.
9 P. A. Webb, D. Ball and T. Thornton, *J. Chromatogr. Sci.*, 21 (1983) 447–453.
10 A. C. J. H. Drouen, H. A. H. Billiet and L. de Galan, *Anal. Chem.*, 56 (1984) 971–978.
11 G. W. Schieffer, *J. Chromatogr.*, 319 (1985) 387–391.
12 W. Flapper, P. J. M. van den Oetelaar, C. P. M. Breed, J. Steenbergen and H. J. Hoenders, *Clin. Chem.*, 32 (1986) 363–367.
13 H. Miklautz, J. Riemann and H. J. Vidic, *J. Liq. Chromatogr.*, 9 (1986) 2073–2093.
14 S. Maezawa and T. Takagi, *J. Chromatogr.*, 280 (1983) 124–130.
15 Y. Hayashi, T. Takagi, S. Maezawa and J. Matsui, *Biochim. Biophys. Acta*, 748 (1983) 153–167.
16 W. W. Yau, personal communication (1987).
17 S.-L. Wu, A. Figueroa and B. L. Karger, *J. Chromatogr.*, 371 (1986) 3–27.
18 X. M. Lu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 19–29.
19 T. S. Reid and D. J. Gisch, *LC–GC, Liq. Chromatogr. HPLC Mag.*, 5 (1987) 986–989.
20 Y. Kato, T. Kitamura, and T. Hasimoto, *J. Liq. Chromatogr.*, 9 (1986) 3209–3224.

21 G. H. Richardson (Editor), *Standard Method for Examination of Dairy Products,* American Public Health Association, Washington, DC, 15th ed., 1985, 17.3–17.5.
22 *Assay for Alkaline Phosphatase,* Boehringr Mannheim, 1986, personal communication.
23 S. C. Krzysko and I. S. Krull, 1986–1988, unpublished results.
24 M. Fosset, D. Chappelet-Tordo and M. Lazunski, *Biochemistry,* 13 (1974) 1783–1787.
25 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519–536.
26 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103–114.
27 H. H. Stuting, R. Blanco, N. Grinberg, I. S. Krull and B. L. Karger, *Anal. Chem.*, (1988) in press.
28 V. Grinshpun and A. Rudin, *J. Appl. Polym. Sci.*, 32 (1986) 4303–4311.