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Size-exclusion chromatography–multiangle laser light scattering analysis of β-lactoglobulin and bovine serum albumin in aqueous solution with added salt

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Abstract

The solution characteristics of β-LGB (β-lactoglobulin) and BSA (bovine serum albumin) are reported as determined by size-exclusion chromatography with on-line multiangle laser light scattering, differential refractive index and UV detection. The order of the three in series placed detectors as well as the interdetector volumes have been carefully pointed out. At concentrations below 2.5 mg/ml and at different values of pH the weight-average molecular mass of both proteins have been obtained. They indicate the appearance of monomers, dimers and higher order multimers. For β-LGB the growth of self-associates could be observed at the isoelectric point over a period of days. The range of applicability of the method is discussed.

Keywords: Detection, LC; Multiangle laser light scattering detection; Lactoglobulin; Albumin; Proteins

1. Introduction

In the last decades protein chromatography has become a wide area of application. Most frequently used are the column separations based on some form of interaction between the protein molecule and the column matrix. So in ion-exchange chromatography [1], the retention mechanism originates from electrostatic interactions, whereas in hydrophobic interaction chromatography [2], the corresponding interactions form the major part. Mixed modes of mechanisms are found in biospecific affinity chromatography [3] and metal chelate interaction chromatography [4]. In size-exclusion chromatography (SEC) the ideal retention mechanism is thought to be a mere sieving by size caused by protein partitioning between the mobile phase and the stationary phase. As usual, a chromatographic partition coefficient K_{SEC}, here for globular proteins, can be introduced which accounts for the retention volume in relation to the total accessible volume of the column and the volume of the mobile phase.

Long ago it was already known that K_{SEC} forms a relationship with the Stokes radius of the protein [5], which later led to the construction of empirical calibration curves involving K_{SEC} and the solvated radii of a series of globular proteins. Although this calibration sometimes seems to work [6], a universal calibration as observed for polymers cannot be made [7–9]. This is not completely unexpected as for

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instance at the isoelectric point a globular protein behaves as a compact sphere, whereas a polymer coil acts more as a soft sphere. Moreover it seems practically impossible to tune the pH and ionic strength such that the calibration proteins are in more or less equal state. The complexity of the situation is also shown by considering the influence of added salt, when the protein and the column matrix are charged. It may enhance either electrostatic protein–column interactions (low salt content) or corresponding hydrophobic interactions (high salt content). Furthermore the ionic strength of the eluent may effect the size of the protein itself [10]. As a result proteins may be eluted more rapidly or slowly then one would expect from pure size-exclusion, which makes the calibration with different protein standards questionable.

These difficulties can be circumvented by measuring on-line the radius or the molecular mass. In this way irregularities in the protein retention become less important and in certain respects (no SEC retention calibration is required) irrelevant. With the coupling of SEC and viscometry the size of the protein can be obtained as a Stokes or viscosity radius [7,9]. Another coupling mode is SEC–low-angle laser light scattering (LALLS), which employs a LALLS photometer to measure directly the molecular mass of the eluted protein [11,12]. This SEC configuration, frequently used in polymer characterization, enabled us to estimate the molecular mass of DNA fragments [13].

In this paper we report the solution properties of β-lactoglobulin (β-LGB) and bovine serum albumin (BSA) as observed by SEC–multiangle laser light scattering (MALLS), a technique we recently applied to polyelectrolytes [14,15]. As the intensity of the scattered light is detected at different angles, the molar mass as well as the radius of gyration can be measured simultaneously. With this method radii larger than about 10 nm can be measured. For most proteins it means that only the size of multimers can be measured with this technique.

In the following we consider first some methodological aspects. In particular we discuss how the MALLS detection can be matched with the concentration detection [refractive index (RI) or UV]. Experimental results are mainly obtained in terms of weight-average molecular masses. Besides the technique detects the presence of dimerization and multimerization of the proteins concerned.

2. Working equations and methods

The MALLS detector measures the intensity of the scattered light with the use of photodiodes placed at specific angles (θ) relative to the incident laser beam. This light intensity is converted to the Rayleigh ratio, $R_\theta(v)$, which is measured as a function of the retention volume v. The equation relating this quantity to other physical properties reads according to Zimm [16]

$$\frac{Kc(v)}{R_\theta(v)} = \frac{1}{M_w(v)} + 2A_2(v)c(v) + \frac{g(v)}{M_w(v)} \cdot \sin^2(\theta/2) \tag{1}$$

where c is the mass concentration of protein, M_w the weight-average molecular mass, A_2 the second virial coefficient, g a quantity proportional to the mean square radius of gyration and K an optical constant. From this equation a Zimm plot can be made, i.e., the left-hand side of Eq. (1) containing the measurable quantities plotted versus $\sin^2(\theta/2)$.

From an extrapolation to zero angle one finds from Eq. (1)

$$\frac{Kc(v)}{R_{\theta\to 0}(v)} = \frac{1}{M_w(v)} + 2A_2(v)c(v) \tag{2}$$

whereas the initial slope of the Zimm curve yields $g(v)$ and therefrom the radius of gyration. Eq. (2) is most conveniently used if $2A_2M_wc$ is much smaller then unity and the right-hand side of Eq. (2) becomes equal to the reciprocal M_w. In spite of the high dilution (small c) in SEC the influence of A_2 may remain in the case of highly charged proteins. Hence it is recommended to perform measurements not too far away from the isoelectric pH and/or to add salt to suppress electrostatic repulsion between protein molecules.

Furthermore it is substantial in the application of MALLS to know the refractive index increment, as the optical constant K is proportional to $(dn/dc)^2$. As

a consequence the measured $M(v)$ is sensible for errors arising in the value of dn/dc with a dependence $(dn/dc)^{-2}$. If, however, $c(v)$ is measured by RI the product $Kc(v)$ depends linearly on dn/dc, whereas the dependence for M_w becomes $(dn/dc)^{-1}$. In this way the discrepancies in the values of dn/dc have less influence on the results. On the other hand it must be noted, that $g(v)$ and thus the on line measured radius of gyration does not depend on the input-value of dn/dc [see Eq. (1) with neglect of the A_2 term].

The SEC–MALLS–RI configuration provides the most detailed information on the protein distribution in solution. It yields the functions $M(v)$ and $g(v)$ representing the on-line measured calibration curves. The absolute calibration in this method is an advantage above usual calibrations with protein standards. Moreover, as far as the protein constituents are separated by size and appear as separate peaks, the corresponding values of M_w of these components can be measured. If the particle size is larger than what is measurable with LS (about 10 nm) the corresponding radius of gyration can be equally well measured. The method allows the detection of very small amounts of protein aggregates $[R_\theta(v)]$.

The above configuration can be more simplified when MALLS is replaced by LALLS. In the latter detection the light scattering signal is observed under a single very low scattering angle. The data can be interpreted with Eq. (2). Hence the particle size cannot be quantified anymore.

Still more information on the protein distribution is lost in the configurations SEC–MALLS or SEC–LALLS without any concentration detection on line. Obviously the amount of injected protein must be known beforehand. In that case one can still observe $R_{\theta\to 0}(v)$ about equal to $c(v)M(v)$ (Eq. (2)), which gives some qualitative insight in the protein distribution. Furthermore the method yields M_w of the whole protein sample.

Finally in batch measurements of concentration series using either MALLS or LALLS without SEC the usual Zimm plots can be obtained. However they provide only information on the solute as a whole. In particular small amounts of aggregates cannot be traced because they do not contribute significantly to M_w.

3. Experimental

3.1. SEC–MALLS

The liquid chromatographic equipment consisted of a Waters 150C high-temperature SEC system connected in series with a MALLS detector (Dawn DSP-F, Wyatt Technology), a UV detector (Gilson) at 280 nm and an interferometric refractive index detector (Optilab 903, Wyatt Technology).

The protein samples were eluted on a TSK G3000SW column (30 cm×7.5 mm I.D.) in 0.17 *M* $NaNO_3$ with a flow-rate of 1 ml/min. The concentration was 2.5 mg/ml; the sample load was 100 μl.

3.2. Solutions

Aqueous solutions of β-LGB (Sigma) were prepared as follows. First the eluent (0.17 *M* $NaNO_3$) was adjusted to pH 5.18 or pH 7 by adding 0.1 *M* HNO_3 or 0.1 *M* NaOH. After dissolving the undried protein the turbid solution was cleared by centrifugation with a speed of 4000 rpm during 2 h. After a careful decantation, leaving behind a negligible amount of material, a clear protein solution was obtained, which was adjusted to the right pH. Prior to injection the solutions were filtered through a 0.45-μm Durapore filter (Millipore). Solutions of BSA (Serva) were prepared only at pH 7 in a similar way. However, the centrifugation was not necessary in this case.

3.3. Concentration determination

Protein concentrations were determined on-line using an interferometric RI detector operating at 28°C with a wavelength of 633 nm. The required refractive index increment (dn/dc) was obtained by an off-line measurement of the undried material followed by a correction for the water content in the used solid protein. For β-LGB at pH 5.2 (isoelectric point) in 0.17 *M* $NaNO_3$ solution we found $dn/dc = 0.180$ ml/g. This value agrees with the value calculated from the data of Perlmann and Longworth [17] under the same conditions (0.183 ml/g). The water content was determined by drying a separate sample

Table 1
Results interdetector measurements with BSA (n = number of measurements)

n	Detector arrangement	Column	Δv_{UV} (ml)	Δv_{RI} (ml)
2	UV→MALLS→RI	G3000SW	−0.123	0.165
2	UV→MALLS→RI	G4000SW	−0.123	0.165
2	MALLS→UV→RI	G3000SW	0.156	0.296
3	MALLS→UV→RI	G4000SW	0.156	0.296

of β-LGB (not further used) to constant mass. Because the correction for the water content in the solid protein BSA turned out to be too large, the calculated value from literature [17] was used for this protein, i.e., $dn/dc = 0.187$ ml/g at pH 7 in 0.17 M $NaNO_3$. The UV detector was calibrated using the RI detector data and provided therefore similar but sometimes more stable results.

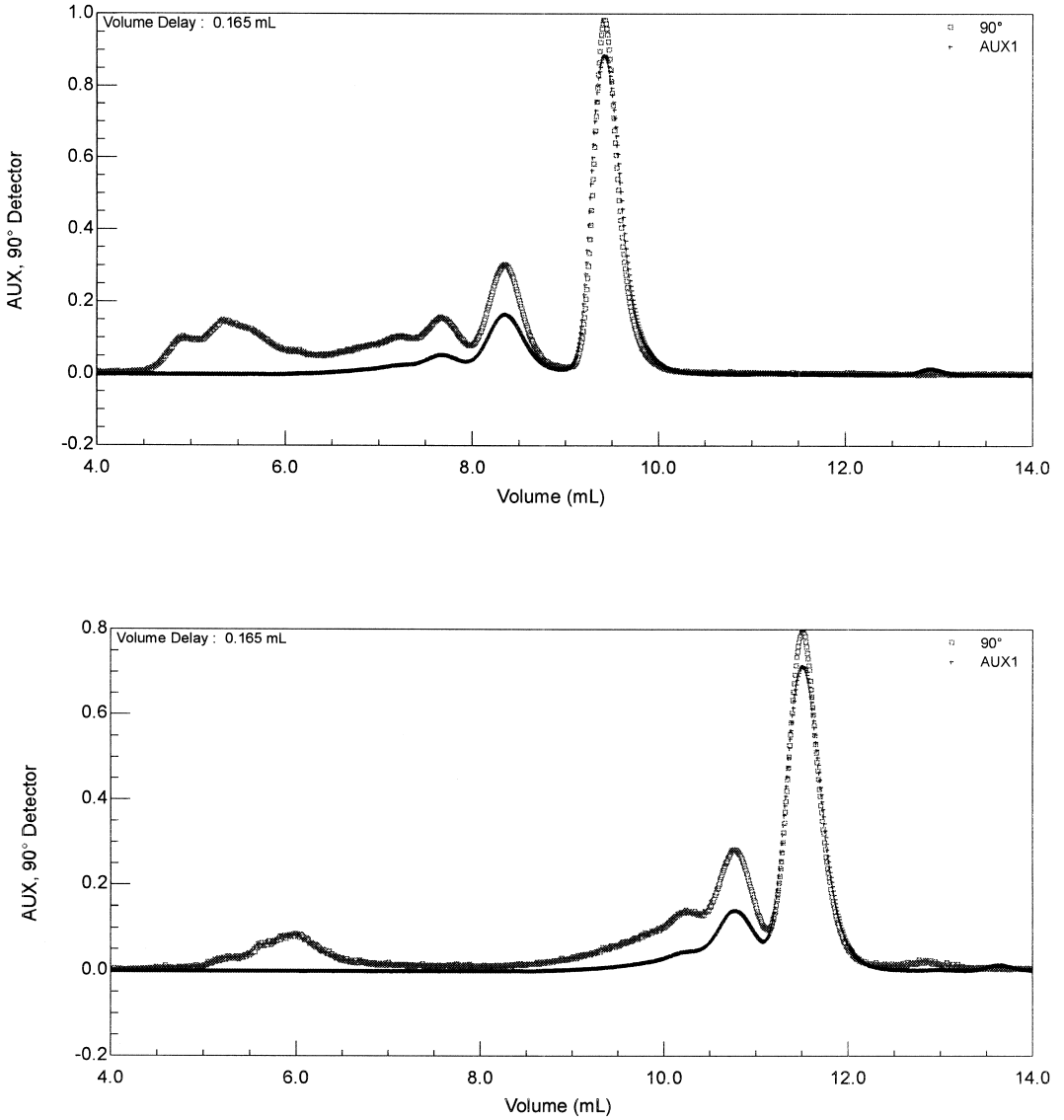

Fig. 1. Alignment of the UV and LS chromatograms on G3000SW (upper) and G4000SW (lower). The MALLS peaks refer to $\theta = 90°$.

3.4. Interdetector volume measurement

Because the molecular mass detection and the concentration detection cannot be performed in the same cell the actual situation, in which a dead volume is present between these detectors, has to be taken into account. Here we used BSA, expected to be rather monodisperse, to determine the interdetector volumes. The MALLS, RI and UV chromatograms are for BSA nicely symmetric. Hence the interdetector volumes could be determined by a simple alignment of the MALLS and concentration peaks.

4. Results and discussion

Using the symmetrical main peaks of the concentration chromatograms of BSA (0.17 *M* $NaNO_3$, pH 7) the interdetector volumes are calculated by aligning them with the MALLS peaks at 90°. In Table 1 the corresponding volumes are shown. Obviously the order of detectors has a significant effect on the observed interdetector volumes but there is no influence of the column. In Fig. 1 two examples of alignment are given. They show that for BSA a better separation is achieved on G3000SW then on G4000SW.

After the above-described alignment of chromatograms a quantitative analysis can be given on the basis of Eq. (2), i.e., $c(v)$ and $R_{\theta\to\to 0}(v)$ can be evaluated from respectively the concentration chromatogram and light scattering chromatogram. It results in $M(v)$ plots, of which an example is given in Fig. 2. In this figure three levels can be observed corresponding to the main peak and the two front peaks in Fig. 1. From the $M(v)$ curve it can be concluded that the BSA solution contains monomers, dimers and trimers. Furthermore the level of the main peak is somewhat curved, which in other cases can be even more pronounced. This phenomenon has been ascribed earlier to secondary peak broadening

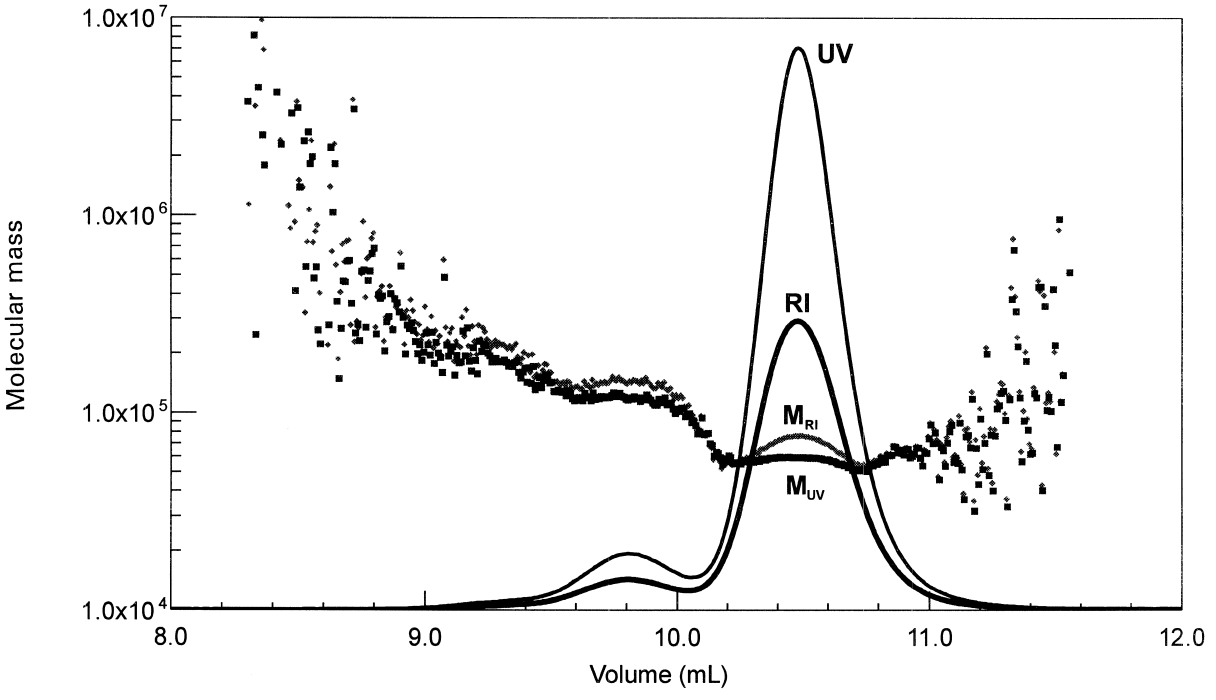

Fig. 2. Molecular mass as a function of elution volume for BSA from SEC–MALLS using RI and UV as concentration detectors. Same conditions as in Table 2.

Table 2
Molecular masses of BSA (pH 7, 0.17 *M* $NaNO_3$) obtained from $M_w(v)$ plots in the configuration MALLS→UV→RI (*n* = number of measurements)

n	Sample	Column	$M_{w,dimer}$	$M_{w,monomer}$
3	Serva 11920	G4000SW	132 000	64 800
4	Serva 11920	G3000SW	150 000	68 100
3	Serva 11924	G4000SW	138 700	68 200
3	Serva 11924	G3000SW	139 000	67 200

resulting from the use of different detectors in series [18].

From Fig. 2 it can be seen that this curvature is more pronounced for the results from the RI detector compared to those from the UV detector in the configuration MALLS–UV–RI. The extra peak broadening is caused by the tubing between the UV detector and RI detector.

Molecular masses obtained from the $M_w(v)$ plots have been compiled in Table 2 for two different BSA samples and the two different types of columns. For the BSA monomer values have been found around the theoretical value 66 500, whereas for the dimer peak the molecular mass indeed turns out to be twice that of the monomer. It must be remarked that the calculated molecular masses are sensible to the values of the dn/dc to be inserted in the calculation. Because $Kc(v)$ appearing in Eq. (2) is proportional to dn/dc, too a large value of dn/dc leads to too a small value of M_w.

β-LGB was processed in the same manner at pH 5.2 (isoelectric point) and pH 7, in both cases in 0.17 *M* $NaNO_3$. The interdetector volumes of Table 1 were used. Typical concentration chromatograms are shown in Fig. 3. There is only a very slight shift of the peak as a function of pH, probably due to hydrophobic interactions of the protein with the column material at the isoelectric point. Another indication for column interaction was an increasing recovery for the first six sample injections which finally retained a stationary value of 96% (pH 5.2). Note that the concentration chromatograms have very flat front peaks.

Molecular masses of β-LGB have been collected in Table 3 for different conditions. These results were obtained with $dn/dc = 0.180$ ml/g ignoring the small pH effect on this value. The effect of the second virial coefficient could also be neglected

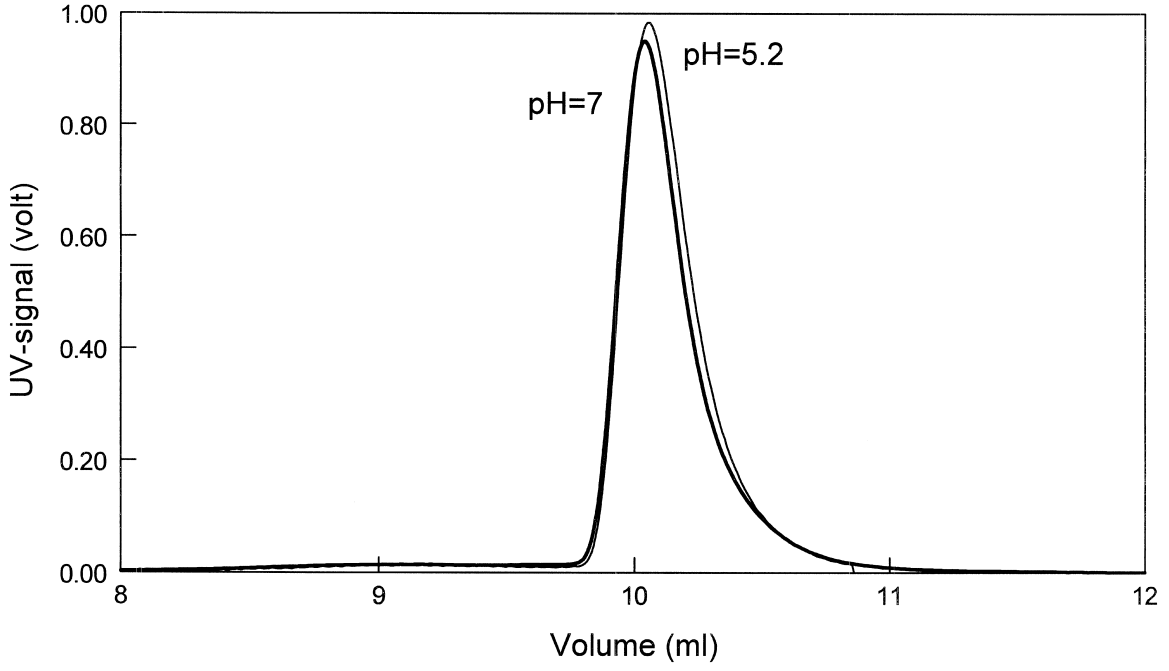

Fig. 3. UV chromatograms of β-LGB at pH 5.2 and pH 7 on G3000SW.

Table 3
Molecular masses of β-LGB as a function of pH and detector arrangement [$M_{monomer}$ = 18 500 g/mol (n = number of measurements)]

n	pH	MALLS→UV→RI		UV→MALLS→RI	
		M_w, total	M_w, main peak	M_w, total	M_w, main peak
6	5.2	39 300±800	35 600±500	35 600±500	
4	7.0			43 800±900	37 200±1000
3	7.0	40 500±1100		35 700±200	

($A_2 = 0$). From the table it can be concluded that β-LGB mainly is in a dimeric form at both pH values. For the whole sample M_w is larger because of the presence of high-molecular-mass material which partly consist of octamers (Fig. 4). The UV–MALLS–RI configuration yields systematically larger molecular mass. The reason for this finding is not clear.

In addition to the above results with freshly prepared β-LGB solutions we investigated the properties of the solutions over a longer period. At the isoelectric point an additional peak appeared in the LS chromatograms near $v = 6$ ml, whereas no peak could be detected in the RI or UV chromatogram at this elution volume. These findings indicate the presence of higher order aggregates, however, in a very small concentration. The growth of the aggregates is seen by the LS peaks increasing in time (Fig. 5). The size of the aggregates varies correspondingly from 40 to 100 nm.

Upon closer inspection of the concentration chromatograms also the lower order multimers are observed to grow during the first measurements. At pH 7 only a small aggregate peak was observed in the signal near $v = 6$ ml which remained univariant in the course of time. Thus the forming of aggregates is enhanced at the isoelectric point, where the hydrophobic interactions are strongest.

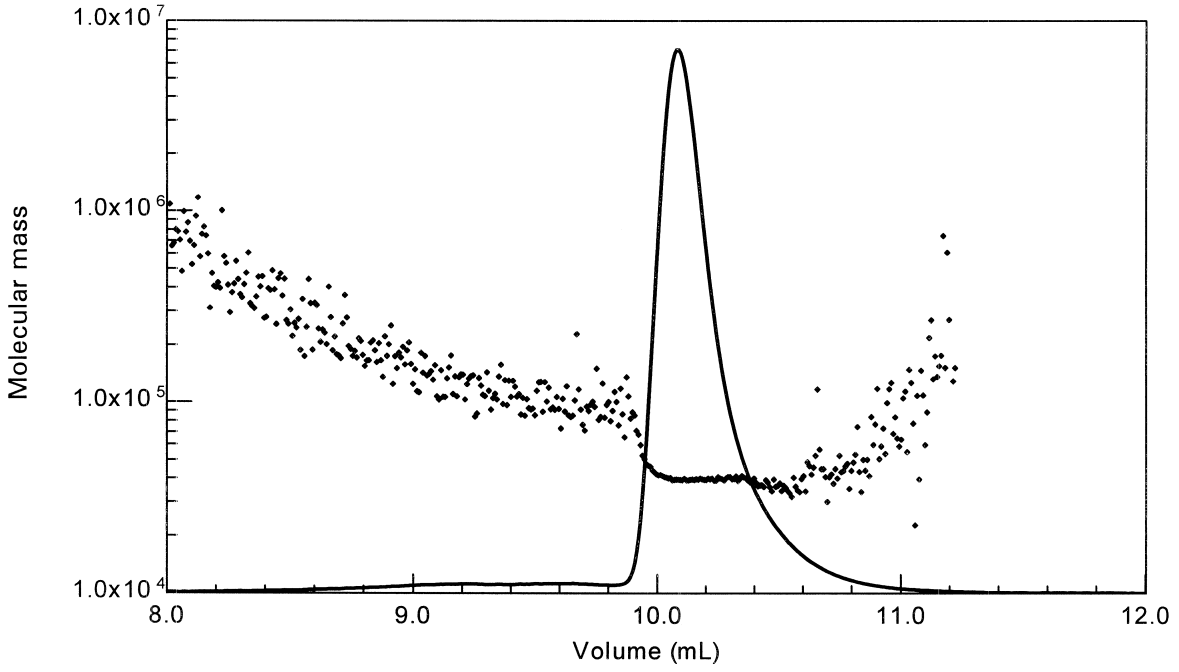

Fig. 4. $M(v)$ for β-LGB calculated with UV as concentration detector. Detector arrangement UV→MALLS→RI. Conditions: pH 5.2 and 0.17 M $NaNO_3$.

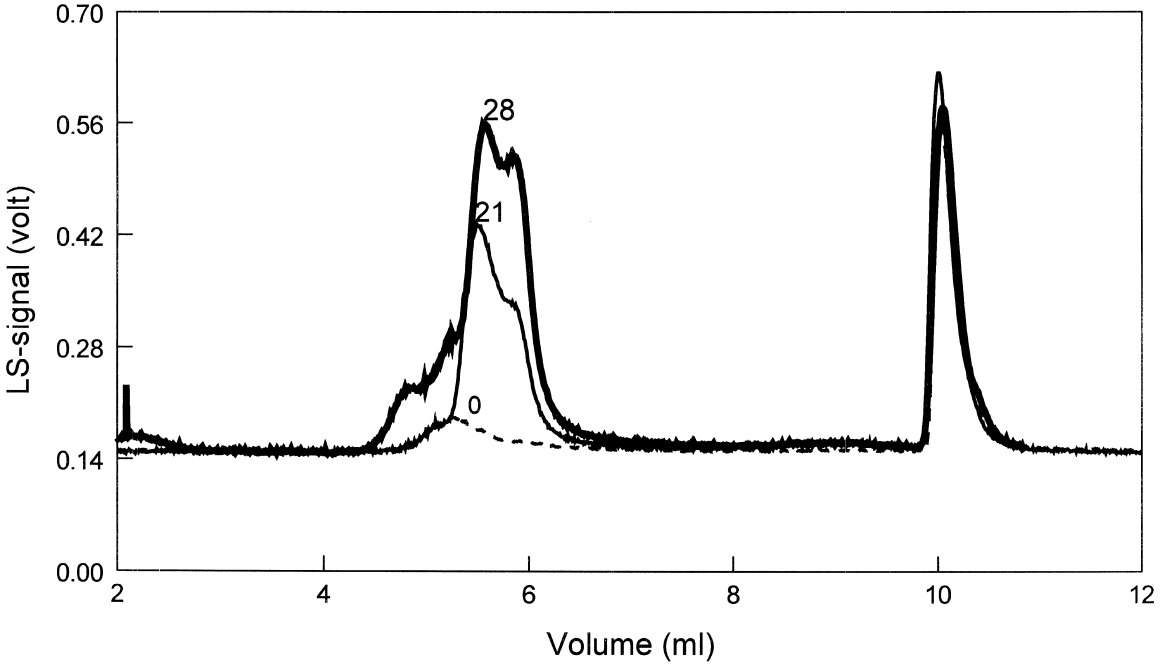

Fig. 5. LS signal (90° detector) in course of time. The numbers indicate hours after the first measurement. Same conditions as in Fig. 4.

5. Conclusions

The SEC–MALLS technique provides a reliable tool to estimate the molecular masses of proteins and the size of large aggregates. The method, however, is restricted to solutions of not more than about 2.5% (w/w) protein. In principle the technique allows the study of dilute solutions of proteins in which the amount of added salt and pH can be varied. The effect of the second virial coefficient A_2 has to be suppressed, which implies that the pH and the amount of added salt must be chosen such, that $2A_2Mc$ is much smaller than unity.

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