Journal of Chromatography, 599 (1992) 205–209
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2528

Determination of molecular mass distributions of whey protein hydrolysates by high-performance size-exclusion chromatography

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ABSTRACT

Two high-performance size-exclusion chromatographic columns (Superose-12 HR 10/30 and Superdex-75 HR 10/30) were compared for their use in the determination of the molecular mass (M_r) distribution of whey protein hydrolysates. For calibration 21 reference compounds of known M_r (ranging from 500 to 68 000) were used by applying two procedures for least-squares curve fitting. Based on the calibration graphs constructed, three apparent-M_r regions were arbitrarily assigned: M_r(app) <5000, 5000–10 000 and $>10\,000$. Different results for the M_r distributions of the whey protein hydrolysates were obtained with the two columns. This was mainly due to a difference in peak resolution, which was better for the Superdex-75 column in the M_r region relevant for the major whey proteins and their hydrolysates. The results were also dependent on the mode of calibration curve fitting used.

INTRODUCTION

To characterize protein hydrolysates for functional or nutritional (dietetic) purposes, it is often desirable to obtain an impression of the molecular mass (M_r) distribution or average peptide chain length of the constituents in the mixtures. For food applications this criterion is connected with the ability of the constituent peptides to be absorbed in the digestive tract, or with the destruction of antigenic determinants during the hydrolytic degradation of food proteins responsible for allergenic reactions [1–4].

Size-exclusion chromatography, particularly when applied in the high-performance mode (HPSEC), is an attractive procedure for investigating peptide profiles in protein hydrolysates if at the same time the molecular size or approximate M_r distribution therein has to be determined. The accuracy of such a method largely depends on the calibration graph constructed for a series of standards of known M_r; these substances should normally have, as far as possible, the same molecular shape and density as the peptides in the hydrolysate under investigation. Hence, the number and diversity of reference compounds should be large enough to compensate for possible deviations of individual standards from the calibration graph and to allow such a graph to be generally applicable to the characterization of different kinds of hydrolysates.

Some years ago, an HPSEC procedure was applied to the determination of M_r distributions in several sources of plant protein hydrolysates [5]; in that study use was made of a silica-based column packing chemically modified with a glycerylpropylsilyl coating (SynChrom). More recently, two HPSEC columns, the silica-based Protein Pack 125 (Waters Assoc.) and the agarose-type Superose-12 HR 10/30 (Pharmacia/LKB), were tested for their applicability in M_r distribution studies of milk protein hydrolysates [6]. Systematically different M_r values were found with these two columns, which was ascribed to hydrophobic interactions between the solute and the gel matrix.

For the investigation of whey protein hydrolysates, we compared the Superose-12 HR 10/30 column with the recently introduced Superdex-75 HR 10/30 column (Pharmacia/LKB). The gel material

0021-9673/92/$05.00

in either column consists of highly cross-linked agarose beads of average size 13 μm, but with the Superdex-75 having dextran chains (covalently) bound to the agarose. Further, we applied two approaches for least-squares fitting in the construction of calibration graphs.

EXPERIMENTAL

Materials

The standards (see Table I) were of analytical-reagent grade. Whey protein hydrolysates were prepared from whey protein concentrate using food-grade enzymes. In the two examples given the degree of hydrolysis, as determined by the pH-stat method [2], was about 14%.

Analysis

The liquid chromatographic equipment consisted of a Waters M 510 pump, a Gilson Model 231/401 automatic sample injector and a Waters Model 481 UV detector operating at 220 nm (0.2 a.u.f.s.). The equipment was linked to a Waters Maxima 820 data acquisition and processing system. Both columns investigated had identical dimensions (300 × 10 mm I.D.).

The sample and elution buffer consisted of 125 m*M* potassium phosphate–125 m*M* sodium sulphate (pH 6.65). Elution was performed at ambient temperature and at a flow-rate of 0.4 ml min^{-1}. The concentrations of standard solutions ranged from 0.05 to 0.2 mg ml^{-1}; the sample load was 50 μl. In some experiments urea was included in the sample solution at a concentration of 6 *M* (pH 6.65). As urea did not have any influence on the elution patterns of the standards and hydrolysates investigated, further separations were carried out without the use of urea. The system pressure was 100 p.s.i.

Based on the calibration graphs constructed, three apparent-M_r regions were arbitrarily assigned: M_r(app) < 5000, 5000–10 000 and > 10 000.

Least-squares fitting was done using Fig. P software (Biosoft, Cambridge, UK).

TABLE I

MOLECULAR MASSES (M_r) AND ELUTION VOLUMES (V_e) OF THE STANDARDS USED FOR THE CONSTRUCTION OF CALIBRATION GRAPHS

Standard	M_r	V_e [a] (ml)	V_e [b,c] (ml)
Serum albumin	68 000	12.56	9.01
Ovalbumin	43 000	13.45	10.07
β-Lactoglobulin A	36 000	13.95	11.22
Carbonic anhydrase	29 000	14.19	11.25
Chymotrypsinogen A	25 000	14.47	11.59
Soybean trypsin inhibitor	21 000	14.81	12.03
α-Lactalbumin	14 400	14.90	12.61
Lysozyme	14 400	15.74	14.01
Cytochrome *c*	12 500	14.55	12.54
Aprotinin	6512	15.65	14.33
Insulin	5734	17.68	16.39
Insulin B-chain (oxidized)	3496	18.92	17.55
α_{s1}-Casein (f1–23)	2764	16.25	14.98
Insulin A-chain (oxidized)	2532	17.11	15.75
β-Casein (f193–209)	1880	17.90	16.85
Bacitracin	1423	18.91	18.33
Vitamin B_{12}	1355	19.91	19.30
Angiotensin (f2–8, 2-Leu)	888	20.91	20.51
β-Casein (f60–66)	790	20.46	19.99
β-Casein (f60–64)	580	20.92	20.57
β-Casein (f60–63)	523	21.57	21.27

[a] Superose-12 HR 10/30 column.
[b] Superdex-75 HR 10/30 column.
[c] Relative standard deviation = 0.5% (n = 13).

RESULTS AND DISCUSSION

The relationship between molecular size [M_r(app)] and elution volume can be expressed by an exponential function, as represented by Fig. 1 for the 21 standards listed in Table I. The shape of the curve obtained by this "exponential" fitting procedure is largely determined by the experimental data for the higher-M_r compounds (left parts of the curves in Fig. 1). Modern computer facilities allow the easy construction of such exponential calibration graphs and the calculation of M_r(app) values of unknown compounds from such graphs by using the appropriate equations. In Fig. 2 the exponential calibration graphs of Fig. 1 are shown on a logarithmic scale for the Superose-12 and Superdex-75 columns (dashed lines) and compared with the corresponding graphs obtained by the commonly used "logarithmic" procedure (solid lines representing log M_r *vs.* elution volume). Theoretically, for ideally behaving standards, the "exponential" and "logarithmic" procedures should lead to the same results. In the (normal) case of non-ideal behaviour, the logarithmic graph should actually be constructed

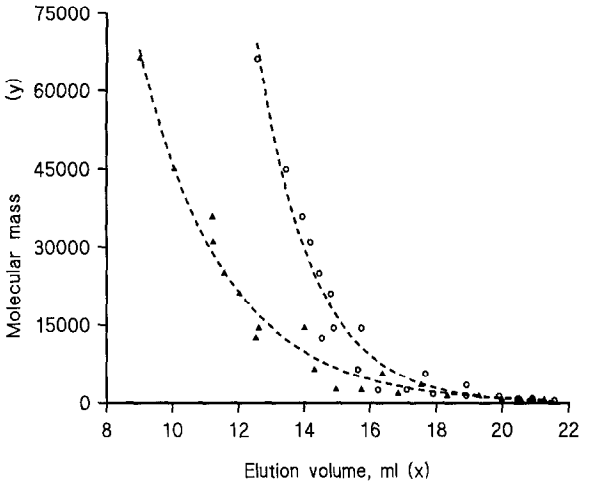

Fig. 1. Exponential curves representing the relationship between molecular mass and elution volume for the 21 standards listed in Table I. (○) Superose-12 HR 10/30; equation: $y = 1.056 \cdot 10^8 \cdot e^{-0.233x}$. (▲) Superdex-75 HR 10/30; equation: $y = 2.194 \cdot 10^6 \cdot e^{-0.154x}$.

by including weighting factors to account for the above-mentioned relative importance of the standards of higher M_r. The fact that such weighting factors are unknown forms an argument for the application of the more direct "exponential" curve-fitting procedure.

As seen in Fig. 2, the calibration graphs constructed for the two columns are significantly different, irrespective of the fitting procedure used.

Applying both curve-fitting procedures we made comparative M_r distribution estimates for several whey protein hydrolysates. Fig. 3 shows a typical example of the separation of one hydrolysate on each of the two columns under the same experimental conditions. In terms of peak resolution in

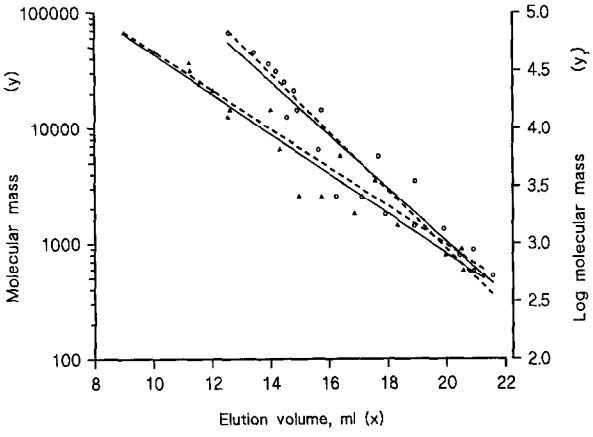

Fig. 2. Linear presentation of the relationship between molecular mass (logarithmic scale) and elution volume for the 21 standards listed in Table I. ○- - -○ = Superose-12 HR 10/30; ▲- - -▲ = Superdex-75 HR 10/30; for the equations of the graphs in their non-linear forms, see the legend of Fig. 1. Comparison with graphs representing log(molecular mass) *vs.* elution volume relationships. ○——○ = Superose-12 HR 10/30; equation: $y_1 = -0.093x + 7.647$. ▲——▲ = Superdex-75 HR 10/30; equation: $y_1 = -0.069x + 6.367$.

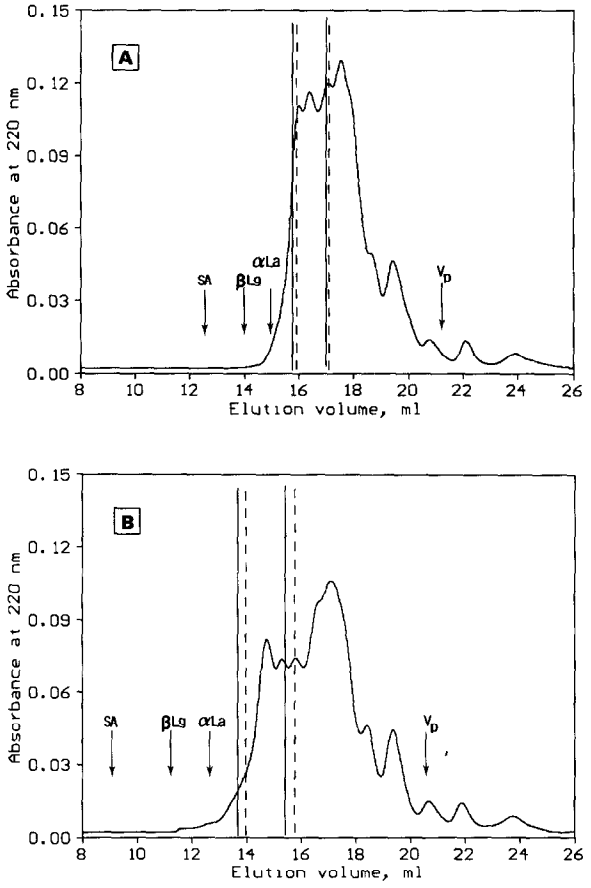

Fig. 3. Peptide profiles of a whey protein hydrolysate (WPH 1 in Table II) fractionated on (A) the Superose-12 HR 10/30 column and (B) the Superdex-75 HR 10/30 column. Positions of the intact whey proteins serum albumin (SA), β-lactoglobulin (βLg) and α-lactalbumin (αLa) and of the column's permeation volume (V_p) are indicated. Vertical lines represent M_r 5000 and 10 000 mass limits as derived from the calibration graphs obtained by "exponential" (dashed lines) and by "logarithmic" (solid lines) curve fitting.

TABLE II

MOLECULAR MASS (M_r) DISTRIBUTION (% OF TOTAL PEAK AREA) OF TWO WHEY PROTEIN HYDROLYSATES, WPH 1 AND WPH 2, AS DETERMINED WITH THE TWO COLUMNS AND BY APPLYING THE EXPONENTIAL AND LOGARITHMIC MODE OF CALIBRATION CURVE FITTING

Sample	Apparent M_r region[a]	Superose-12		Apparent M_r region[a]	Superdex-75	
		Exponential	Logarithmic		Exponential	Logarithmic
WPH 1	a	12	9	a	8	6
	b	29	33	b	25	21
	c	59	59	c	67	73
WPH 2	a	22	18	a	18	16
	b	27	30	b	23	21
	c	52	51	c	59	63

[a] (a) M_r(app) > 10 000; (b) 5000 < M_r(app) < 10 000; (c) M_r(app) < 5000.

the M_r region relevant for the major whey proteins (α-lactalbumin and β-lactoglobulin) and their hydrolysates, the Superdex-75 column is better; this is in line with its recommended M_r fractionation range for globular proteins (3000–70 000) as compared with that of the Superose-12 column (1000–300 000). From Fig. 3 it is also apparent that M_r regions as derived from the two curve-fitting procedures are slightly different (*cf.*, the vertical lines representing the M_r 5000 and 10 000 limits). Consequently, M_r distribution determinations based on these two methods lead to systematic differences in the results (Table II); however, such differences are not necessarily always of practical importance.

By using an elution buffer of elevated ionic strength we have as far as possible circumvented non-size-exclusion effects caused by electrostatic solute–matrix interactions. However, as can be seen for instance in Fig. 3, adsorption effects (probably hydrophobic) could not be fully eliminated. A possible remedy for this could be the inclusion of an organic solvent in the elution buffer or elution under denaturing conditions [5,7–10]. However, such procedures are not always satisfactory; also, the presence of (high concentrations of) denaturing agent, or impurities therein, may interfere with detection at low wavelengths (especially relevant to non-aromatic peptides).

The quantitative aspects of the present calculations are based on the determination of peak areas obtained by absorbance measurements at 220 nm. It should be realized that this method does not take account of differences in absorptivities between the various constituents of the hydrolysates (a drawback which would also have been encountered with detection at any other wavelength). At 220 nm a general underestimation of the low-M_r compounds (peptides and amino acids) can be expected in M_r distribution estimates of this kind. An alternative could be refractive index detection, which, however, has a lower intrinsic sensitivity than detection at 220 nm; moreover, when applied in combination with an elevated ionic strength of the eluent, baseline drift is observed [11].

CONCLUSIONS

In the determination of M_r distributions of whey protein hydrolysates, the Superdex-75 HR 10/30 column shows better resolution than the Superose-12 HR 10/30 column. Generally, by applying the HPSEC procedure, only apparent values for M_r distributions can be expected, which are dependent on the nature of the column and on the mode of calibration curve fitting used. In principle, the "exponential" fitting procedure is better than the commonly used "logarithmic" method in which no weighting factors are employed.

The HPSEC method for M_r distribution determination has its limitations as long as spectrophotometric detection is applied. Nevertheless, the method can be used for comparative purposes.

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