Journal of Chromatography, 282 (1983) 487–494
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 163

PROBLEMS IN MOLECULAR WEIGHT DETERMINATION OF PROTEINS BY GEL CHROMATOGRAPHY AND APPLICATIONS OF HIGHER ORDER DERIVATIVE SPECTROMETRY

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SUMMARY

Sometimes in gel chromatography proteins give rise to irregularities in molecular weight determinations if their p*I* values are higher than 8. Optimal conditions and standard lines are given for trypsin, chymotrypsin and their oligomeres. Second derivatives of the elution diagrams are used for fine resolution. Oligomers are identified by taking fourth-order derivatives of their fundamental spectra.

INTRODUCTION

In order to study intermolecular cross-linking of proteins to definable oligomers[1], it was found necessary to separate the reaction products and to determine their molecular weights (MWs) as exactly as possible. In addition to ultracentrifugation and light scattering, gel chromatography is a simple but powerful method for attaining this goal.

Although bovine serum albumin (BSA), ovalbumin (OA), pepsin (P), ribonuclease (RNase) and many other proteins behave normally, chymotrypsin (CHTR) and especially trypsin (TR) give distinctly smaller MWs on gel chromatography. Porath[2] and Gelotte[3] described a number of observations where substances showed widely different behaviour to that expected from their molecular size. Janson[4] reported adsorption phenomena for Sephadex and Peaucellier[5] pointed out that proteins with p$I \geqslant 8$, *e.g.*, trypsin (p*I* 10.8) and chymotrypsin (p*I* 8.8), have smaller apparent MWs than those calculated from the amino acid sequences if Ultrogel® AcA 54 is used for the MW determination. These results were reinvestigated in this work and the elution conditions were optimized. For the fine resolution of the elution diagrams and for the identification of the oligomers, higher order derivatives were employed.

EXPERIMENTAL

Materials

Chymotrypsin (CHTR) was purchased from Boehringer (Mannheim, F.R.G.);

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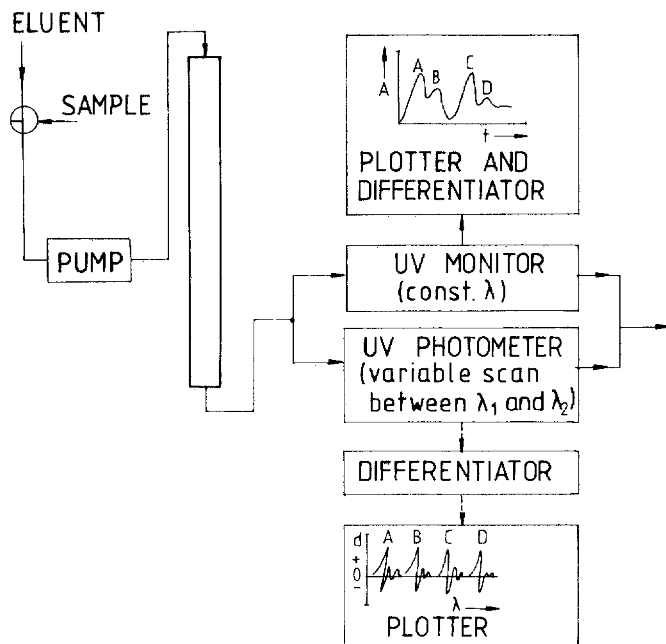

Fig. 1. Flow diagram of the separation apparatus with connected differentiators to resolve elution diagrams and to identify selected fractions.

trypsin (TR) recrystallized twice), ovalbumin (recrystallized five times), bovine serum albumin (BSA) ($>99\%$), pepsin (recrystallized twice) and ribonuclease (recrystallized five times) were supplied by Serva (Heidelberg, F.R.G.).

The elution buffers (0.05 *M*) were phosphate (pH 7.5), citrate (pH 4.0) and Tris (pH 6.2–7.5), and the column packings were Sephadex® G-100 (Pharmacia, Freiburg, F.R.G.) and Ultrogel AcA 44 and AcA 54 (LKB, Bromma, Sweden).

Chromatographic apparatus and conditions

The separation apparatus (Fig. 1) consisted of an LKB Model Microperpex 2132 pump, an LKB column (500 × 16 mm I.D.), Uvicord 8303A and Uvicord S II UV monitor (LKB; λ 280 nm) and a Redirac 2112 fraction collector (LKB). For fine resolution of the elution diagrams the UV monitor was connected to a Model 8110 "intelligent plotter", (Bascom-Turner Instruments, Newton, MA, U.S.A.), which was suitable for manipulation of the spectra (averaging, regression analysis), for computing derivatives and for the long-term storage of the results on floppy disks.

It was advantageous to divide the eluent into two parallel flows, one being used to record the elution diagram and the other to take scans of the eluent between 320 and 220 nm (slit width, 1 nm; scan rate, 2–5 nm/sec; volume of the flow cell, 8 or 100 μl).

To compute higher order derivatives at selected points of the elution diagram, a TLB 6000 analog computer[6,7] (Lucius & Baer, Geretsried, F.R.G.) or a digital computer (*e.g.*, Model 8110 plotter) was connected on-line to an SP 8-100 UV–visible spectrophotometer (Pye Unicam, Cambridge, U.K.).

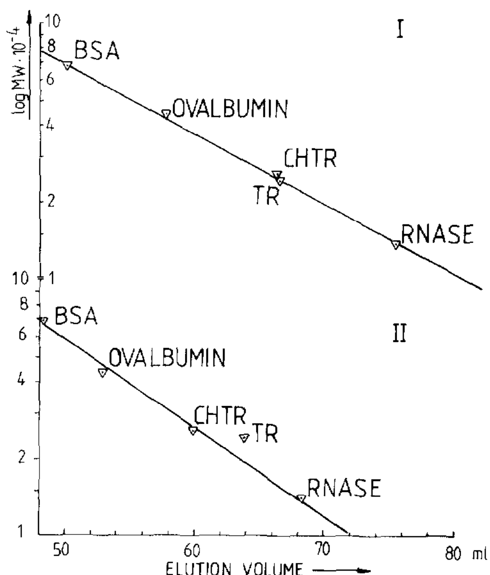

Fig. 2. Calibration graphs for MW determination of trypsin, chymotrypsin and their oligomers. I, Sephadex G-100, 0.05 *M* Tris buffer (pH 6.2), 0.2 *M* KCl; $r = -0.999$; II, Ultrogel AcA 44, 0.05 *M* Tris buffer (pH 6.7), 0.2 *M* KCl; $r = -0.985$; used only for chymotrypsin and its oligomers.

RESULTS AND DISCUSSION

To determine the molecular weights of trypsin, chymotrypsin and their oligomers[1] by gel chromatography we used Ultrogel AcA 44 and 54 and Sephadex G-100 as column packings under various conditions. Although the ionic interaction between the gel and the protein is minimal when the pH of the eluent corresponds to the isoelectric point (p*I*) of the protein, with CHTR and TR it was impossible to maintain these conditions, because the enzymes are very unstable at pH $\geqslant$ 8 owing to autolysis. Therefore, a pH of about 6–7 and elution temperatures near zero must be used. Some selected examples are summarized in Table I.

The results showed that Ultrogel gives sharper separation and Sephadex yields the best MW values (Table I, No. 6). The salt concentration must be very low [0.2 *M* (KCl)] and the pH 6–7. Under these conditions, the mean deviations for all proteins tested did not exceed $\pm 3\%$.

Two calibration graphs for the MW determination of CHTR oligomers are shown in Fig. 2. The values calculated from it agree very well with the expected MWs. (Table II). The number-average molecular weight ($\bar{M}_n$) of the dimeric CHTR was also checked by electron microscopy of freeze-etched preparations[1,8], which method is based on counting the number of molecules per unit volume of the frozen solution[9]. BSA was used as the standard. For monomeric CHTR $\bar{M}_n$ was found to be 25,000 daltons $\pm 5\%$ and for the dimeric protein 55,000 daltons $\pm 10\%$ (theoretical value, 50,000 daltons).

Enhancement of analytical detail from normal liquid chromatograms[6,10–12] and high-performance liquid chromatograms[13–15] by taking derivatives of the output sig-

TABLE I

VARIOUS CONDITIONS FOR MW DETERMINATION OF DIFFERENT PROTEINS

BSA = bovine serum albumin; OA = ovalbumin; P = pepsin; CHTR = chymotrypsin; TR = trypsin; RNase = ribonuclease; LFR = linear flow-rate; p*I* = isoelectric point; MW = molecular weight; Exp. = experimentally; Theor., theoretically (computed from amino acid sequence). Maximal error of reproducibility: ±5%. Column length, 50 cm; I.D., 16 mm.

No.	*Gel*	*Buffer*	*Salt*	*pH*	*LFR (cm/h)*	*Protein*	*pI*	*MW · 10^{-3}*		*Mean deviation (%)*
								Exp.	*Theor.*	
1	Ultrogel AcA 44	0.05*M* phosphate	–	7.5	5.4	BSA	4.9	72.0	67.0	+7.5
						OA	4.7	42.0	43.0	+2.3
						P	2.9	32.0	36.0	−11.1
						CHTR	8.8	22.5	25.0	−10.0
						TR	10.8	12.0	23.8	−49.6
						RNase	8.9	15.9	13.7	+16.1
2	Ultrogel AcA 44	0.05 *M* Tris	–	7.5	4.9	BSA	4.9	58.0	67.0	−13.4
						OA	4.7	42.5	43.0	−1.2
						P	2.9	46.0	36.0	+27.8
						CHTR	8.8	21.9	25.0	−12.4
						TR	10.8	13.8	23.8	−42.0
						RNase	8.9	14.6	13.7	+6.6
3	Ultrogel AcA 54	0.05 *M* Tris	–	7.5	4.5	BSA	4.9	56.0	67.0	−16.4
						OA	4.7	41.0	43.0	−4.7
						P	2.9	42.0	36.0	+16.7
						CHTR	8.8	23.5	25.0	−2.7
						TR	10.8	16.0	23.8	−32.8
						RNase	8.9	16.5	13.7	+20.4
4	Ultrogel AcA 44	0.05 *M* citrate	–	4.0	5.05	BSA	4.9	66.0	67.0	−1.5
						OA	4.7	46.5	43.0	+8.1
						CHTR	8.8	22.0	25.0	−12.0
						TR	10.8	13.4	23.8	−43.7
						RNase	8.9	15.0	13.7	+9.5

5	Sephadex G-100	0.05 *M* Tris	0.1 *M* KCl	7.5	8.81	BSA	4.9	68.0	67.0	+1.5
						OA	4.7	43.0	45.0	−4.4
						CHTR	8.8	24.0	25.0	−4.0
						TR	10.8	18.5	23.8	−22.3
						RNase	8.9	14.1	13.7	+2.9
6	Sephadex G-100	0.05 *M* Tris	0.2 *M* KCl	6.2	9.0	BSA	4.9	68.0	67.0	+3.0
						OA	4.7	42.5	43.0	−1.2
						CHTR	8.8	24.5	25.0	−2.0
						TR	10.8	24.0	23.8	+0.8
						RNase	8.9	14.0	13.7	+2.2
7	Sephadex G-100	0.05 *M* Tris	0.5 *M* KCl	6.4	9.1	BSA	4.9	69.0	67.0	+3.0
						OA	4.7	42.5	43.0	−1.2
						CHTR	8.8	24.0	25.0	−4.0
						TR	10.8	23.5	23.8	−1.3
						RNase	8.9	14.2	13.7	+3.6
8	Ultrogel AcA 44	0.05 *M* Tris	0.2 *M* KCl	6.7	6.0	BSA	4.9	64.0	67.0	−4.5
						OA	4.7	45.0	43.0	+4.7
						CHTR	8.8	26.0	25.0	+4.0
						TR	10.8	19.0	23.8	−20.2
						RNase	8.9	13.5	13.7	−1.5

TABLE II

RESULTS OF THE MW DETERMINATION OF CHYMOTRYPSIN OLIGOMERS

Conditions as in Table I, No. 6.

Chymotrypsin	$MW \cdot 10^{-3}$		*Mean deviation (%)*
	Exp.	*Theor.*	
Monomer	24.5	25.0	−2.0
Modified monomer	27.5	—	
Dimer	52.0	50.0	+4.0
Trimer	73.5	75.0	−2.0
Tetramer	105.0	100.0	+5.0
Higher oligomers	⩾130.0	—	

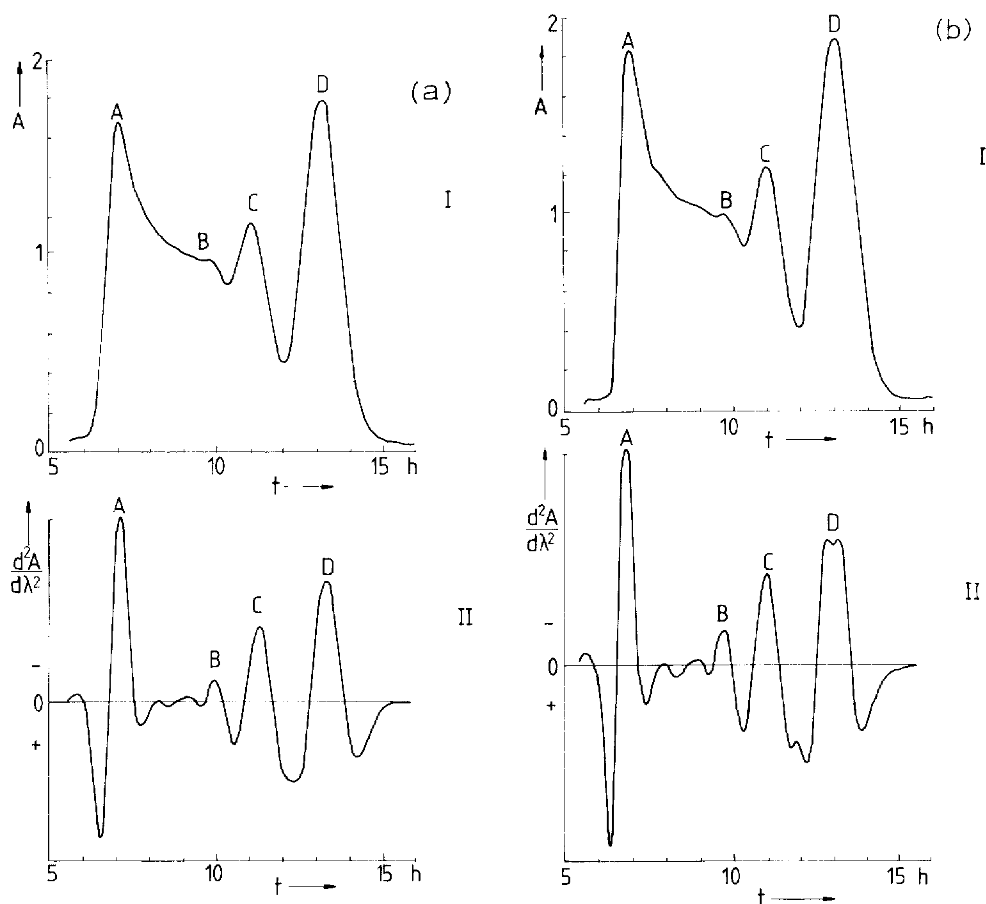

Fig. 3. (a) Elution diagram of chymotrypsin oligomers. I, Basic curve; II, 2nd derivative. For conditions, see Table I, No. 6. A, Oligomers ($n \geqslant 4$); B, trimer; C, dimer; D, modified monomer. (b) Elution diagram of chymotrypsin oligomers as shown in Fig. 3a. Both diagrams (Figs. 3a, I, and Fig. 3b, II) are very similar but the 2nd derivative (Fig. 3b, II) of the basic curve (Fig. 3b, I) has a split minimum between C and D and a split peak D, which means that the separation of the dimer from the monomer was not optimal.

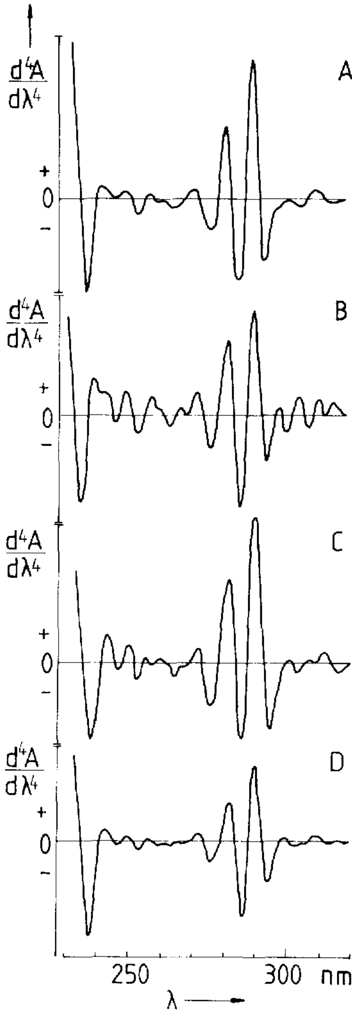

Fig. 4. Fourth derivatives of fractions A, B, C and D in Fig. 3a. If the four substances were identical, the diagrams would have to be identical.

nals has been reported previously. Differentiation of the elution diagram is very helpful in judging whether the chromatographic fractions are well separated. When the peaks are not symmetrical or show shoulders, it helps to resolve such irregularities and to eliminate unwanted background.

In our studies we used a UV monitor with constant wavelength and, in parallel, a UV–visible spectrophotometer (Fig. 2). The elution diagram was differentiated for fine resolution and quantitative evaluation of the fraction. When the separation was unsatisfactory the peaks were split by higher derivatives (Fig. 3a and b). Higher order derivative spectrophotometry was found suitable for identifying the oligomers using "fingerprints" (Fig. 4). To eliminate the concentrations of the protein solutions, the logarithms of the spectra were first taken and then the fourth derivatives were com-

puted. We have reported on this derivative spectrophotometric technique elsewhere[16,17].

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Deutsche Forschungsgemeinschaft and the Max-Buchner Forschungsstiftung. The authors are also grateful to Miss Franziska Reisinger for technical help.

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