Journal of Chromatography, 238 (1982) 193–202
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,534

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS: ANALYTICAL APPLICATIONS*

K. BUCHHOLZ and B. GÖDELMANN
Dechema-Institute, D-6000 Frankfurt 97 (G.F.R.)
and
I. MOLNAR*
Knauer, Hegauer Weg 38, D-1000 Berlin 37 (G.F.R.)
(First received February 23rd, 1981; revised manuscript received November 10th, 1981)

SUMMARY

Analysis of proteins in solution by high-performance liquid chromatography is presented with respect to structural changes in solution, adsorption processes and differentiation concerning specific activities. Trypsin and cellulases were taken as examples.

INTRODUCTION

Radioimmunoassay, ultracentrifugation, electrophoresis, gel filtration, size-exclusion, ion-exchange and affinity chromatography, and membrane osmometry are commonly used methods for the characterization of proteins. These techniques are, however, time consuming and difficult to automate, and they have, therefore, often been replaced by high-performance liquid chromatographic (HPLC) methods, which offer advantages in these respects.

Aqueous size-exclusion HPLC is increasingly being used for the separation and characterization of biologically important macromolecules[1–4]. In particular, improvements in column technology and the introduction of stationary phases based on chemically modified silica gel[5–10], which are mechanically stable under high pressures, have advanced the use of this technique although its specificity and resolution are still relatively low. In this paper we describe some applications of size-exclusion HPLC to the analysis of proteins in solution. It will be shown that this method is a useful tool for the rapid characterization of proteins and for monitoring the degradation of enzymes in solution.

In addition, size-exclusion HPLC was used for studying the hydrolysis of cellulose by cellulases, complex mixtures of enzymes of different activity which catalyse the successive reaction steps in this process[11,12]. The development of rapid methods

* Dedicated to Professor G. Manecke on the occasion of his 65th birthday.

0021-9673/82/0000–0000/$02.75

for analysing the adsorption of these enzymes on their substrate, their separation and the identification of the active fractions is an important topic in enzyme technology and reaction engineering[13]. Although the separation of cellulases by conventional methods has recently been improved[11,14,15], a correlation of the activity and the molecular size of the cellulase components, measured by HPLC, has to our knowledge so far not been reported. In this paper, we report a study of this correlation and compare the separation of cellulases on DEAE-Sephadex with that obtained on LiChrosorb DIOL columns.

EXPERIMENTAL

Apparatus

The high-pressure size-exclusion chromatograph was obtained from Knauer (Berlin, G.F.R.).

Fast screening of proteins was performed with a double-head reciprocating high-pressure pump (Type 52.00, Knauer), and a high-pressure sample introduction valve (RH 71-25, Knauer). The column (250 × 4.6 mm) was packed with LiChrosorb DIOL (5 μm) with an average pore size of 100 Å (103.07.16.005, Knauer). As mobile phase we used 0.1 *M* phosphate buffer, pH 2.1, at a flow-rate of 2.0 ml/min. Proteins (20 μg per injection) were detected with a spectrophotometer operating at 200 nm (Type 87.00, Knauer). The flow cell of the detector had a volume of 8 μl. The elution volume was accurately measured with an electronic volumeter (Type 68.00, Knauer) with a cell volume of 8.51 μl. This instrument generates a small heat impulse which is carried by the eluent flow and is detected by a small thermocouple. When the voltage of the thermocouple exceeds a threshold level, a new heat impulse is generated electronically and the cycle repeats itself as long as the eluent flows through the cell. A

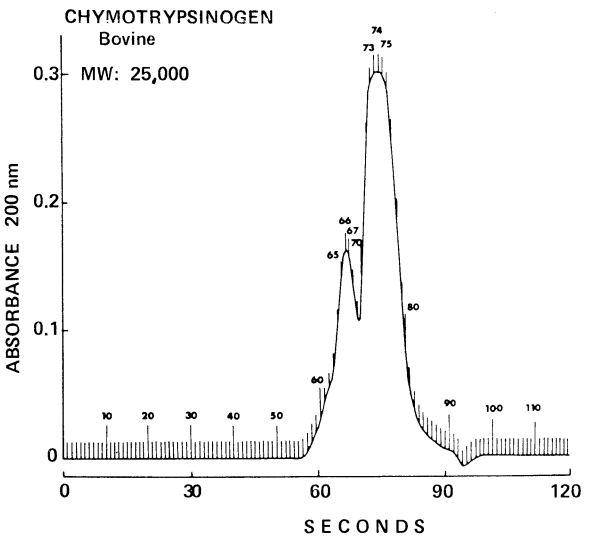


Fig. 1. High-speed, high precision separation of bovine chymotrypsimogen by size-exclusion HPLC. For fast screening conditions see Experimental section.

chromatogram using electronic volume measurement is shown in Fig. 1. Each mark on the chromatogram corresponds to cell volumes.

For trypsin analysis a 5-μm LiChrosorb DIOL column (250 × 4.6 mm) (103.07.16.005, Knauer) was used, eluted with 0.1 *M* phosphate buffer, pH 5, at a flow-rate of 1 ml/min and a pressure drop of 100 bar. Proteins were detected spectrophotometrically at 200 nm.

For adsorption studies of cellulases five columns of LiChrosorb DIOL, 5 μm, as specified above, were operated in series. The flow-rate was 0.5 ml/min, the pressure drop 300 bar, and the sample volume 20 μl. Buffer and detector were the same as above.

For activity studies and correlation of enzyme activity with HPLC data two LiChrosorb DIOL columns (250 × 16 mm) (105.09.16.005, Knauer) were coupled in series. The flow rate was 1 ml/min, the pressure *ca.* 2 bar, while other conditions were as above, except for the sample volume, which was 100 μl.

Materials

Protein samples of different molecular weight were purchased from Serva (Heidelberg, G.F.R.).

Trypsin (crystalline) and buffers were obtained from E. Merck (Darmstadt, G.F.R.). Cellulase from *Penicillium funiculosum* were a gift from J.a.E. Sturge (Selby, Great Britain): two preparations with different activities were used. Avicel (microcrystalline cellulose) was from Serva.

Methods

Trypsin inactivation. This was investigated in solution at a concentration of 0.05 g/l, 25°C, pH 8.2, 0.3 m*M* Tris buffer, 1 m*M* NaCl. The activity was determined from the kinetics of the hydrolysis of 1 m*M* N-α-benzoyl–arginine–*p*-nitroanilide, under the same conditions as before. The release of product was measured by monitoring its absorbance at 410 nm.

Adsorption of cellulases. Adsorption was investigated by incubation of the protein (1 g/l) with Avicel (0, 10, 50 g/l) for 10 min or for various times at 40°C, in 0.05 *M* citrate buffer, pH 5.

Chromatography of cellulases. Chromatography of cellulases (100 mg) on DEAE-Sephadex A-50 (15 g) was performed in a column with a volume of 0.96 l, and a length of 48 cm, which was eluted with 0.01 *M* citrate buffer, pH 4.3, at 1 ml/min. Samples of 10 ml were collected.

Determination of cellulase activities. Glucanases were analysed by incubation with Avicel (25 g/l) at 40°C, in 0.05 *M* citrate buffer, pH 4.5. Samples were taken at various times, centrifuged and analysed for glucose and cellobiose by HPLC, using LiChrosorb NH_2 (103.07.17.010, Knauer), acetonitrile–water (70:30), 2 ml/min, and a differential refractometer as detector (Type 98.00, Knauer). β-Glucosidases were analysed by incubation with cellobiose, 5 m*M*, under similar conditions as before.

RESULTS AND DISCUSSION

In size-exclusion chromatography, proteins are eluted according to their molecular weights. However, as discussed by Schmidt *et al.*[1], deviations may occur,

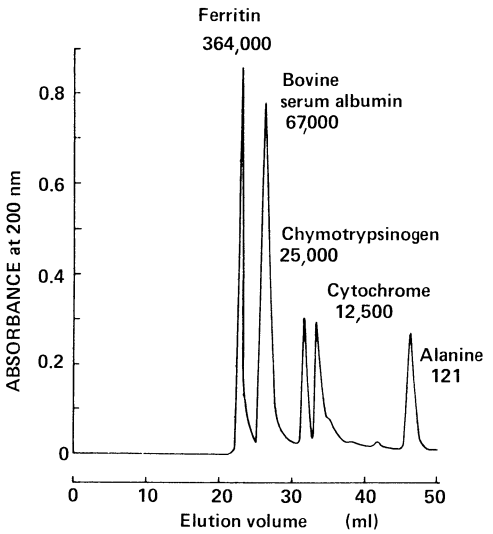
mainly as a result of interactions of the charged proteins with dissociated residual silanol groups on the silica surface. Negatively charged proteins will tend to elute faster than expected owing to repulsion by the negatively charged surface of the stationary phase. As a result, the calculated molecular weights will be higher than the real molecular weights. Positively charged proteins, on the other hand, will be retained more strongly by the stationary phase, resulting in too low values for the calculated molecular weights. For a meaningful correlation between elution volumes and molecular weight, therefore, pH control and complete coverage of the silica surface are essential. In LiChrosorb DIOL the latter requirement is achieved by covering the surface with covalently bound $-O-CH_2-CHOH-CH_2OH$ groups.

To investigate the retention characteristics of this stationary phase for proteins, a mixture of standard proteins of known molecular weight was chromatographed on a LiChrosorb DIOL column (Fig. 2).

The relationship between the molecular weights and the elution volumes on a 250 × 4.6 mm column packed with 5-μm LiChrosorb DIOL is shown in Fig. 3, which demonstrates the validity of the function

$$\log MW = A - BV_e$$

(where MW = molecular weight, V_e = elution volume of the protein, A and B = constants) even under conditions of high speed and low pore volume.



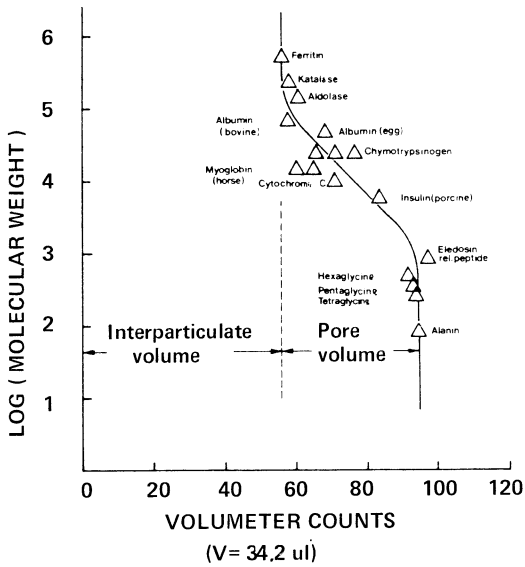


Fig. 2. Separation of protein molecular weight standards by size-exlusion HPLC. Column: prepacked with LiChrosorb DIOL, 5 μm (Knauer, 105.09.16.005) (250 × 16 mm); mobile phase: 0.1 M phosphate buffer, pH 5; flow-rate: 2.5 ml/min; low pressure drop; detection: 200 nm, spectrophotometer; sample amount: 20 μg each.

Fig. 3. Correlation between elution volume and molecular weight. For fast screening conditions see Experimental section.

As a demonstration of the high speed of this type of analysis, Fig. 1 shows a chromatogram of bovine chymotrypsinogen, which was obtained in less than 100 sec on a column with a liquid volume of *ca.* 3.3 ml. The shoulder preceding the main peak in the chromatogram is probably due to the formation of dimers or polymers as it disappeared upon dilution of the sample solution. In experiments with bovine trypsin it was shown that the reproducibility of this type of "molecular-size screening" is excellent.

Degradation of trypsin

Trypsin is in solution subject to autolysis, the kinetics of which depend on the experimental conditions[16].

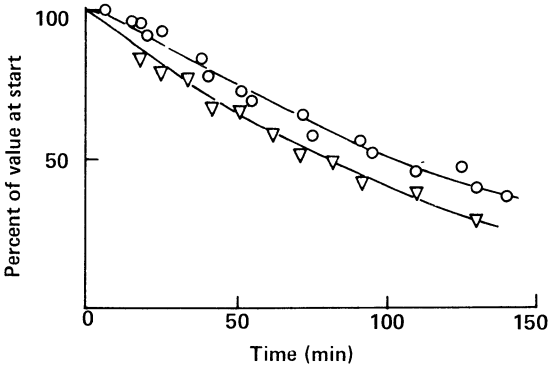


Fig. 4. Degradation (autolysis) of trypsin in solution. For conditions see Experimental section. O, Activity test (BAPNA); △, first peak in HPLC.

Fig. 4 shows the decrease in trypsin activity with time as well as the decrease in the height of the first peak in the HPLC chromatogram. As can be seen, both lines run parallel, suggesting that the first peak in HPLC corresponds to the integral active enzyme.

Adsorption of cellulases on cellulose

Adsorption of the enzymes on the substrate is the first step in enzymic cellulose hydrolysis. This process is also important in technical processes, where the active enzyme and the substrate are kept together within the reactor. We investigated by HPLC the adsorption of cellulases on Avicel, which served as a model substrate.

Fig. 5a shows a chromatogram of the cellulases. Fig. 5b presents the superimposed HPLC chromatograms of two fractions which had been separated previously on DEAE-Sephadex. Fig. 5c shows a chromatogram of cellulase preparation can be identified. Fig. 6a and b shows the decrease in peak heights (concentration in the supernatant) after adsorption of the enzyme on increasing amounts of Avicel. The components with α values of 0.68 and 0.92 are not, or only weakly, adsorbed ($\alpha = V_e/V_0$; V_e and V_0 = elution volumes of protein and buffer, respectively). The main peaks with α values of 0.72 and 0.75 are strongly adsorbed, but not completely. Even

at high concentrations of Avicel the peak heights were still about 20% of the values found in the absence of Avicel, suggesting that these peaks correspond to more than one protein each. One component ($\alpha = 0.87$) was adsorbed only moderately.

Fig. 7a, b and c shows that the adsorption equilibrium is established within *ca.* 10 min, and that part of the enzymes is released into solution after a considerable reaction time. For a detailed characterization of the cellulase system preparation No. 2 was separated by ion-exchange chromatography on DEAE-Sephadex (Fig. 8a). Fractions (10 ml each) were taken and were further investigated as described below.

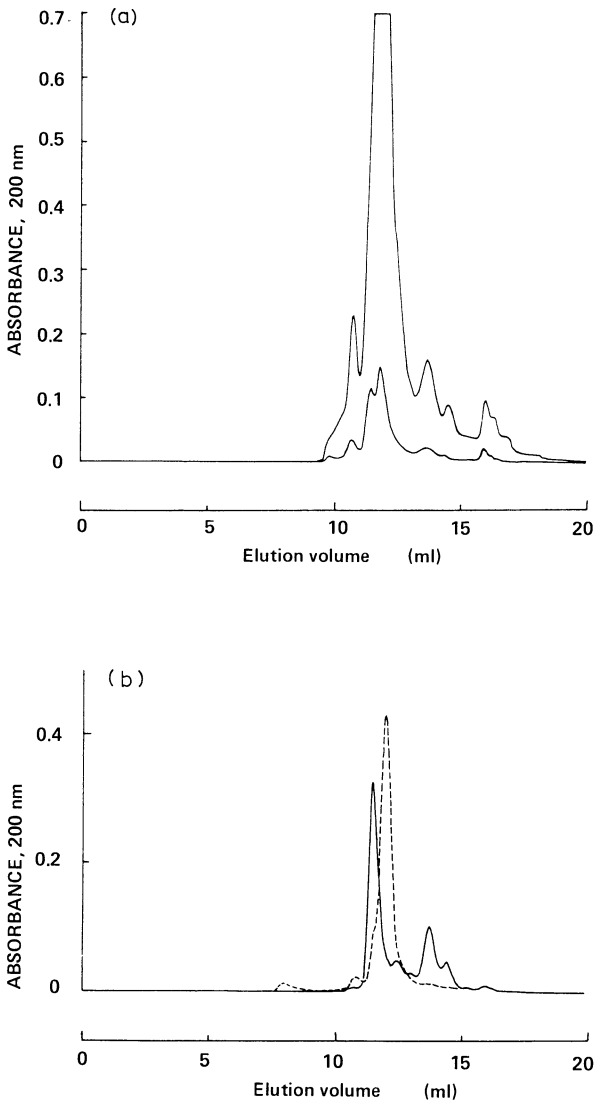


Fig. 5.

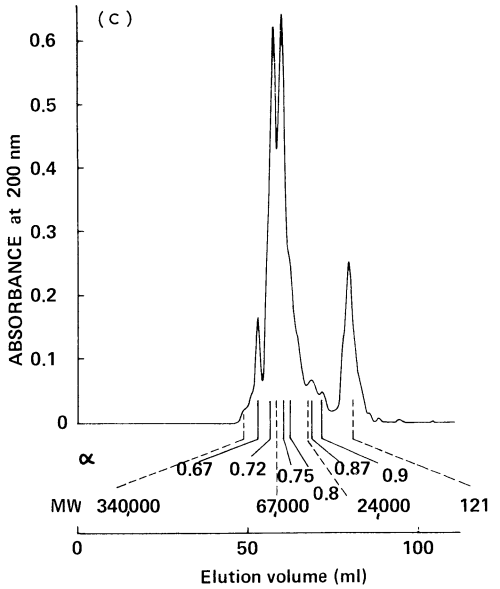


Fig. 5. (a) HPLC of cellulase, preparation No. 1 (5 g/l and 0.5 g/l, respectively); five columns with 4.6 mm diameter; for conditions see Experimental section. (b) Same as in (a); superimposition of chromatograms of two main fractions which had been separated by chromatography on DEAE-Sephadex; two columns with 16 mm diameter; for conditions see Experimental section. (c) HPLC of cellulases, preparation No. 2. For conditions see Experimental section. Retention indices ($\alpha = V_e/V_0$) are given, those of molecular weight standards are indicated.

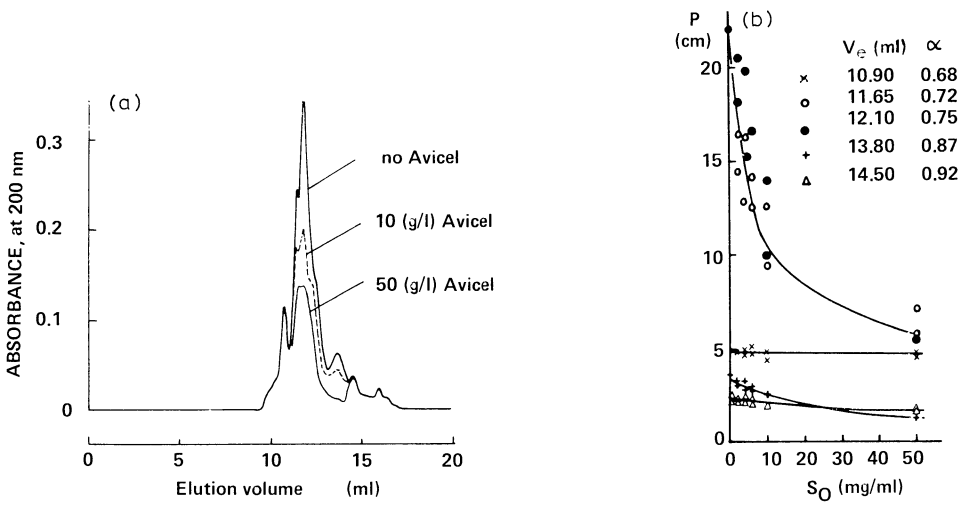


Fig. 6. Adsorption of cellulases (1 g/l) (a) On cellulose (Avicel, 0, 10 and 50 g/l) after 10 min, 40°C, pH 5, citrate buffer 0.05 *M*; HPLC from the supernatant. (b) Peak height (*P*) as a function of cellulose concentration (S_0), conditions as in (a).

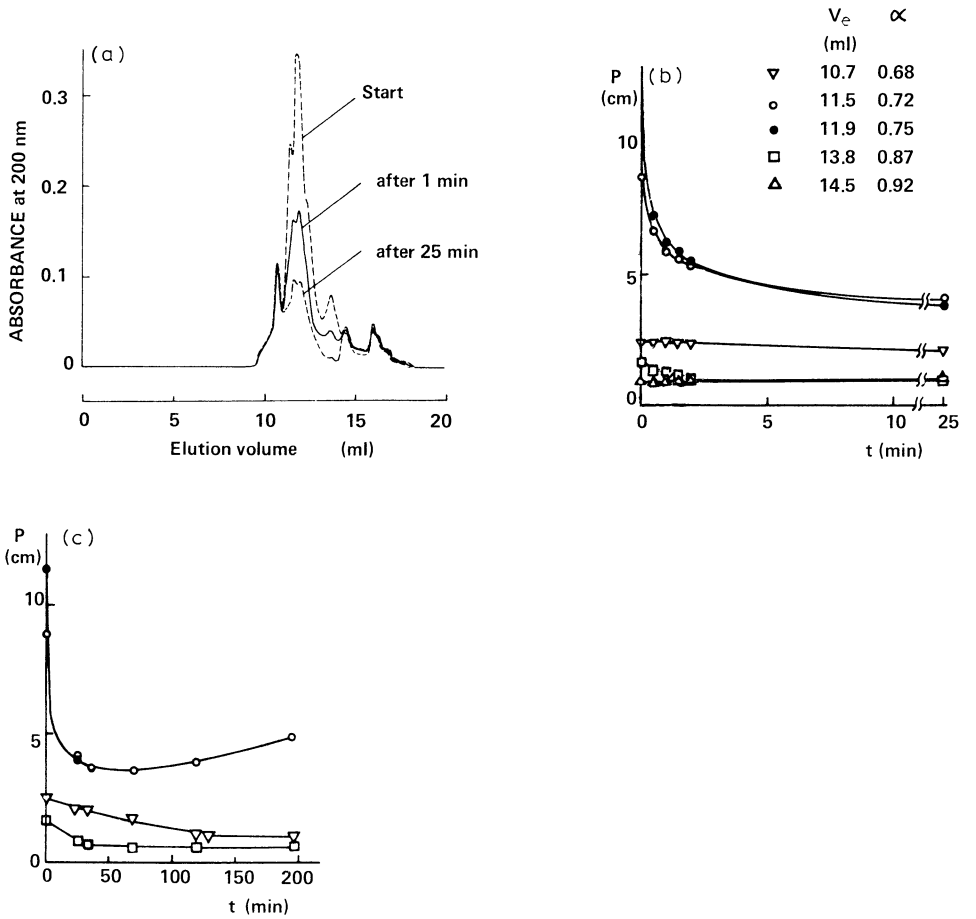


Fig. 7. (a) Time course of cellulase adsorption (0, 1, 25 min), conditions as in Fig. 6a (b, c) Peak height in HPLC as a function of time (0.5 g/l protein, 25 g/l Avicel). Conditions as in Fig. 6a.

Measurements of (β-glucosidases)$_{E2}$ and (glucanases)$_{E1}$ activities

endo-Glucanases randomly split bonds between glucose units in a cellulose chain. *exo*-Glucanases split cellobiose units from the end of a cellulose chain. Their activity was measured with Avicel (consisting mainly of crystalline cellulose) as substrate which can only be attacked by combined action of *endo*- and *exo*-glucanases. β-Glucosidases hydrolyse the cellobiose into two glucose units. The activity was measured with cellobiose as substrate.

Cellobiose and glucose were separated on LiChrosorb-NH_2 (see Experimental section). The activities of the cellulase fractions separated on DEAE-Sephadex are shown in Fig. 8b and c.

Relationship between fractions separated on DEAE-Sephadex and their molecular size, estimated by HPLC on LiChrosorb DIOL

Each fraction collected from the DEAE-Sephadex column was injected on two

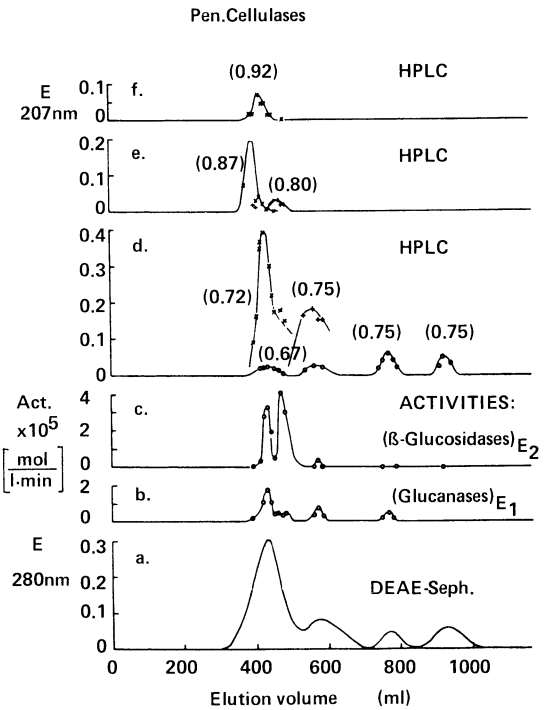


Fig. 8. (a) Chromatogram of cellulases (0.1 g) on DEAE-Sephadex (15 g, 0.96 l, h = 48 cm, citrate buffer 0.01 *M*, pH 4.3, Q = 1 ml/min). (b, c) Activity determination in fractions from (a). E_1 = activity with Avicel as substrate. E_2 = activity with cellobiose as substrate. Note: several different proteins with similar activity are present. (d–f) HPLC of the fractions from (a). The peak height of different peaks in HPLC (ordinate) is given for the fractions from (a) (abscissa). For conditions see Experimental section.

LiChrosorb DIOL columns (16 mm diameter). The fractions consisted of more than one component, as was expected. The first peak of the DEAE-Sephadex chromatogram was shown by HPLC to contain five different species with approximate molecular weights of 80,000 (α = 0.67), 50,000 (α = 0.72), 24,000 (α = 0.80), 12,500 (α = 0.87) and some low-molecular-weight species (α = 0.92). Three further peaks contained proteins with a molecular weight of *ca.* 37,000 (α = 0.75). For clarity these results are shown in three different figures (Fig. 8d, e and f).

The first two peaks in HPLC (α = 0.67 and 0.72) (Fig. 8d), which eluted at V_c = 430 ml from DEAE-Sephadex, appear to be associated with glucanases and a β-glucosidase activity.

Two species, both with α = 0.75 in HPLC, and with V_E = 570 and 770 ml, respectively, in the chromatogram obtained on DEAE-Sephadex, exhibited glucanase activities. Since they produced cellobiose they are most probably *exo*-glucanases. There was furthermore an inactive protein with α = 0.75 and with V_E = 950 ml. The peak (α = 0.80) normally hidden as a shoulder under the second main component of the HPLC separation seems to be a β-glucosidase since it corresponds to one of the two maxima in this activity (Fig. 8e and c).

In Fig. 8e and f two HPLC peaks (α = 0.87 and 0.92) seem to be due to inactive

material: the first peak ($\alpha = 0.87$) does not correlate with any activity, and the second peak is of low molecular weight ($\alpha = 0.92$). Similarly one main peak ($\alpha = 0.75$) apparently contains inactive protein as stated before.

This assignment of activities, though tentative, is expected to be a valuable tool for the localization of activities in enzyme reactors, as it can be performed much more readily than other tests.

CONCLUSIONS

The main advantage of size-exclusion HPLC is its speed without the need for regeneration of the system, enabling the rapid monitoring of alterations in protein mixtures due to degradation or adsorption processes.

Even from a complex mixture of enzymes, information on the activities of the different components can be obtained using relatively simple methods. The main activities in the cellulase system were found to be associated with the main HPLC peaks ($\alpha = 0.72$ and 0.75) and with a minor one ($\alpha = 0.80$). These components are rather strongly adsorbed on to the substrate, a finding that is important for reaction engineering in cellulose hydrolysis[14].

Chromatograms from DEAE-Sephadex columns show peaks which often contain several species of different molecular weight (Fig. 8). On the other hand peaks of different retention volume on DEAE-Sephadex can be proteins of the same molecular weight (see also Fig. 8).

ACKNOWLEDGEMENT

Support by the Federal German Ministry for Research and Technology is gratefully acknowledged.

REFERENCES

1 D. E. Schmidt, Jr., R. W. Giese, D. Conron and B. L. Karger, *Anal. Chem.*, 52 (1980) 177.
2 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 142 (1977) 311.
3 P. Roumeliotis and K. K. Unger, *J. Chromatogr.*, 185 (1979) 445.
4 C. T. Wehr and S. R. Abbott, *J. Chromatogr.*, 185 (1979) 453.
5 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
6 K. K. Unger and N. P. Becker, *28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, 1977, Cleveland, OH*, Abstr. No. 171.
7 D. Mathes and H. Engelhardt, *Naturwissenschaften*, 66 (1979) 551.
8 I. Molnar, *Chromatographia*, 12 (1979) 371.
9 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 185 (1979) 305.
10 J. F. K. Huber and F. Eisenbeiss, *J. Chromatogr.*, 149 (1978) 127.
11 T. K. Ghose, in T. K. Ghose, A. Fiechter and N. Blakebrough (Editors) *Advances in Biochemical Engineering*, Vol. 6, Springer, Berlin, 1977, pp. 39–76.
12 P. Linko, *Chem.-Ing.-Techn.*, 50 (1978), 655.
13 K. Buchholz, J. Puls, B. Gödelmann and H. H. Dietrichs, *Process Biochemistry*, 16 (1980/81) 37.
14 F. J. Bissett, *J. Chromatogr.*, 178 (1979) 515.
15 S. P. Shoemaker, R. D. Brown, *Biochim. Biophys. Acta*, 523 (1978) 133, 147.
16 D. Gabel, V. Kasche, *Acta Chem. Scand.*, 27 (1973) 1971.