Journal of Chromatography, 148 (1978) 237–245

CHROM. 10,407

RAPID CHROMATOGRAPHIC SEPARATION OF TECHNICAL ENZYMES ON SPHERON ION EXCHANGERS

O. MIKEŠ, P. ŠTROP and J. SEDLÁČKOVÁ

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

SUMMARY

New types of ion exchangers with a hydroxyalkyl methacrylate matrix (Spheron) that are suitable for the sorption and high-performance liquid chromatography of technical enzymes are described. Their use is illustrated by examples of the sorption and desorption of a microbial protease and by examples of the rapid, semi-preparative chromatography of technical enzymes (protease, glucose oxidase, pectinase).

INTRODUCTION

The rapid separation of technical enzymes by reversible sorption and modern high-performance liquid chromatography (HPLC) is important from the viewpoint of the development of industrial bioengineering. A necessary prerequisite of this development is the availability of suitable ion exchangers. The main requirements which these materials must fulfil are summarized in Table I.

Recently, we reported[1–3] on novel ion exchangers with a Spheron matrix, which have been employed successfully for the chromatography of biopolymers and their fragments, *i.e.*, proteins, peptides, amino acids, nucleic acids, oligonucleotides and nucleotides. We found later that the diethylaminoethyl derivative of Spheron is also suitable for the chromatography of sugars[4]. These Spheron ion exchangers, based on a hydroxyalkyl methacrylate matrix, conform to the requirements given in Table I. We have attempted to illustrate the possibilities of their application in the field of technical enzymes by an example of reversible adsorption of protease and by the chromatography of several other enzymes.

EXPERIMENTAL

Materials

The ion exchangers used are summarized in Table II. They were prepared in our laboratory from Spheron P-300, particle size 20–40 μm (Lachema, Brno, Czechoslovakia) on a small scale by methods described elsewhere[1–3]. Samples of the ion

TABLE I

REQUIRED CHARACTERISTICS OF ION EXCHANGERS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND REVERSIBLE SORPTION OF TECHNICAL ENZYMES

Required characteristic	*Reason*
(1) *Macroporosity*, allowing penetration of enzymes into beads	In the case of *microporous resins* only the functional groups situated on the surface of beads are available for chromatography of enzymes
(2) A sufficiently *hydrophilic character*, which guarantees that the native conformation of the enzymes and thus their activity are retained	Many ion-exchange resins with a *hydrophobic* matrix denature enzymes
(3) *Resistance* of matrix to *enzymes* and to *microbes*	Culture liquids and technical enzymes often attack *polysaccharide matrices* of conventional ion exchangers
(4) *Rigidity of matrix*, which ensures resistance of particles to pressure	HPLC and manipulation during sorption of enzymes require *ion exchanger particles not deformable whose abrasion is minimal*
(5) *Spherical shape* and *appropriate size* of ion exchanger particles	Spherical particles give *best flow-rates*. HPLC needs fine particle size, technical application coarse size
(6) Possibility of *repeated use* without loss of capacity and *chemical resistance*	Regeneration of ion exchangers after separation of crude technical enzymes often calls for *solutions at extreme pH values* or for *organic solvents*
(7) *Minimal changes in volume* as result of changes in pH and ionic strength	Regeneration, cycling, equilibration and sorption of enzymes must be effected *in the same column* without a substantial bed change

TABLE II

ION EXCHANGERS USED

Designation	*Composition*	*Functional group*	*Capacity for small ions (mequiv./g)*
Cation exchangers			
CM-Spheron P 300	Carboxymethyl-Spheron	–COOH	2.0
S-Spheron P 300	Sulphonyl-Spheron	$-SO_3H$	1.0
Anion exchanger			
DEAE-Spheron P 300	Diethylaminoethyl-Spheron	$-N(C_2H_5)_2$	2.0

exchangers are commercially available*. The types of technical enzymes and their sources are shown in Table III.

Preparation of ion exchangers

Fresh, dry ion exchangers were suspended in water before use, the suspension

* Small batches of Spheron ion exchangers are prepared by Lachema, Brno, Czechoslovakia. The Spheron gels and their derivatives are distributed by Koch-Light Labs., Colnbrook, Great Britain.

TABLE III

TECHNICAL ENZYMES USED

Enzyme	*Characteristic*	*Source*
Proteolytic	Crude protease from *Aspergillus sojae*	Japan*
Glucose oxidase	Technical preparation of fungal origin (*Aspergillus niger*)	Boehringer, Mannheim, G.F.R.**
Pectolytic	Leozym, technical preparation of fungal origin (*Aspergillus niger*)	Product of Lihovary a Konservárny, Leopoldov, Czechoslovakia***

* Courtesy of Dr. J. Turková, of this Institute.
** Courtesy of Dr. J. Čoupek, Laboratorní přístroje, Prague.
*** Experimental sample, courtesy of Dr. L. Rexová, Institute of Chemistry, Slovak Academy of Sciences, Bratislava.

was deaerated *in vacuo* of a water aspirator and the resin was allowed to settle several times. All ion exchangers (volume always 10–11 ml) were stirred before and after use with 50 ml of the regenerating solution on a glass filter; the solution was aspirated off after 10 min. This procedure was repeated several times. The sequence of the regenerating solutions was as follows: 2 *M* sodium chloride solution, water, 2 *M* sodium hydroxide solution (with cation exchangers), water, 2 *M* hydrochloric acid, water; anion exchangers were washed first with the acid, then with water and lastly with the hydroxide. Ion exchangers in the H^+ or OH^- form were equilibrated with the first eluting buffer by an appropriate procedure[5].

Chromatography

The ion exchangers treated as described were packed into a jacketed column of the type used in amino acid analyzers; a 20 × 0.8 cm column was formed. The column was cooled with tap water. The enzyme was always dissolved in 0.2–0.4 ml of the first eluting buffer and the solution placed under a buffer layer on top of the column. The buffers were supplied from the gradient mixing device to the column by a two-piston proportional pump (Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), operating with minimum pulses. The emergence of the effluent components was recorded at 285 and 254 nm by means of two through-flow cells connected in series. The absorbance was recorded in EZ 4 and TZ 21 S line recorders (Laboratorní přístroje, Prague, Czechoslovakia), simultaneously recording also the collection of fractions by a collector operated on a time principle. The pH of the fractions was measured with a compensating pH meter (Instrument Development Workshops) and the conductivity with a Type OK-102/1 conductivity meter (Radelkis, Budapest, Hungary).

Sorption and desorption

CM-Spheron (*ca.* 10 g) for the sorption was prepared as described above; subsequently the resin was placed on a glass filter (S_2, diameter 5 cm) where a *ca.* 2.5-cm column had formed; the latter was covered with a disc of filter-paper.

Technical protease (0.5 g) was dissolved in 200 ml of cold water, a small amount of sediment was centrifuged off and the pH of the solution was adjusted to 4.5 with 10% acetic acid. This solution was passed by gravity through the CM-

Spheron column at 4°; fractions of 20 ml were collected (*cf.*, Fig. 2). After the application of 200 ml of the supernatant, the glass filter was washed with 200 ml of equilibrating buffer; the components that were not sorbed were thus displaced. Subsequently, desorption was effected by elution of the ion exchanger with the desorption buffer; fractions of 10 ml were collected. All fractions were tested by measurement of the absorbance at 280 nm, conductivity, pH and proteolytic activity.

The active fractions desorbed were pooled (80 ml), frozen with stirring to a crushed ice form and mixed with 120 ml of cold acetone at −20°. The precipitate thus formed was centrifuged for 30 min at −10° and 1100 *g*. The sediment was freed from acetone at 4° *in vacuo* on a water aspirator.

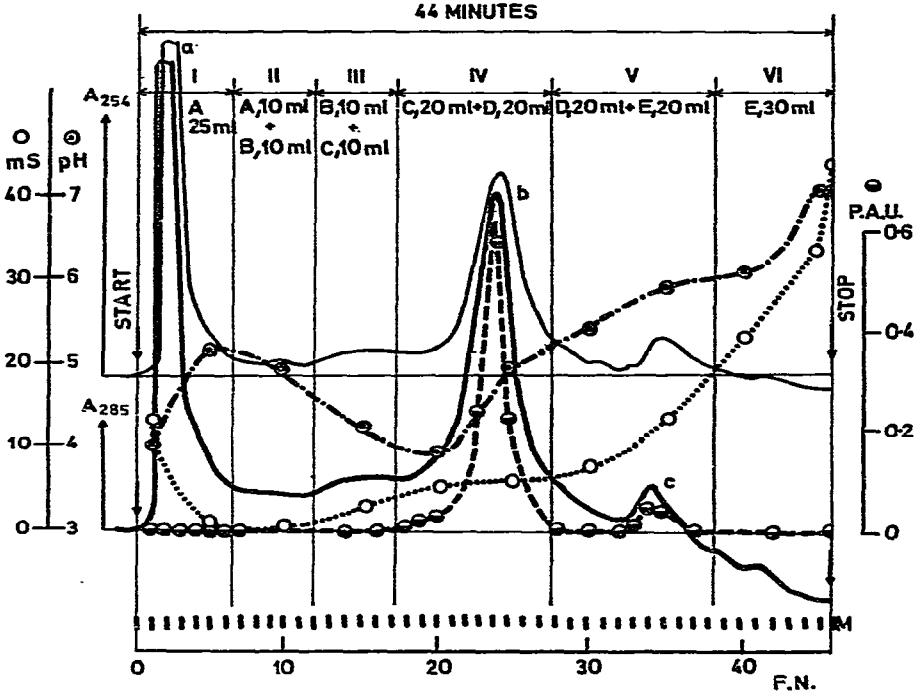

Fig. 1. Chromatography of crude protease on CM-Spheron. The ion exchanger was equilibrated with buffer A. Load: 20 mg of enzyme in 0.3 ml of the same buffer. I: buffer A without gradient. Subsequently linear gradients II (A + B), III (B + C), IV (C + D) and V (D + E) were applied. VI: buffer E without gradient. Flow-rate, 4.07 ml/min; fractions, 4.24 ml; temperature, 10°; counter pressure, 5–10 atm. Buffers: A, 0.005 *M* ammonia + formic acid, pH 4.5; B, 0.05 *M* ammonia + formic acid, pH 4.5; C, 0.25 *M* ammonia + acetic acid, pH 6.0; D, 0.5 *M* ammonia + acetic acid, pH 8.0; E, buffer D, 1.0 *M* in NaCl, pH 8.0. Full lines (———), record of effluent absorbance at 285 and 254 nm by two flow-through cells connected in series; chart speed, 5 mm/min. The broken line (– – –) shows proteolytic activity measured on 0.5-ml aliquots; the activity is given in absorbance units (280 nm) of trichloroacetic acid filtrate of digested haemoglobin (a proteolytic activity unit P.A.U. corresponds to absorbance 1.0). The position of the values was corrected to account for the retardation of fractions with respect to the record by a shift corresponding to the tubing volume + half the volume of the fraction. The fractions were also subjected to measurement of pH (–·–·–) and conductivity (.....), expressed in millisiemens (mS). a, Inactive substances; b, neutral proteinases; c, basic proteinases. M represents automatic marking of fraction collection. F.N. = Fraction number.

Determination of enzymatic activities

Aliquots taken from individual fractions were assayed by the following methods. Proteolytic activity was determined in terms of haemoglobin cleavage using a modification[6] of Anson's method[7]. The activity of glucose oxidase was determined manometrically according to oxygen uptake[8], and polygalacturonanase activity by the method of Somogyi[9], *i.e.*, by the determination of the increase in reducing saccharides after cleavage of pentagalacturonic acid.

RESULTS

The chromatography of the sample of microbial protease (Fig. 1) showed that

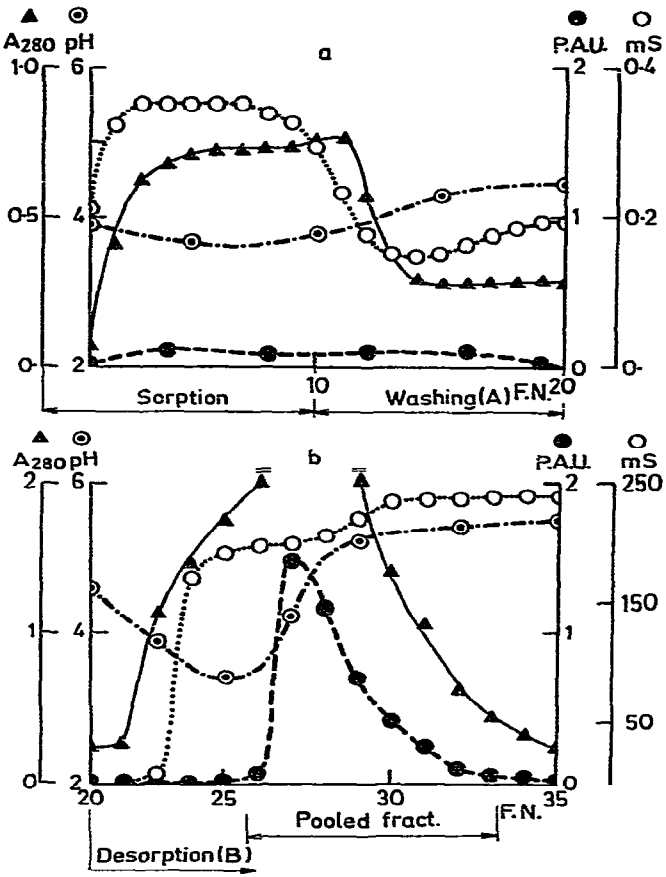

Fig. 2. Course of sorption (a) and desorption (b) of crude protease to CM-Spheron. (a) A solution of 0.5 g of the enzyme preparation in 200 ml of water, pH 4.5, was filtered by gravity through a 5 × 2.5 cm column of ion exchanger formed on a glass filter and equilibrated with buffer A. Fractions (20 ml) were collected at 3-min intervals. The ion exchanger was then washed with 200 ml of buffer A at the same rate. (b) Desorption was effected by washing with 150 ml of buffer B; 10-ml fractions were collected at 2-min intervals. The fractions were subjected to all measurements described in Fig. 1. Buffers: A, 0.005 *M* ammonia + formic acid, pH 4.5; B, 0.25 *M* ammonia + acetic acid, pH 6, 0.5 *M* in NaCl. The operation was carried out at 4°. F.N. = Fraction number; P.A.U. = proteolytic activity units.

proteolytic enzymes are reversibly sorbed to the carboxymethyl derivative of Spheron. This ion exchanger could therefore serve well for the technical sorption and desorption of these enzymes. To check this possibility, the experiment illustrated in Fig. 2 was carried out; the Büchner funnel was replaced with a glass filter. The product desorbed was precipitated with acetone in the cold and analysed by chromatography on DEAE-Spheron (Fig. 3). The solution applied was brown because of the pigments present in the protease; these pigments had adsorbed on the protease during the acetone precipitation. These pigments were eluted from the resin virtually in one band together with the main zone of the protease. The experiment showed that CM-Spheron can be used for reversible sorption of basic and neutral enzymes. It also illustrates the possibility of using this ion exchanger and also of DEAE-Spheron for the rapid, semi-preparative analysis of technical proteases.

The chromatography of technical glucose oxidase (Fig. 4) represents another example of use of DEAE-Spheron for similar purposes when more complicated mixtures are to be analyzed. The chromatography of the same enzyme preparation on the sulphonic acid derivative S-Spheron can be effected in 25 min, as reported earlier (without testing the activity of individual fractions; ref. 1, Fig. 8).

Leozym, a pectolytic technical enzyme, was analyzed chromatographically as

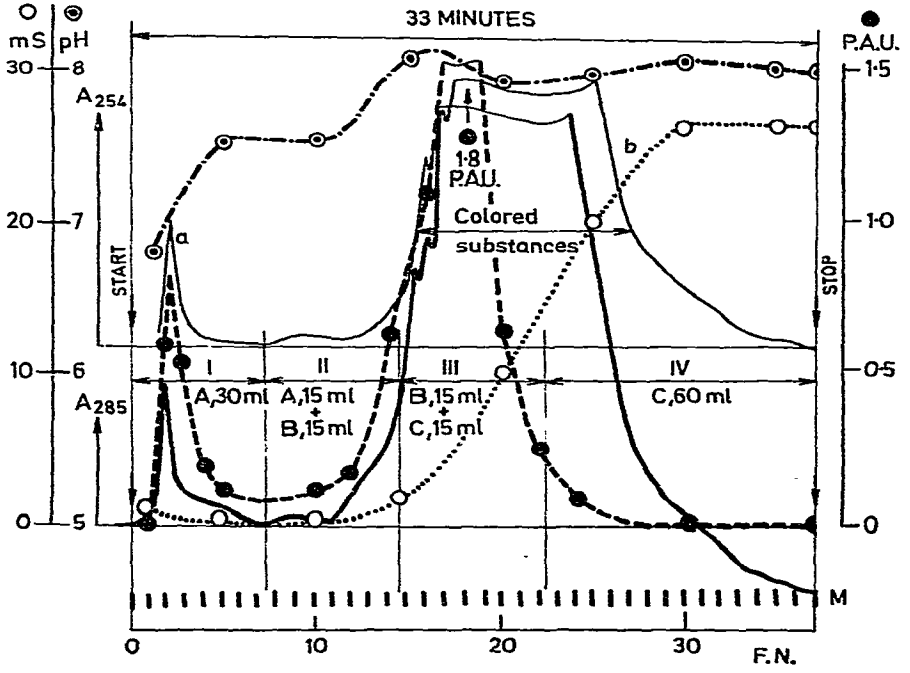

Fig. 3. Chromatogram of proteolytic enzyme desorbed from CM-Spheron (*cf.*, Fig. 2) on a column of DEAE-Spheron. The ion exchanger was equilibrated with buffer A. Load: about half of the sediment after acetone precipitation of desorbed protease, dissolved in 0.4 ml of buffer A. I: buffer A without gradient, followed by linear gradients II (A + B) and III (B + C). IV: buffer C without gradient. Flow-rate, 4.5 ml/min; 4.05-ml fractions; temperature, 10°; counter pressure, 5–10 atm; other parameters and tests of fractions as in Fig. 1. Buffers: A, 0.01 *M* acetic acid + sodium hydroxide, pH 6.8; B, 0.2 *M* acetic acid + sodium hydroxide, pH 6.6; C, buffer B, 1 *M* in NaCl, pH 6.5. F.N. = Fraction number; P.A.U. = proteolytic activity units. a = Basic, and b = neutral substances.

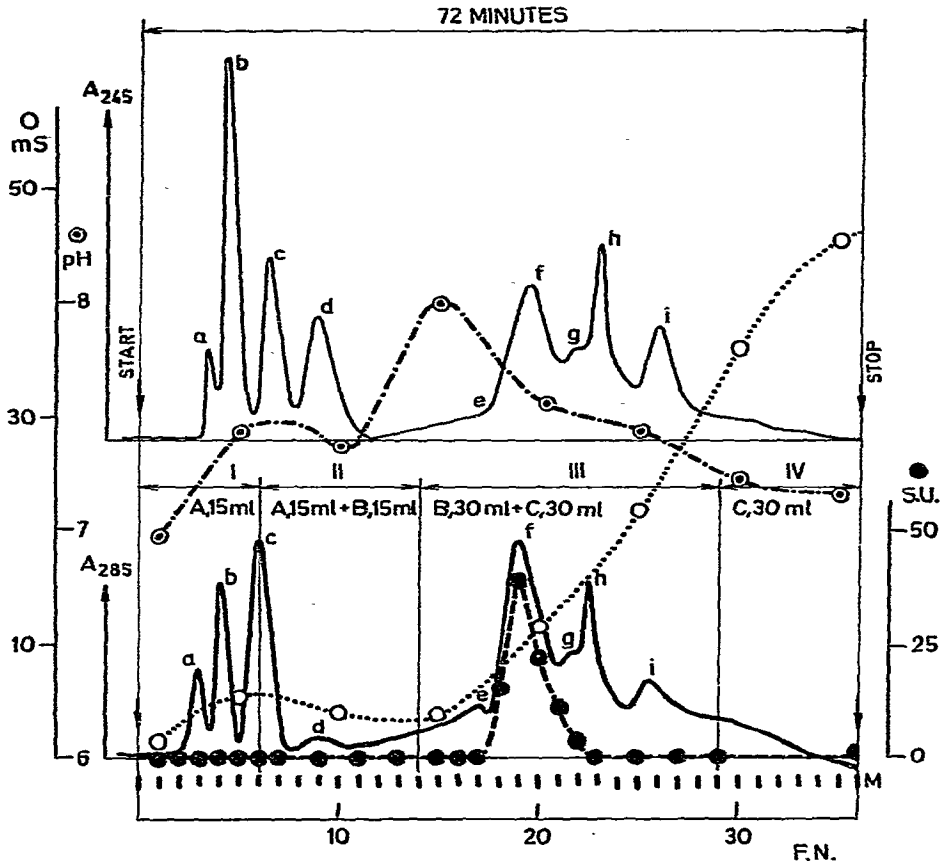

Fig. 4. Chromatography of technical glucose oxidase on DEAE-Spheron. Load: 15 mg of preparation in 0.2 ml of buffer A. The ion exchanger was equilibrated with buffer A. I. buffer A without gradient. Linear gradients II (A + B) and III (B + C). IV, buffer C without gradient. Flow-rate, 2 ml/min; 4-ml fractions; temperature, 14°; counter pressure, 3–7 atm; chart speed, 2 mm/min. Buffers: A, 0.01 *M* acetic acid + NaOH, pH 6.8; B, 0.3 *M* acetic acid + NaOH, pH 5.5; C, buffer B, 1 *M* in NaCl, pH 5.3. Broken line, glucose oxidase activity of effluent in Sarret units (S.U.; 1 S.U. corresponds to the consumption of 600 μl of oxygen at 30°) with correction of shift of values (see legend to Fig. 1 for explanation). The remaining testing of fractions and details of description are identical with those described in the legend to Fig. 1. Corresponding peaks in both parts of the chromatogram are designated by a–i. The UV spectrum of the chromophore of compound d differed significantly from the remaining ones. F.N. = Fraction number.

shown in Fig. 5 and a rapid and satisfactory separation of the main components was achieved.

DISCUSSION

The rate of separation, illustrated by the examples given in Figs. 1 and 3–5, does not represent the limit of applicability of Spheron ion exchangers. An up-to-date liquid chromatograph was not used in the experiments described above. The apparatus was built of readily available components not designed for HPLC and therefore better parameters could not be achieved. Likewise, the particle size of the resin,

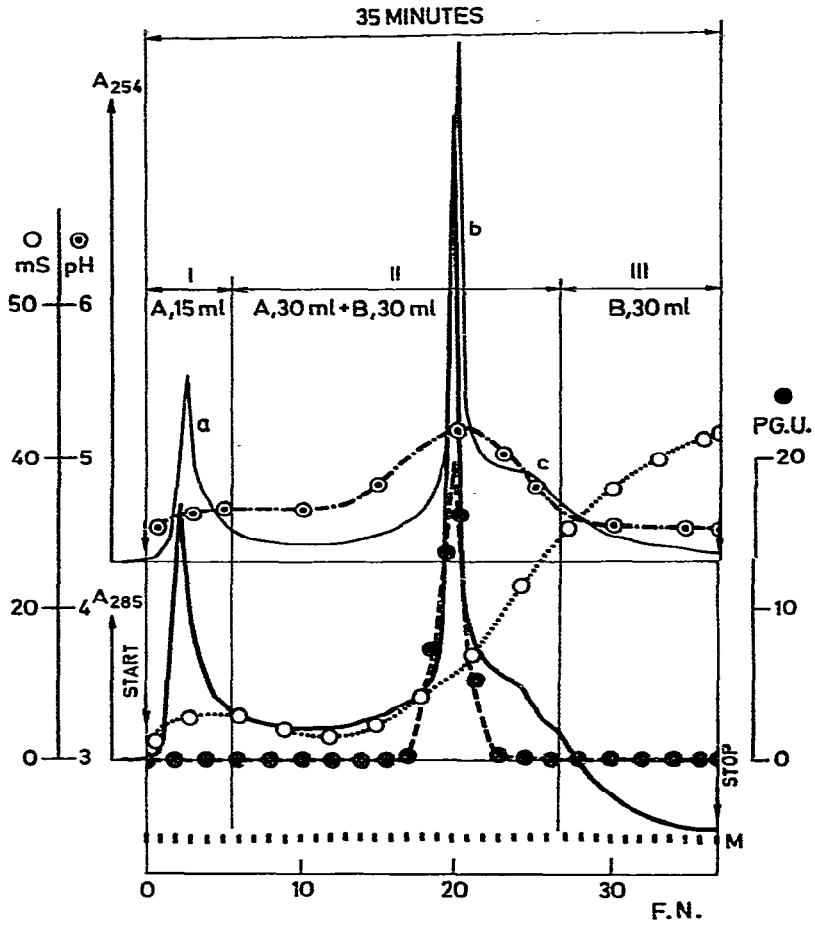

Fig. 5. Chromatogram of technical pectolytic enzyme on DEAE-Spheron. The ion exchanger was equilibrated with buffer A. *Load*: 25 mg in 0.3 ml of the same buffer. I: buffer A without gradient. II: linear gradient of buffer A + B. III: buffer B without gradient. Flow-rate, 3.0 ml/min; 2.84-ml fractions; temperature, 16°; counter pressure, 6–10 atm. Buffers: A, 0.3 *M* acetic acid + sodium hydroxide, pH 4.5; B, buffer A, 1 *M* in NaCl. Broken line: activity of polygalacturonase in units (PG.U.) μmole · min^{-1} · ml^{-1} × 10^3, corrected for the shift of fractions as described in the legend to Fig. 1. F.N. = Fraction number. a and c, Balast proteins; b, polygalacturonase.

20–40 μm, is not optimal for the chromatography of biopolymers. A smaller size of the beads of Spheron resins than are developed at present will permit shorter diffusion times and thus provide new possibilities for speeding up the chromatography of biopolymers to an extent comparable with the HPLC of other types of compounds.

An important factor in the use of Spheron ion exchangers in bioengineering and in the analysis of technical enzymes is that these resins are so hydrophilic that they do not denature the enzymes. They can therefore be widely applied not only in separation procedures based on sorption but also for the immobilization of enzymes in enzyme reactors. Their mechanical and chemical stability and the possibility of their repeated use without loss of their original properties make them very suitable

for this purpose. The synthetic matrix of these resins, moreover, is not digested by any enzyme and they are not likely to be attacked by microbial infection.

The experiment illustrated in Fig. 2 shows that these ion exchangers, even when used in the form of a low and wide bed of a glass filter, sorb very well; the course of the experiment is essentially that of chromatography, even though less perfect than in a column arrangement. The re-calculation of flow-rates used with Spheron ion exchangers during chromatography in our columns (*i.e.*, 8 ml/cm^2 · min) for columns used in laboratories operating on the kilogram scale and pilot plants gives values of tens of litres per minute and even higher. These values are of technical importance. The development of Spheron ion exchangers should therefore also be directed to materials suitable for this purpose.

ACKNOWLEDGEMENTS

The authors thank Dr. Ljuba Rexová-Benková for the determination of polygalacturonase activity, Dr. Lada Huderová-Svobodová for her help with the determination of glucose oxidase activity, and the colleagues mentioned in Table III for the samples of the enzymes.

REFERENCES

1 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339.
2 O. Mikeš, P. Štrop and J. Čoupek, *J. Chromatogr.*, in press.
3 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, in preparation.
4 O. Mikeš, Z. Chytilová, J. Farkaš, P. Vrátný and P. Štrop, *Czechoslovak Federation Biochemical Meeting, Starý Smokovec (Czechoslovakia), May 4–6, 1977, Abstr. No. 8.14, Bull. Czech. Biochem. Soc.*, 5 (1977) 89.
5 O. Mikeš, in Z. Deyl, K. Macek and J. Janák (Editors), *Liquid Column Chromatography*, Elsevier, Amsterdam, 1975, p. 355.
6 J. Turková and O. Mikeš, *Collect. Czech. Chem. Commun.*, 36 (1971) 2739.
7 M. L. Anson, *J. Gen. Physiol.*, 22 (1938) 79.
8 A. Kleinzeller, *Manometrické Metody*, Stát. Zdravotnické Vydavatelství, Prague, 1954, pp. 10 and 21.
9 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19.