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HYDROXYALKYL METHACRYLATE GELS DERIVATIZED WITH EPICHLOROHYDRIN AS SUPPORTS FOR LARGE-SCALE AND HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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SUMMARY

The preparation of hydroxyalkyl methacrylate copolymers derivatized with epichlorohydrin (epoxide group content 140–1770 μmol/g) has been accomplished. The coupling of various amino derivatives on to derivatized carriers has been investigated as a function of pH, time of coupling, concentration of the compound bound, degree of epoxidation of the carrier and specific structural features of amino compounds. The unreacted epoxide groups were eliminated using hydrolysis with 0.1 *M* perchloric acid.

For the preparation of a specific sorbent of carboxylic proteinases, a procedure for the synthesis of ε-aminocaproyl-L-Phe-D-Phe-OCH_3 and for its binding on to an epoxide carrier has been devised. The sorbents prepared were used in affinity chromatography of a raw sample of pepsin and proteinases from *Aspergillus oryzae*. The same efficiency was achieved with pepsin solutions, the enzyme concentration differing within the range of two orders of magnitude. The mechanical stability of the support made possible an analytical application of the high-performance liquid affinity chromatography of pepsin, by which the pepsin concentration in solution can be determined within 30 min with high sensitivity.

INTRODUCTION

The use of microbial enzymes as biocatalysts in industry is promising because of the abundance of their sources. Many microorganisms are able to produce enzymes at high concentrations in the cultivation medium. The method of choice for obtaining individual enzymes from such mixtures is affinity chromatography. Of course, the simultaneous presence of enzymes of various types (proteinases, amylases, cellulases, etc.) in media makes specific demands both on the solid support and on the

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affinity ligand. The support must not only be macroporous and mechanically and chemically stable, but also resistant to the action of enzymes. A suitable support is the macroporous spherical hydroxyalkyl methacrylate carrier derivatized with epichlorohydrin. The hydroxyalkyl methacrylate gel is prepared by the copolymerization of hydroxyalkyl methacrylate with alkylene dimethacrylate[1]. With respect to interactions with biopolymers, it has many properties in common with agarose, which at present is the support most frequently used in affinity chromatography[2]. Using the cyanogen bromide activation method elaborated by Porath and co-workers[3,4], trypsin inhibitor and chymotrypsin were successfully bound on the hydroxyalkyl methacrylate gel[5,6]. Both of these compounds have been used in the affinity chromatography of chymotrypsin or its inhibitors[5]. Similarly, binding by means of benzoquinone, introduced by Brandt *et al.*[7], has been applied successfully[8] on hydroxyalkyl methacrylate gel.

In this study we utilized another method developed in Porath's laboratory[9–12] and by Matsumoto *et al.*[13], *viz.*, modification of the carrier with epichlorohydrin. We investigated both the preparation of the derivatized hydroxyalkyl methacrylate gel with various contents of epoxide groups and the binding of various amino compounds as a function of time, pH of the reaction medium and concentration of reactive groups. As an example of the preparation of the carrier for affinity chromatography, a specific sorbent formed by binding ε-aminocaproyl-L-Phe-D-Phe-OCH_3 on the epoxidized hydroxyalkyl methacrylate gel was studied in greater detail. We examined its usefulness not only in the isolation of carboxylic proteinases, but also in their high-performance affinity chromatography.

EXPERIMENTAL

Materials

The hydroxyalkyl methacrylate gels Separon H 300 (molecular weight exclusion limit 300,000, specific surface area *ca.* 90 m^2/g, particle size 125–200 μm) and Separon H 1000 (molecular weight exclusion limit 1,000,000, specific surface area *ca.* 30 m^2/g, particle size 100–200 μm) were prepared by a procedure described earlier[1].

Porcine pepsin with a specific activity of 14 units/mg was obtained from Léčiva (Dolní Měcholupy, Czechoslovakia). A crude enzymatic preparation of *Aspergillus oryzae* (Amylorizin P1OX) with a specific activity of 0.25 units/mg was obtained from the All-Union Research Institute of Biotechnology (Moscow, U.S.S.R.).

Reaction of Separon with epichlorohydrin

(a) For the preparation of maximally derivatized Separon H 1000 E_{max}, Separon H 1000 (10 g) was swollen in 50% potassium hydroxide solution (50 ml) at a temperature below 10°C for 15 h. The support was then filtered by suction on a glass filter and transferred into a flask with epichlorohydrin (40 ml). The temperature of the stirred mixture rose to the boiling point of epichlorohydrin within 1 h. After completion of the reaction, saturated aqueous potassium bromide solution (10 ml) was added, and the mixture was boiled with stirring for 3 h. On cooling, the sorbent was removed by filtration, washed three times with acetone on the glass filter (100 ml each time), with water (100 ml each time) to a neutral reaction of the filtrate, then washed with acetone again, and finally with diethyl ether (three times, 100 ml each time). The

material was dried in a vacuum drying box at room temperature for 24 h. The content of epoxide groups was 1770 μmol/g. Separon H 300 E_{max} prepared in a similar way contained 1550 μmol/g.

(b) Medium derivatized Separon H 1000 E_{med} was prepared from the same amount of the gel as in (a). The swollen gel was suspended in dioxan (20 ml), and a solution of epichlorohydrin (25 ml) in dioxan (25 ml) was added to the suspension. The mixture was stirred at room temperature for 4 h, the sorbent was removed by filtration, washed and dried as in (a). The content of epoxide groups was 800 μmol/g. Separon H 300 E_{med} prepared similarly contained 600 μmol/g.

(c) Minimally derivatized Separon H 1000 E_{min} was prepared by procedure (b), with the difference that a solution of 1.6 ml of epichlorohydrin in 25 ml of dioxan was used in the derivatization, and the reaction time was 3 h. The content of epoxide groups was 140 μmol/g. Separon H 300 E_{min} prepared similarly contained 240 μmol/g.

Methods

Epoxide groups were determined by addition of bromide anion in anhydrous acetic acid followed by titration of the released base with a solution of perchloric acid in acetic acid, with visual indication using crystal violet or quinaldine red[14]. The amounts of derivatized gel used varied between 30 and 300 mg, the solution of perchloric acid was 0.01–0.1 *M* according to the content of epoxide groups. The amount of tetraethylammonium bromide or cetylpyridinium bromide was five times that of the theoretically required amount, *i.e.*, in a single determination it was 7 ml of a freshly prepared solution of ammonium salt (0.1–0.005 *M*) in glacial acetic acid neutralized in advance with perchloric acid to the same colour of the indicator as in the titration itself. The reaction mixture was titrated after standing for 6–8 h. The reproducibility varied in the range ±3–4 μmol/g if 0.01 *M* perchloric acid was used. The method was checked on a sterically hindered model compound, 3-*tert.*-butyl-1-cyclohexene epoxide, where it yielded results within the limits of ±0.35%.

The coupled amino derivatives were determined by amino acid analysis or by determination of the nitrogen content by the Kjeldahl method. Prior to analysis, the sample of the modified gel was washed with 6 *M* guanidinium chloride, water and acetone, or with water, ethanol, *n*-butanol and ethanol, dried to constant weight at 105°C and hydrolysed in 6 *M* hydrochloric acid at 110°C for 20 h.

The proteolytic activity was determined by a modified method suggested by Anson[15,16]. The activity unit was defined as the amount of proteolytic activity (measured at pH 2 for pepsin and at pH 4 for carboxylic proteinase from *Aspergillus oryzae*) which yields an absorbance at 280 nm equal to unity, related to an incubation time of 1 min at 37°C.

Coupling of amino derivatives on Separon H 1000 E_{max}. The gel (1 g) was suspended in 5 ml of aqueous ammonia, 1,2-diaminoethane, 1,4-diaminobutane or 1,6-diaminohexane solution, concentration 0.5 *M*. The pH of the mixture was adjusted to 11.5 by adding hydrochloric acid or sodium hydroxide solution. The suspension was stirred at room temperature for 6 or 40 h. On completion of the coupling reaction, unreacted epoxide groups were blocked by reaction with 2-aminoethanol at room temperature for 24 h. The results are shown in Fig. 1.

Coupling of 2-aminoethanol on Separon H 1000 E_{max} as a function of pH and

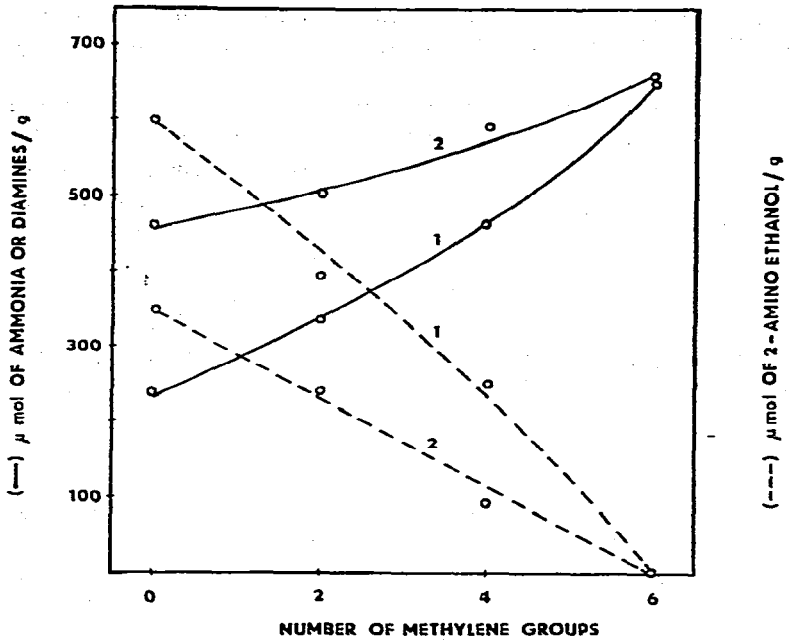


Fig. 1. Coupling of ammonia and diamines [$H_2N(CH_2)_nNH_2$] (solid lines) on Separon H 1000 E_{max} followed by blocking of unreacted epoxide groups with 2-aminoethanol (broken lines). Base solutions (concentration 0.5 *M*) in water, pH adjusted to 11.5, coupling at room temperature. Curves 1, coupling 6 h; curves 2, coupling 40 h.

coupling time. The gel (0.1 g) was suspended in 5 ml of a solution of 2-aminoethanol (concentration 0.5 *M*) in Britton–Robinson buffer (pH 3, 5, 7, 9, 11) (additional adjustment of pH by means of phosphoric acid is necessary; in the case of pH 9 an aqueous solution was used, the pH of which was also adjusted with phosphoric acid).

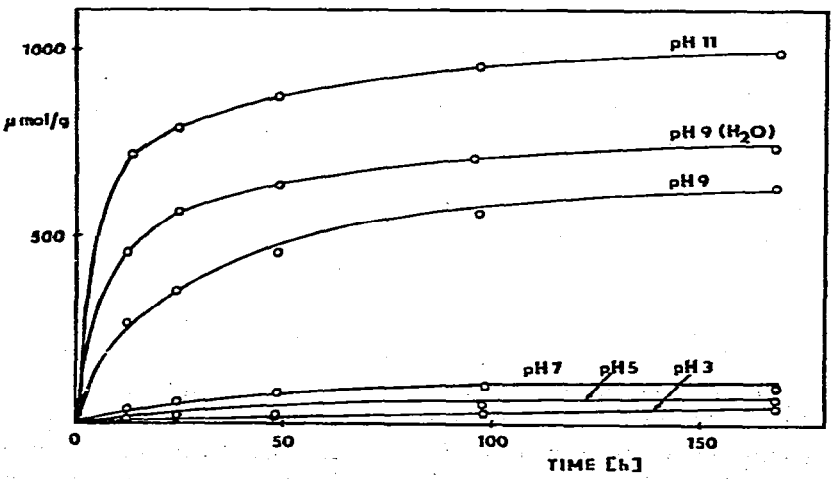


Fig. 2. Coupling of 2-aminoethanol (in μmol per gram of dry conjugate) bound on Separon H 1000 E_{max} as a function of coupling time and pH. Britton–Robinson buffers were used for pH 3, 5, 7, 9 and 11, with the exception of pH 9, where the aqueous solution was also used. Coupling at room temperature.

The suspension was shaken at room temperature for 12, 24, 48, 96 or 162 h. The results are shown in Fig. 2.

Coupling of ε-aminocaproic acid, ethylamine, 1,2-diaminoethane and 1,6-diaminohexane on Separon H 300 E_{max}. (a) The gel suspension (0.5 g) in 2.5 ml of a 1 *M* aqueous solution of ε-aminocaproic acid (pH 3–12, adjusted with hydrochloric acid or sodium hydroxide solution) was shaken at room temperature for 120 h.

(b) The gel suspension (0.1 g) in 2 ml of ethylamine solution (1 *M*) in citrate–phosphate buffer (pH 7) or in an aqueous solution of pH 9 or 11 (pH adjusted with hydrochloric acid or sodium hydroxide solution) was shaken at room temperature for 72 or 144 h, respectively.

(c) The gel suspension (0.2 g) in 2 ml of 1,2-diaminoethane solution (1 *M*) in citrate-phosphate buffer (pH 3, 5, 7, pH adjusted with phosphoric acid or sodium hydroxide solution) and in water (pH 9 and 11, adjusted with hydrochloric acid or sodium hydroxide solution) was shaken at room temperature for 144 h.

(d) The gel suspension (0.2 g) in 2 ml of 1 *M* aqueous solution of 1,6-diaminohexane (pH 3–12, adjusted with hydrochloric acid) was shaken at room temperature for 72 h.

The results of the experiments in (a)–(d) are summarized in Fig. 3.

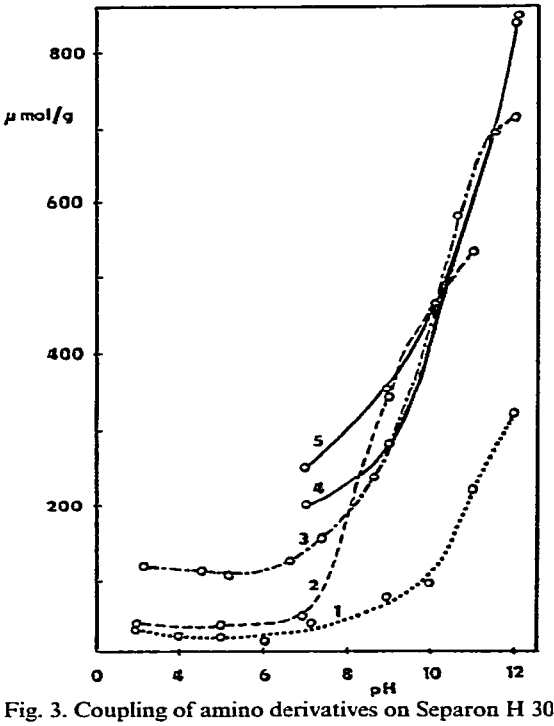


Fig. 3. Coupling of amino derivatives on Separon H 300 E_{max} as a function of pH. Curve 1, coupling of ε-aminocaproic acid, 120 h, aqueous solution of concentration 1 *M*, pH 3–12; curve 2, coupling of 1,2-diaminoethane, 144 h, solution of concentration 1 *M*, pH 3, 5, 7 in citrate–phosphate buffers, pH 9 and 11, aqueous solution adjusted to the required value; curve 3, coupling of 1,6-diaminohexane, 72 h, aqueous solution of concentration 1 *M*, pH 3–12; curve 4, coupling of ethylamine, 72 h; curve 5, coupling of ethylamine, 144 h, both for a solution of concentration 1 *M*, pH 7 citrate–phosphate buffer, pH 9 and 11, aqueous solution adjusted to the required value. Coupling at room temperature.

Coupling of ε-aminocaproic acid on the gel as a function of the degree of epoxidation and of the concentration of the ligand. The gel (0.5 g; Separon H 300 E_{max}, Separon H 300 E_{med}, Separon H 300 E_{min}, Separon H 1000 E_{max}, Separon H 1000 E_{med}, Separon H 1000 E_{min}) was suspended in 2.5 ml of an aqueous solution of ε-aminocaproic acid (0.05, 0.1, 0.25, 0.5 and 1.0 *M*) at pH 11 (adjusted with sodium hydroxide solution). The suspension was shaken at room temperature for 120 h. The results are shown in Fig. 4.

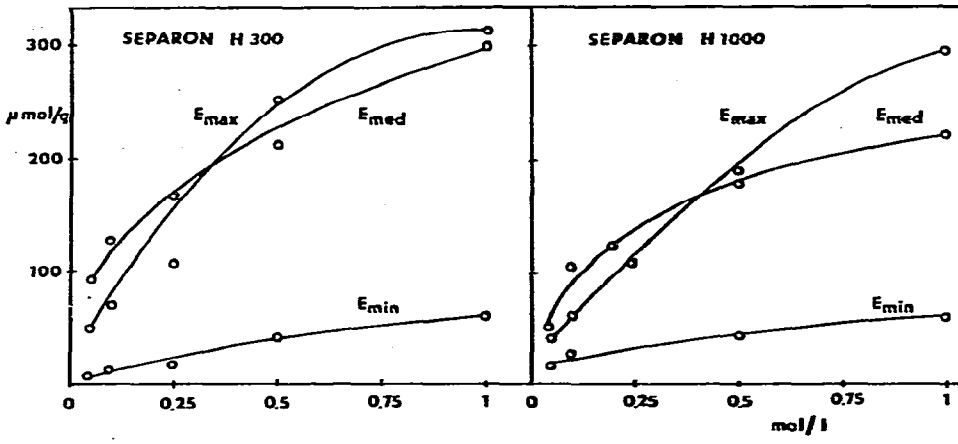


Fig. 4. Coupling of ε-aminocaproic acid on Separon 300 and H 1000 (in μmol per gram of dry conjugate) as a function of the concentration of ε-aminocaproic acid in the reaction medium and of the degree of epoxidation of Separon. Aqueous solution, pH 11; room temperature.

Coupling of glycyl-D-phenylalanine and glycyl-D-leucine as a function of coupling time and pH. Dipeptide (20 mg) was dissolved in Britton–Robinson buffer of pH in the range 4–11.5 (in buffers of pH 8 and higher, the final pH was adjusted by adding 0.2 *M* sodium hydroxide solution). The gel (100 mg) was added to the solution, and the suspension was shaken at room temperature for 12, 24 and 48 h. The results are shown in Fig. 5.

Synthesis of N-(2-nitrobenzenesulphenyl)-ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester. N-(2-Nitrobenzenesulphenyl)-ε-aminocaproic acid was prepared as follows. To a mixture of ε-aminocaproic acid (48 g), 2 *M* sodium hydroxide solution (183 ml) and dioxan (450 ml), 76.8 g of 2-nitrobenzenesulphenyl chloride were added with stirring at 17°C within 30 min. The pH of the mixture was maintained within the range 11.5–12.5 by adding 2 *M* sodium hydroxide solution (pH meter). After the whole amount had been added, the mixture was stirred at 30°C for 1 h, poured into water (4 l), freed from solid fractions by filtration, and the aqueous solution was acidified to pH 2–3 with 0.5 *M* sulphuric acid and shaken with ethyl acetate. On drying (sodium sulphate), the organic extracts were mixed with dicyclohexylamine (69.5 ml) and 450 ml of diethyl ether. The dicyclohexylammonium salt which separated by standing at 0°C was filtered by suction, washed with diethyl ether (twice with 50 ml each time) and dried. The yield was 146 g (86%), m.p. 137–138.5°C. For $C_{24}H_{39}N_3O_4S$ (mol. wt. 465.7): calculated, C 61.91, H 8.44, N 9.02; found, C 62.04, H 8.54, N 8.86%.

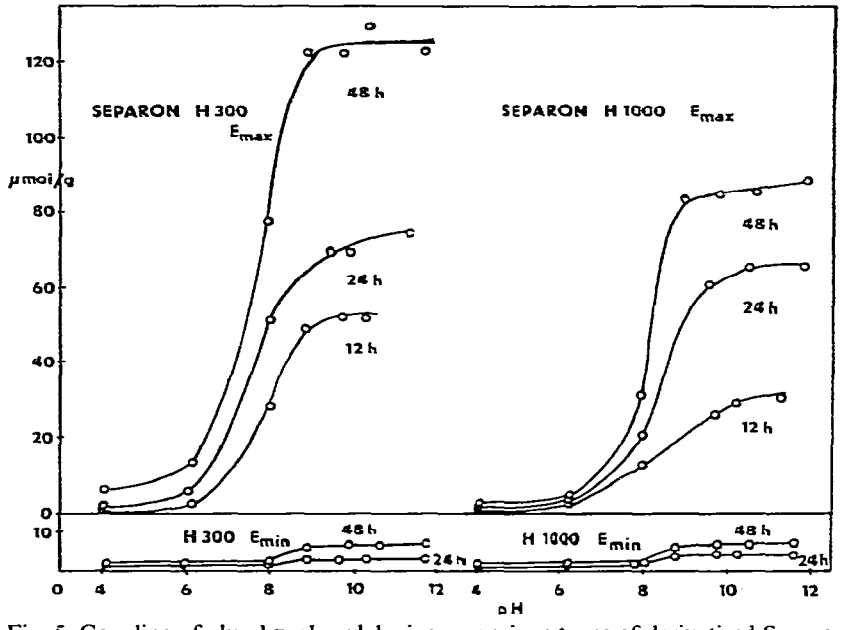


Fig. 5. Coupling of glycyl-D-phenylalanine on various types of derivatized Separons as a function of pH and time.

The tripeptide was prepared as follows. To a solution of L-phenylalanyl-D-phenylalanine methyl ester (36.3 g) and N-(2-nitrobenzenesulphenyl)-ε-aminocaproic acid (46.6 g, dicyclohexylammonium salt) in chloroform (500 ml) were added 23 g of N,N'-dicyclohexylcarbodiimide at −10°C. The mixture was stirred at −10°C for 1 h and left to stand overnight at 0°C. After filtration of the mixture, evaporation of chloroform, dissolution of the residue in ethyl acetate (1200 ml), washing with 0.5 *M* sodium hydroxide solution, water, 0.5 *M* sodium hydrogen carbonate solution and water, drying and evaporating, 46.7 g (79%) of a product with m.p. 155–157°C (ethyl acetate–diethyl ether) were obtained. For $C_{31}H_{36}N_4O_6S$ (mol. wt. 592.8): calculated, C 62.91, H 6.12, N 9.45%; found, C 62.57, H 6.31, N 9.80%.

ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester hydrochloride. Protected tripeptide (2 g) was suspended in methanol (50 ml), 1 *M* hydrochloric acid in methanol (5 ml) was added and the mixture was left to stand at 20°C for 10 min (checked by thin-layer chromatography in benzene). The reaction mixture was then evaporated, triturated with diethyl ether and the solid fraction after filtration was dissolved in methanol and precipitated with diethyl ether. The yield was 1.3 g (80%), m.p. 177–178°C. For $C_{25}H_{34}ClN_3O_4$ (mol. wt. 476.0): calculated, C 63.08, H 7.20, Cl 7.45, N 8.83%; found, C 62.89, H 7.15, Cl 7.32, N 8.15%.

Coupling of ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester on Separon H 1000. (a) Tripeptide hydrochloride (3.75 g) was dissolved in dimethylformamide (300 ml) and triethylamine (1.14 ml) and Separon H 1000 E_{med} (60 g) were added. The mixture was shaken for 48 h (with breaks at night), filtered and the polymer was washed with dimethylformamide, water, ethanol and diethyl ether and dried. Amino acid analysis (phenylalanine determined) revealed the presence of 0.85

μmol of tripeptide per gram of gel. In similar experiments with a triple concentration of tripeptide in dimethylformamide and a corresponding amount of triethylamine (in some parallel experiments, the equivalent of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was also added), sorbents containing 3.0–4.5 μmol of tripeptide per gram of gel were prepared. Unreacted epoxide groups were removed either by coupling them with a 1 *M* solution of 2-aminoethanol (room temperature, 24 h), or by treatment with 0.1 *M* perchloric acid (room temperature, 12 h).

(b) Tripeptide hydrochloride (125 mg) and triethylamine (50 μl) in dimethylformamide (10 ml) were shaken with 2 g of Separon H 1000 E_{max} at room temperature

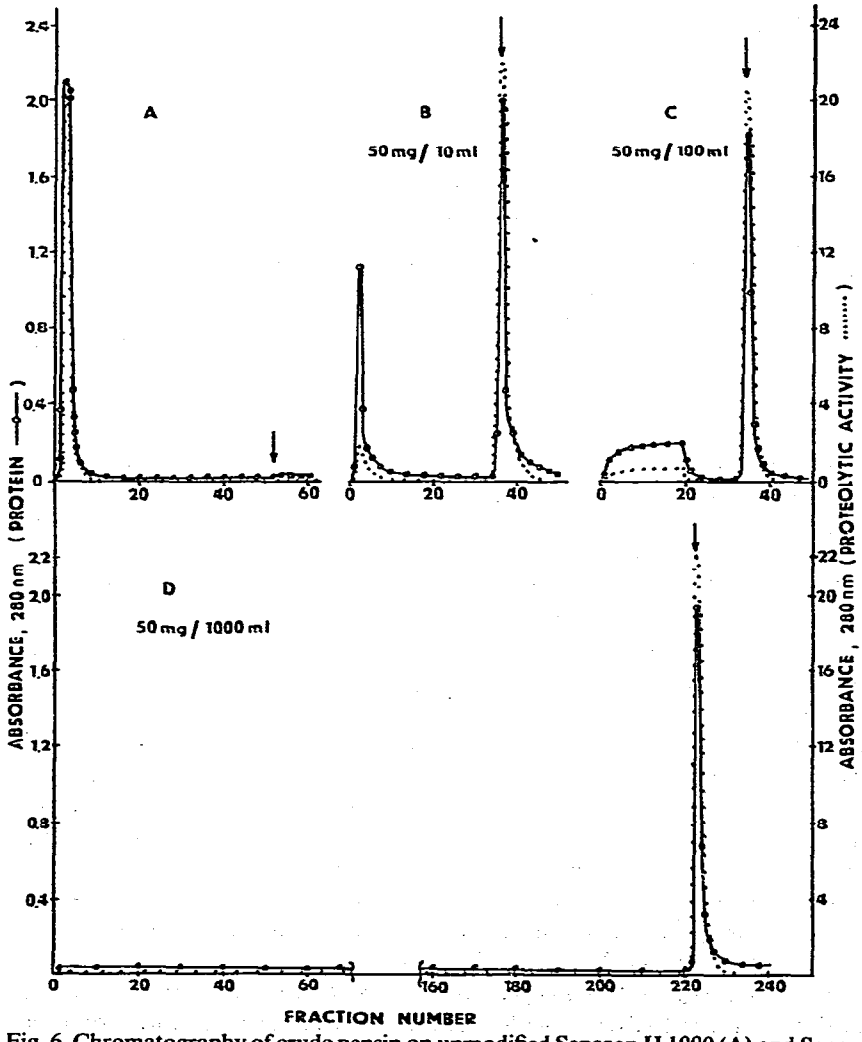


Fig. 6. Chromatography of crude pepsin on unmodified Separon H 1000 (A) and Separon H 1000 E_{med} with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (B, C, D). Sample, 50 mg of crude pepsin (A, B in 10 ml, C in 100 ml, D in 1000 ml of 0.1 *M* sodium acetate buffer, pH 4.5) washed first with the same buffer, starting from the spot denoted with an arrow washed with the same buffer containing 1 *M* sodium chloride. Fractions, 5 ml in 4-min intervals; room temperature.

for 48 h. The product was treated as in (a). The resulting sorbent contained 2.0 μmol of tripeptide per gram. Separon H 1000 (particle size 10 μm) with coupled ε-aminocaproyl-L-Phe-D-Phe-OCH_3 prepared in a similar way contained 0.5 μmol of tripeptide per gram.

(c) Tripeptide hydrochloride (125 g) was dissolved in a mixture of 5 ml of buffer (pH 10.5, pH adjusted with 0.1 *M* sodium hydroxide solution) and 5 ml of dimethylformamide. Separon H 1000 E_{max} was added to the solution and the suspension was shaken for 24 h. After treatment as in (a), a sorbent with 72 μmol of tripeptide per gram was obtained.

Chromatography of proteinases on Separon H 1000 with bound ε-aminocaproyl-L-Phe-D-Phe-OCH_3. (a) Dry porcine pepsin was dissolved in 10 ml (Fig. 6A and B), or 100 ml (Fig. 6C) or 1000 ml (Fig. 6D) of 0.1 *M* sodium acetate buffer (pH 4.5) and introduced into a column (9 × 0.9 cm I.D.) of Separon H 1000 E_{med} with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (1.5 g dry weight of 4.5 μmol of tripeptide per gram), which was equilibrated with 0.1 *M* sodium acetate (pH 4.5). The column was first washed with the equilibrating buffer and then with the same buffer containing 1 *M* sodium chloride. The chromatographic run is shown in Fig. 6B–D. A similar experiment was also carried out on a column with unmodified Separon H 1000 (Fig. 6A).

(b) Crude enzyme preparation from *Aspergillus oryzae* (100 mg) was dissolved in 10 ml of 0.1 *M* sodium acetate buffer (pH 4.5) and applied to a column with the same sorbent as in (a). The chromatogram is shown in Fig. 7.

High-performance (liquid) affinity chromatography. A stainless-steel column (100 × 4 mm I.D.) packed with Separon H 1000 E_{max} with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (0.5 μmol/g, particle size 10 μm) was used.

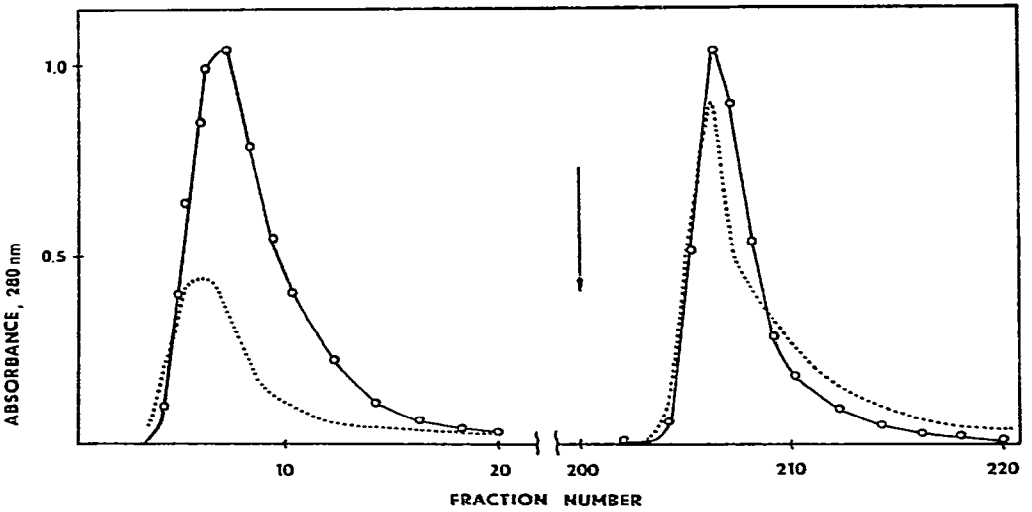
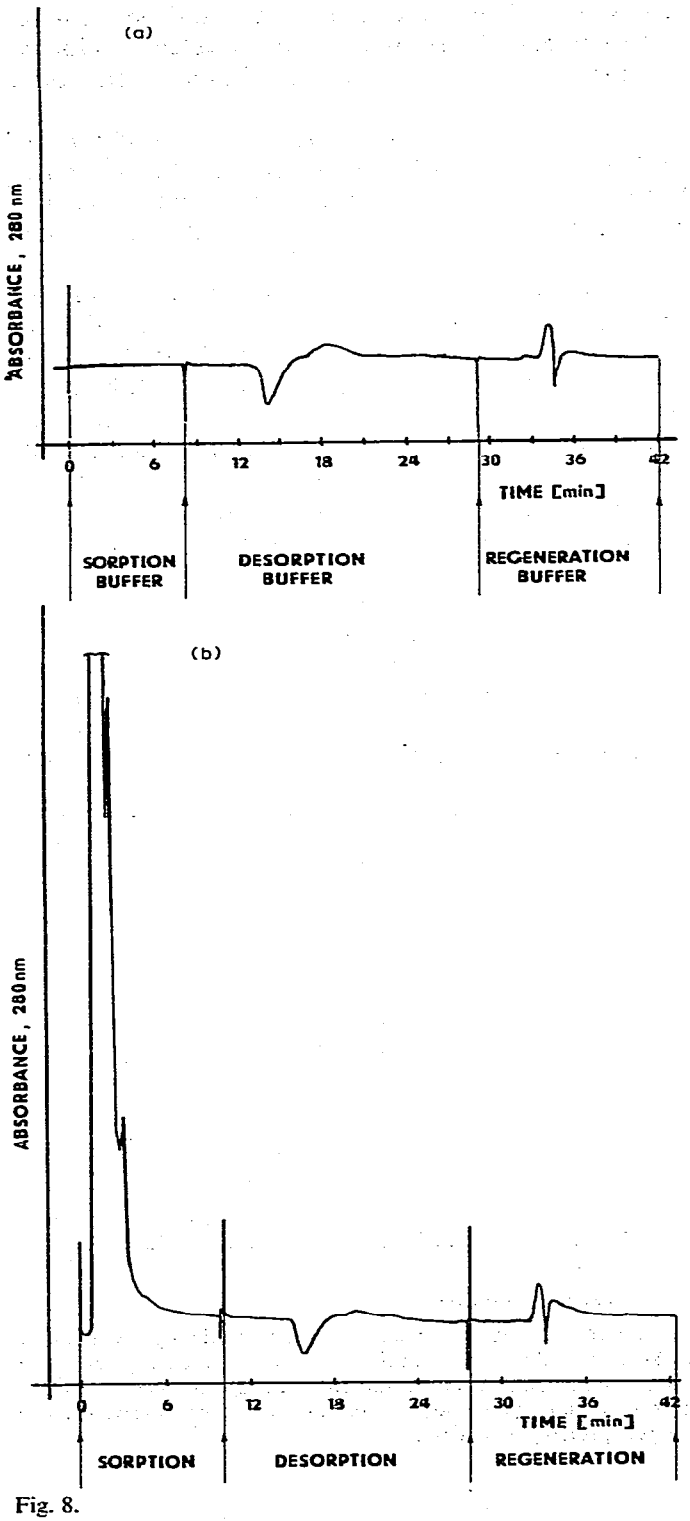


Fig. 7. Affinity chromatography of proteinase from *Aspergillus oryzae* on Separon H 1000 E_{med} with bound ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester. Sample of proteinase (100 mg) in 0.1 *M* sodium acetate buffer (pH 4.5) washed first with the same buffer, starting from the spot denoted with arrow washed with the same buffer containing 1 *M* sodium hydroxide. Fractions, 4 ml in 2-min intervals; room temperature. Solid line, protein; broken line, proteolytic activity.


(a)
ABSORBANCE, 280 nm
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(b)
ABSORBANCE, 280nm
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30
36
42
TIME [min]
SORPTION
DESORPTION
REGENERATION

Fig. 8.

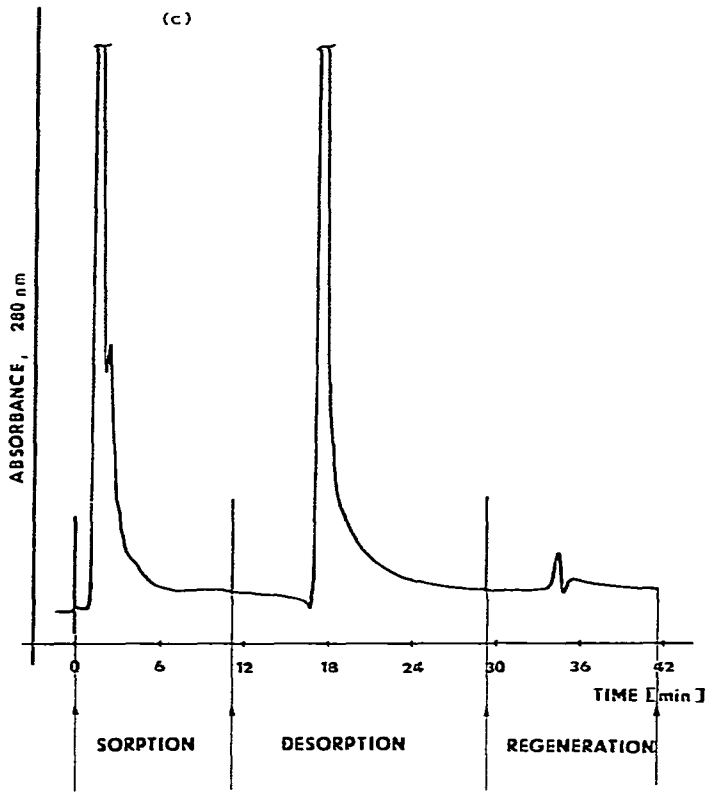


Fig. 8. High-performance affinity chromatography of pepsin. (a) Baseline characteristics of the high-performance affinity chromatographic system. Column: 100 × 4 mm I.D. Sorbent: Separon H 1000 modified (c), mean particle diameter 10 μm. Detector: UV (280 nm). Mobile phase: sorption buffer, 0.1 *M* sodium acetate (pH 4.5); desorption buffer, 0.1 *M* sodium acetate (pH 4.5) containing 1 *M* sodium chloride; regeneration buffer, 0.1 *M* sodium acetate (pH 4.5) containing 10% 2-propanol. Injection: 10 μl of sorption buffer without protein. (b) Chromatogram of pepsin sample on non-modified carrier. Column: 100 × 4 mm I.D. Sorbent: Separon H 1000, mean particle diameter 10 μm. Detector: UV (280 nm). Mobile phase: see (a). Injection: 10 μl of pepsin solution in sorption buffer (0.5 mg/μl). Desorption and regeneration characteristics as in (a). (c) Chromatogram of pepsin sample on specific sorbent. Column: 100 × 4 mm I.D. Sorbent: Separon H 1000 modified with 0.5 μmol of ε-aminocaproyl-L-Phe-D-Phe-OCH_3 per gram of carrier. Detector: UV (280 nm). Mobile phase: see (a). Injection: 10 μl of pepsin solution in sorption buffer (0.5 mg/μl).

Comparative measurements were performed on the same column packed with unmodified sorbent. The apparatus consisted of an MP 2501 membrane pump (Laboratory Instruments Works, Prague, Czechoslovakia), UV LC 3 variable wavelength detector (Pye Unicam, Cambridge, Great Britain) and a TZ 4221 recorder (Laboratory Instruments Works).

The columns were packed by the following procedure. A suspension prepared from 30 ml of dry sorbent in 250 ml of water was stirred ultrasonically and decanted several times until it sedimented with a sharp phase boundary. Packing was effected with a pressure packing apparatus (maximum pressure 5 MPa) at a constant flow-rate of water (1 ml/min) for 2 h. The efficiency of both 10-cm long columns reached about

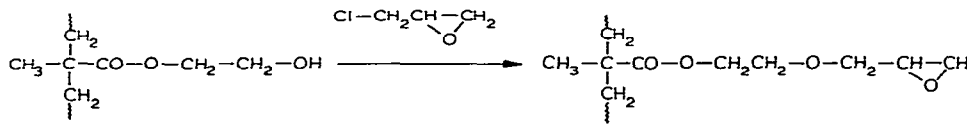
1000 theoretical plates at a flow-rate of 0.5 ml/min (tested with a 5% glucose solution in water; injection 10–25 μl).

The following mobile phases were used in the chromatographic runs: (1) sorption —0.1 *M* sodium acetate (pH adjusted to 4.5 by adding acetic acid); (2) desorption —the same buffer, containing 1 *M* sodium chloride; (3) regeneration (washing) —the same buffer, containing 10% 2-propanol. The flow-rate of the mobile phase was 0.5 ml/min, and detection was effected at 280 nm. After the baseline had been established, the mobile phases were gradually changed in the order 1, 2, 3. The changes in the baseline thus produced are shown in Fig. 8A.

Under the same conditions, 10 μl of pepsin solution (500 mg in 1 ml of 0.1 *M* sodium acetate buffer, pH 4.5) were injected into both columns in mobile phase 1. Mobile phase 2 was used in the desorption and mobile phase 3 was used in the final washing. In both the sorption and desorption processes samples for the enzyme activity determination were taken at the maxima of the peaks. The proteolytic activities of fractions in Fig. 8c were 0.02, 0.07 (proteinase of a different type) and 9.25 units/ml (pepsin). The results are summarized in Fig. 8A–C.

RESULTS AND DISCUSSION

Copolymerization of hydroxyalkyl methacrylate with alkylene dimethacrylate yields heavily cross-linked microparticles which by aggregation give rise to macroporous spheroids. The hydroxy groups of the copolymer react in an alkaline medium with epichlorohydrin according to the equation



The amount of epoxide groups may be controlled to a great extent by the reaction conditions during the modification. The decisive role seems to be played by the amount of epichlorohydrin in the reaction mixture (which may be diluted with a non-polar solvent) and by the reaction temperature. The preparation procedures described under Experimental concerning the maximum, medium and minimum substituted carrier should meet the requirements of various other applications.

The epoxide groups of the derivatized support may react with the amino, carboxy, hydroxy and sulphydryl groups and with some aromatic nuclei, such as indole and imidazole. In the coupling of peptides or proteins the most important bond seems to be that obtained by means of amino groups, both with respect to their frequent presence in the ligands and also, more important, owing to the extraordinary stability of the links thus formed. This is why this coupling was studied with special attention. No pH extreme was observed in the coupling of glycyl-D-phenylalanine on the maximally and minimally derivatized gel (Fig. 5). The same behaviour occurs with glycyl-D-leucine, unlike the coupling of the same dipeptide on the glycidyl methacrylate support, obtained by the direct copolymerization of the component containing the epoxide group[17], where maximum coupling was observed at pH 9.7. An increase in pH leads to an increase in the amount of the coupled amino component of

various structural types (*cf.*, Fig. 3). The same character of the curves was also found when studying the coupling of a model dipeptide Ac-L-Lys-Gly-OCH_3 on the glycidyl methacrylate support obtained by direct copolymerization[18]. As demonstrated with ethylamine, an increase in pH leads to an increase in the coupling rate. It is also affected by the structure of the bound amino component. Fig. 1 shows the amounts of ammonia, 1,2-diaminoethane, 1,4-diaminobutane and 1,6-diaminohexane which were bound on derivatized Separon during 6 and 40 h. The coupling reaction is very fast with 1,6-diaminohexane, being completed after 6 h. Subsequent saturation with 2-aminoethanol did not take place (unlike the coupling reactions of other compounds shown in Fig. 1). The cause might be a local increase in the concentration of 1,6-diaminohexane on the surface of the support due to hydrophobic interactions, which mainly with the hexamethylene chain must play the most important role. The dependence of coupling on the character of the component bound and on the character of the support has been observed earlier in the process of coupling of some proteins on various epoxide-containing supports[19].

The amount of the component bound may be affected to a certain extent by an increased concentration of the component in the reaction mixture. Fig. 4 shows this finding, using the coupling of ε-aminocaproic acid in aqueous buffers as an example. In contrast, in non-aqueous media an increase in the concentration was not reflected in an increase in the coupling. It seems that if high yields of coupled amino components are to be achieved, it is more important to work at higher pH (10.5 and above), and preferably in dimethylformamide–aqueous buffer mixtures. Under such conditions, the reaction with the amino group is sufficiently fast to compete with the parallel hydrolytic reaction. In purely non-aqueous media the pH that can be attained by means of tertiary organic bases is obviously not sufficiently high.

Of course, in all instances a great difference was observed between the content of epoxide groups in the initial derivatized support and the final yield of the bonded amino component. Analyses of unreacted epoxide groups in sorbents with completed coupling show that part of the epoxide rings are split hydrolytically during coupling if this is carried out in the presence of water, and/or during the subsequent washing of the gel. Let us give as an example the coupling of ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester on Separon H 300 E_{med} in the mixture dimethylformamide–water (*cf.*, Experimental). The product obtained contained 72 μmol/g of bound tripeptide and the remaining 366 μmol/g of epoxide groups. Thus, about 25% of the epoxide groups were destroyed during the coupling and subsequent treatment. In the incubation in a buffer of pH 10, the derivatized Separon H 300 E_{max} exhibited a decrease of *ca.* 12% of the original content of the epoxide groups after the first 2 h. This decrease did not change further in the period from 2 to 24 h. Obviously, further treatment of the sorbent, especially washing with a dilute solution of a mineral acid, leads to a further decrease in the content of the epoxide functional groups of the carrier.

For complete removal of the epoxide groups, it is widely recommended[2] that additional coupling of 2-aminoethanol be used. We employed this procedure in order to check the completeness of coupling in addition to the determination of the epoxide groups. Our results indicate, however, that such a procedure is not satisfactory: the reaction also proceeds with the intact derivatized Separon incompletely and slowly, and moreover requires a high concentration of 2-aminoethanol and a high pH (*cf.*,

Fig. 2). We therefore concentrated our attention on complete removal of the epoxide groups in the acidic medium, which ought to proceed more quickly and more completely[20] and be useful at least when the bound component does not contain functional groups that readily undergo acid splitting. On standing with 0.1 *M* perchloric acid (aqueous medium, room temperature, 15 h), Separon H 300 E_{med} yielded a material containing only 10 μmol of epoxide groups. The hydrolysis of Separon H 1000 E_{max} with a high content of epoxide groups (residual content 16 μmol/g) proceeded similarly. This material was used in the coupling of tripeptide ester under the conditions described above. The amino acid analysis revealed only 0.16 μmol/g of the peptide component. Standing with 0.1 *M* perchloric acid destroyed the remaining epoxide rings via the hydrolytic mechanism also in the Separon H 300 E_{med} sample mentioned above with the bound tripeptide ester; the value of 366 μmol/g epoxide groups decreased to 10 μmol/g. The residual content of the epoxide groups found in the sorbents after treatment with 0.1 *M* perchloric acid lies at the boundary of determination by employing the analytical method described. It does not seem likely, however, that such a content of non-reacted epoxide groups could have any negative effect on the use of sorbents in their application in affinity chromatography.

In an attempt to demonstrate the practical applicability of the technique, we prepared a sorbent specific for the interaction with carboxylic proteinases[21,22]. However, as the non-derivatized Separon appeared to be suitable for the hydrophobic chromatography of some proteins[23], we first examined the behaviour of crude porcine pepsin on a column of unmodified Separon H 1000 under the conditions of affinity chromatography (Fig. 6A). In this instance the whole proteolytic activity was washed in the front of the fraction obtained by elution with the equilibration buffer. The following fraction with a high ionic strength (1 *M* sodium chloride) no longer contained a protein material. On the contrary, desorption at a high ionic strength carried out on a column of Separon H 1000 modified with ε-aminocaproyl-L-phenylalanyl-D-alanine methyl ester yielded a sharp peak with a high activity of pepsin (Fig. 6B, C and D). From the practical point of view, it seems important that pepsin could be obtained with the same efficiency from solutions having an enzyme concentration that differed by up to two orders of magnitude, and thus with very different volumes of the sample taken for the treatment. With increasing dilution of the solution of enzyme the peak of the first fraction undergoes spreading (and completely disappears at the lowest concentration). In contrast, the peak of the pepsin fraction maintains its sharpness in all three instances. The suitability of the suggested affinity chromatographic procedure for the isolation of microbial proteinases has been demonstrated using the isolation of proteinase of the carboxylic type from *Aspergillus oryzae* (*cf.*, Fig. 7).

Affinity chromatography was also employed analytically under conditions of high-performance liquid chromatography (Fig. 8). The specific sorbent prepared for this purpose (Separon H 1000, particle size 10 μm, and with 0.5 μmol/g of ε-aminocaproyl-L-Phe-D-Phe-OCH_3) appears to be very suitable for fast and very efficient separations. The results allow us to envisage applications of high-performance affinity chromatographic techniques in the clinical analysis of enzymes.

To summarize, the hydroxyalkyl methacrylate supports, medium-derivatized with epichlorohydrin, are suitable for the preparation of sorbents with bound low-molecular-weight amino components, in particular peptide inhibitors, intended for

the efficient affinity chromatography of proteolytic enzymes, in both the classical and the high performance arrangement. The advantage of such sorbents is their chemical and mechanical stabilities, small extent of non-specific interactions (perhaps as a consequence of the large number of surface hydroxyl groups due to the hydrolytic opening of unreacted epoxide rings), their ability to undergo almost complete regeneration[24] and the possibility of using high pressures in column or microcolumn techniques.

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REFERENCES

1 J. Čoupek, M. Křiváková and S. Pokorný, *J. Polym. Sci., Polym. Symp.*, 42 (1973) 182.
2 J. Turková, *Affinity Chromatography*, Elsevier, Amsterdam, 1978, pp. 246–318.
3 J. Porath, R. Axén and S. Ernback, *Nature (London)*, 215 (1967) 1491.
4 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
5 J. Turková, O. Hubálková, M. Křiváková and J. Čoupek, *Biochim. Biophys. Acta*, 322 (1973) 1.
6 J. Turková, *Methods Enzymol.*, 44 (1976) 66.
7 J. Brandt, L. O. Anderson and J. Porath, *Biochim. Biophys. Acta*, 386 (1975) 196.
8 N. Stambolieva and J. Turková, *Collect. Czech. Chem. Commun.*, 45 (1980) 1137.
9 J. Porath, J.-C. Janson and T. Låås, *J. Chromatogr.*, 60 (1971) 167.
10 J. Porath and L. Sundberg, *Nature (New Biol.)*, 238 (1972) 261.
11 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87.
12 J. Carlsson, R. Axén and T. Unge, *Eur. J. Biochem.*, 59 (1975) 567.
13 I. Matsumoto, Y. Mizuno and N. Seno, *J. Biochem.*, 85 (1979) 1091.
14 W. Bathe, J. Janecke and H. Meerwein, in E. Müller (Editor), *Methoden der organischen Chemie (Houben-Weyl)*, Vol. II, Thieme-Verlag, Stuttgart, 4th ed., 1973, p. 430.
15 M. L. Anson, *J. Gen. Physiol.*, 22 (1938) 79.
16 J. Turková, O. Mikeš, K. Gančev and M. Boublik, *Biochim. Biophys. Acta*, 178 (1969) 100.
17 J. Turková, K. Bláha, M. Malaníková, D. Vančurová, F. Švec and J. Kálal, *Biochim. Biophys. Acta*, 524 (1978) 162.
18 J. Turková, in L. Vitale and V. Simeon (Editors), *Industrial and Clinical Enzymology*, Pergamon Press, Oxford, 1980, p. 65.
19 I. Zemanová, J. Turková, M. Čapka, L. A. Nakhapetyan, F. Švec and J. Kálal, *Enzyme Microb. Technol.*, 3 (1981) 229.
20 R. E. Parker and N. S. Isaacs, *Chem. Rev.*, 59 (1959) 737.
21 V. M. Stepanov, G. I. Lavrenova and M. M. Slavinskaya, *Biokhimiya*, 39 (1974) 384.
22 V. M. Stepanov, G. I. Lavrenova, K. Adly, M. V. Gonchar, G. N. Baladinova, M. M. Slavinskaya and A. Ya. Strongin, *Biokhimiya*, 41 (1976) 294.
23 P. Štrop and D. Čechová, *J. Chromatogr.*, 207 (1981) 55.
24 J. Turková and A. Seifertová, *J. Chromatogr.*, 148 (1978) 293.