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CROWN ETHERS AS LIGANDS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS AND NUCLEIC ACIDS

DJURO JOSIĆ* and WERNER REUTTER
Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33 (Dahlem) (F.R.G.)
and
JOACHIM REUSCH
Säulentechnik Dr. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37 (F.R.G.)

SUMMARY

By immobilization of the crown ether 1,10-diaza-18-crown-6 to different porous and non-porous, epoxy-activated supports, a chromatographic sorbent was prepared, which, mediated by potassium ions, can be used for the separation of both nucleic acids and proteins. Model experiments have been carried out with ribonucleic and deoxyribonucleic acids. In experiments with standard proteins the influence of pH and the role of loading of the column with potassium ions were demonstrated. The column was used for separating complex protein mixtures, such as serum and plasma membrane extracts, in the presence of detergents.

INTRODUCTION

Crown ethers are neutral, macrocyclic polymers. In 1967 Pedersen[1] demonstrated for the first time that the cyclically arranged ethylene glycol units can form stable and stoichiometrically defined complexes with alkali-metal cations. In the presence of such cations, all C–O–C structure elements point inwards, thus coordinating them by means of ion–dipole interaction. As a consequence of this orientation, all CH_2 groups point outwards and, in a way, the cation is coated by a lipophilic environment. Therefore, alkali-metal salts can be dissolved in lipophilic media[2] or channeled in complex form through lipophilic membranes, *e.g.*, into a cell[3]. The formation of complexes takes place in an highly selective manner. A potassium ion with an ionic radius of 2.66 Å, for example, will fit well into the inner cavity of an 18-crown-6 molecule, which has an diameter of 2.70 Å. However, a sodium ion, with its smaller diameter, will not take up all the space, and consequently will form less stable complexes.

As crown ethers not only coordinate metal ions, but also ammonium, diazonium and guanidinium ions, corresponding asymmetric ligands should allow chiral separations as well. A kind of made-to-measure cavity architecture provides a close adaption to the structural characteristics of the target molecule[4,5]. Specific interac-

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tions of crown ethers and nucleic acids was first observed by Pitka and Smid[6]. They found that, in the presence of potassium ions, polyadenylate formed a water-insoluble complex with the water-soluble poly-vinylbenzo-18-crown-6). In turn, polyadenylate immobilized on Sepharose can bind poly(vinylbenzo-18-crown-6). The highest concentration of two crown ether molecules per nucleotide is reached at a concentration of 0.1 *M* potassium ions.

The crown ether 1,10-diaza-18-crown-6 contains two secondary amino groups in its molecule and can therefore be immobilized on activated rigid supports, such as silica gel or organic polymers. In order to provide better interaction between the immobilized crown ether and the ligate, sufficient space between the ligand and matrix is necessary. This is achieved through a spacer[7,8]. Fig. 1 shows the course of such reaction.

In this paper, the synthesis of chromatographic sorbents with immobilized crown ethers is described, together with their use in high-performance liquid chromatography (HPLC) of nucleic acids and proteins.

EXPERIMENTAL

Chemicals

All chemicals were of analytical reagent grade from Merck (Darmstadt, F.R.G.) or Sigma (München, F.R.G.). Bacterial RNA and DNA samples were a gift from Dr. B. Wittig (Institut für Molekularbiologie und Biochemie, Freie Universität, Berlin, F.R.G.). Liver plasma membranes were isolated and fractionated by stepwise extraction, as described elsewhere[9].

Immobilization of ligands

The 1,10-diaza-18-crown-6 was immobilized on epoxy-activated silica gel (Eu-

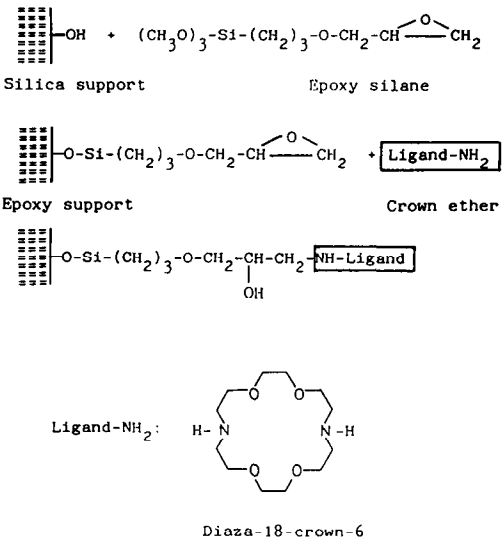

Fig. 1. Route for the preparation of ligand-substituted silica or polymer support.

rochrom; Säulentechnick Knauer, Berlin, F.R.G.) or polymethacrylamide (Eupergit, Röhm Pharma, Weiterstadt, F.R.G.), as described elsewhere[7,8]. The characteristics of the silica gel support were: particle size 7 μm and pore size 300 Å. The synthetic support, Eupergit C 30N had 30-μm particles and 450-Å pores, the non-porous Eupergit C 1Z had 1-μm particles.

HPLC

The HPLC system consisted of two pumps, a programmer, a spectrophotometer with a deuterium lamp, a loop injection valve (all from Knauer, Berlin, F.R.G.) and a Frac-100 fraction collector (Pharmacia-LKB, Freiburg, F.R.G.). The chromatographic conditions are given in the Figure legends.

Protein recovery was determined by measuring the protein concentration before and after separation according to the procedure of Lowry *et al.*[10]. The recovery of dipeptidylpeptidase IV (DPP IV) was determined by measuring its enzymatic activity[11].

Columns

All analytical columns, with dimensions of 60 mm × 4.0 mm, and all semipreparative columns, with dimensions of 120 mm × 8.0 mm, were produced and packed by Säulentechnik Knauer.

Buffers

The following buffers were used: eluent A was 5 m*M* Tris–HCl (pH 6.8), containing 20 or 50 m*M* KCl. The elution buffer (eluent B) was eluent A with 1 *M* NaCl added. In some experiments 0.1% (w/v) detergent was added to both buffers.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Dialysed and freeze-dried samples were dissolved in 62.5 m*M* Tris–HCl buffer (pH 6.8), containing 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue and boiled for 3 min. SDS-PAGE was performed by the Laemmli method[12]. An amount of 100–150 μg of protein was applied to each track when standard equipment was used (Pharmacia-LKB). Between 5 and 15 μg of protein were applied to each track when working with a mini-system (Bio-Rad, München, F.R.G.).

RESULTS AND DISCUSSION

Production of chromatographic supports with immobilized crown ethers

To the epoxy-activated silica gel or the synthetic support, Eupergit, 1,10-diaza-18-crown-6 is added to excess. It is assumed that one ligand has bound to each epoxy group (see Fig. 1). The activated silica gel contains between 250 and 300 μmol epoxy groups per 1 g of dry gel while Eupergit C 30N contains about twice as much. Non-reacted epoxy groups are blocked with mercaptoethanol by shaking for 2 h, a procedure which is routinely used for the binding of ligands. The capacity of the silica-based support with crown ether ligands is about 25 mg of 5S RNA per g (ref. 1), and about 40 mg of 5S RNA per 1 g of Eupergit C 30N support. The capacity of non-porous Eupergit C1Z crown ether support is 5–10% of the capacity of Eupergit

C 30N crown ether. This is explained by the fact that the specific surface area of this support is smaller, and the amount of available ligand therefore lower.

Separation of nucleic acids

The interaction between nucleic acids and crown ethers, as observed by Pitka and Smid[6], is the basis of their chromatographic separation. The separation is reproducible, the retention time depending on the size of the nucleic acid. As is seen in Fig. 2, the smaller 5S rRNA is eluted before the larger 23S rRNA. Fig. 3 shows the separation of denatured and coiled DNA from a plasmid of *Escherichia coli*. The only difference between the two components is that the denaturated DNA is unfolded; it is exposing more phosphate groups for interaction with the potassium ions, immobilized in crown ether, than the non-denaturated coiled DNA. Consequently, the retention time for denaturated DNA is longer. The smaller by-products, which have resulted from cleavage of the DNA during the isolation process, have a shorter retention time, or are even excluded in the dead-volume (see smaller peaks in the front in Fig. 3). Nucleotides are not bound, which suggests that several free phosphate groups are necessary for strong binding to the support (Fig. 4). This is confirmed by the experiment shown in Fig. 5. A thymus DNA sample was separated before and after digestion with DNAse on an Eupergit C1Z crown ether column with 1-μm non-porous particles. In this way, the progress of digestion can easily be controlled since the chromatographic analysis takes only 10 min.

Fig. 4 shows a model representing the interaction between the nucleic acids and the potassium ions, immobilized on the crown ethers. It is shown that the interaction

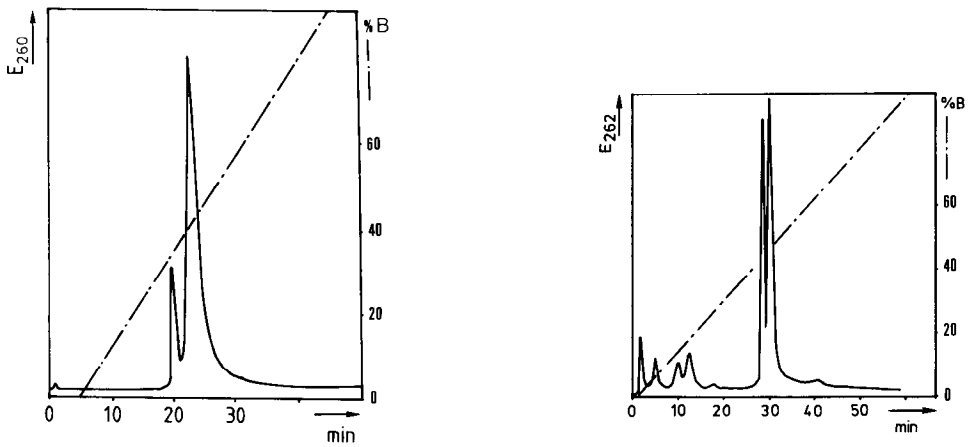

Fig. 2. Separation of 5S and 23S rRNA from *Bacillus stearothermophilus*. An 0.5-mg sample was applied to a 60 mm × 4.0 mm silica-based crown ether column. The content of KCl in the loading buffer (A) was 50 m*M*; the pH of both buffers was 7.6. Other chromatographic conditions: flow-rate, 1 ml/min; pressure, 6.0 MPa; room temperature. The gradient is shown in the chromatogram. For other conditions see Experimental.

Fig. 3. Separation of denaturated and coiled DNA of a plasmid from *Escherichia coli*. A 25-μg sample in 20 μl of eluent A was loaded on the column. The first peak is denatured DNA, the second is non-denatured, coiled DNA. Products resulting from the cleavage are either excluded or appear at the beginning of the gradient. Chromatographic conditions as in Fig. 2.

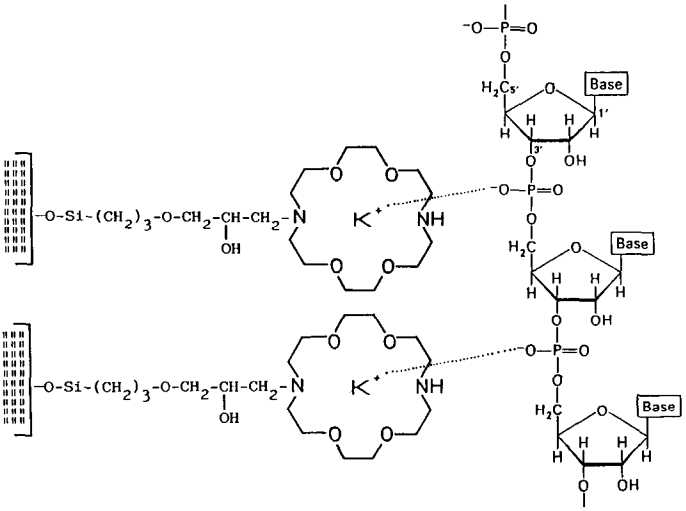

Fig. 4. Interaction between the nucleic acids and the potassium ions immobilized on crown ethers.

with the ligate and, consequently, its retention time increases with the number of free phosphate groups available on the molecule surface.

Separation of proteins

Some proteins also interact with immobilized ethers. This interaction is mediated by potassium ions and is pH-dependent (see Fig. 6 a–c for the pH dependency of such separations). Of the standard proteins, transferrin and ovalbumin are hardly retarded at all at pH 4.2. If, however, the column is previously saturated with potassium ions by injecting 1 ml of 0.5 *M* KCl, a fundamental change is observed. The retention time of all three standard proteins, transferrin, ovalbumin and ferritin, is extended (*cf.*, the dashed line in Fig. 6a). A similar, though less distinct effect is seen

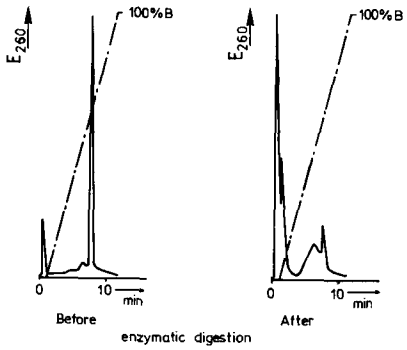

Fig. 5. Separation of a thymus DNA sample before and after DNAse digestion. For each experiment, 25 μg of sample were applied to the column. DNA is retarded on the column; the short-chain products after digestion are excluded. Chromatographic conditions: column, 60 mm × 4.0 mm; packed with the non-porous support Eupergit C 1Z crown ether (particle size 1 μm); flow-rate, 0.5 ml/min; pressure, 8.0–12.0 MPa; room temperature. The gradient is shown in the chromatogram. For other conditions see Fig. 2.

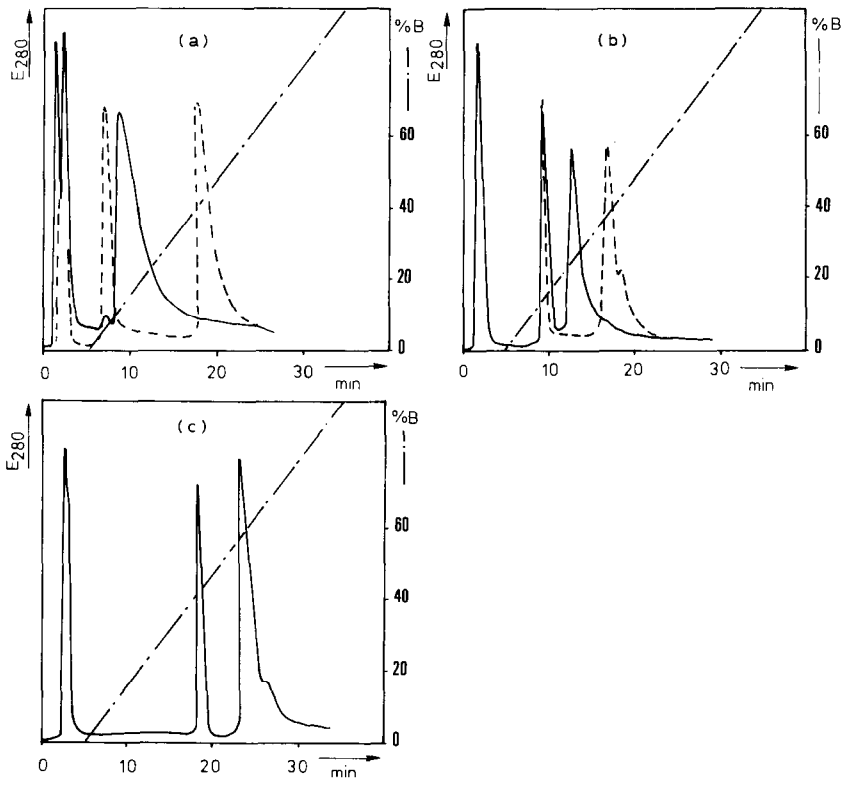

Fig. 6. Influence of pH on the separation of the standard proteins transferrin, ovalbumin and ferritin: pH 4.2 (a); 5.8 (b) and 6.8 (c). (———) Without loading of the column with 1 ml of 0.5 *M* KCl; (– – –) after loading the column with 1 ml of 0.5 *M* KCl. For chromatographic conditions see Fig. 2.

during the separation at pH 5.8 (see Fig. 6b). Here, the retention of ferritin is prolonged considerably. If the separation is carried out at pH 6.8, the previous saturation with potassium ions has no effect at all on the retention time of the standard proteins used (see Fig. 6c). This suggests that the pH dependency affects the loading of the crown ethers with potassium ions rather then the interaction between the ligates and the already immobilized potassium ions. At pH 6.8 a concentration of 50 m*M* potassium in the equilibration buffer (eluent A) is sufficient to saturate the immobilized crown ethers. Such a saturation is not provided by 50 m*M* potassium at lower pH.

The columns with synthetic supports (Eupergit C 30N and C 1Z) show a slightly different behaviour in this respect. Retention times are similarly pH dependent; the column loading with potassium ions has to be carried out in all cases, regardless of the pH investigated. This is shown in Fig. 7 in the case of the Eupergit C 1Z column. If the column is not saturated with KCl, all three standard proteins are excluded, even at pH 6.8 (*cf.*, the chromatogram on the left in Fig. 7). Retention of standard proteins and their separation is achieved only after saturation of the column with 1 ml of 0.5 *M* KCl (chromatogram on the right of Fig. 7).

The model for the interaction between nucleic acids and potassium ions immobilized on crown ether, as shown in Fig. 4, cannot be applied to proteins without some

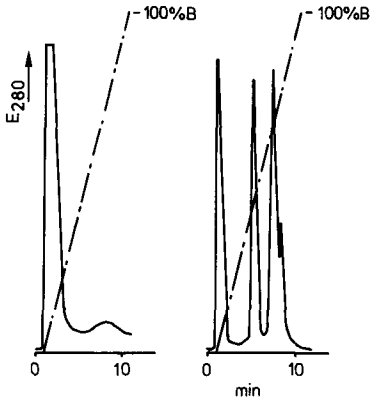

Fig. 7. Separation of the standard proteins transferrin, ovalbumin and ferritin on the Eupergit C 1Z crown ether column with 1-μm non-porous particles. Buffer pH, 6.8. On the left, no loading of the column with an injection of 0.5 *M* KCl; on the right, the column was loaded with 200 μl 0.5 *M* KCl. Other conditions as in Fig. 5.

modification. Here, the non-phosphorylated proteins, such as serum albumin, are also retarded (not shown). The basic proteins such as trypsin inhibitor, lysozyme and cytochrome *c* do not bind to the crown ether column, and neither does immunoglobulin G (IgG). A comparison between crown ether sorbents and anion exchangers shows that the crown ether columns behave completely different in protein separation. Both trypsin inhibitor and IgG are bound by most anion exchangers at low salt concentrations[13], but not by crown ether sorbents under identical conditions. For the interaction of crown ethers with nucleic acids as well as with proteins, the immobilized potassium ion has an important function. Without loading with KCl, none of the listed substances can be bound (*cf.*, Fig. 7).

Further investigations were carried out with complex protein mixtures, such as serum samples and plasma membrane extracts. The object of these experiments was to find out whether the columns can also be used under complex conditions, *e.g.*, with the application of detergents and with samples that contain less water-soluble components.

Fig. 8 shows the separation of a sample containing rat serum on a crown ether column with silica gel as the support. Under the conditions used for the separation of standard proteins (see above, Fig. 6c), most sample components do not bind to the column. Even serum albumin is excluded, although it was retarded in other experiments, when a salt gradient had to be used for elution (see Fig. 8b, left). However, when 0.1% (w/v) detergent is added, *e.g.*, octyl glucoside or decanol-N-methylglucamide (MEGA-10), the separation is markedly improved (Fig. 8b, centre and right). In the presence of 0.1% MEGA-10, transferrin (peak 1), serum albumin (2) and a protein with an apparent molecular weight in SDS-PAGE of 200 000 daltons (probably fibronectin, 3) were isolated.

As serum albumin tends to aggregate with itself and other proteins, the improved separation in the presence of a detergent can be explained by the prevention of this kind of interaction. The non-ionic and zwitterionic detergents, *e.g.*, CHAPS, do not adversely affect column life and column performance.

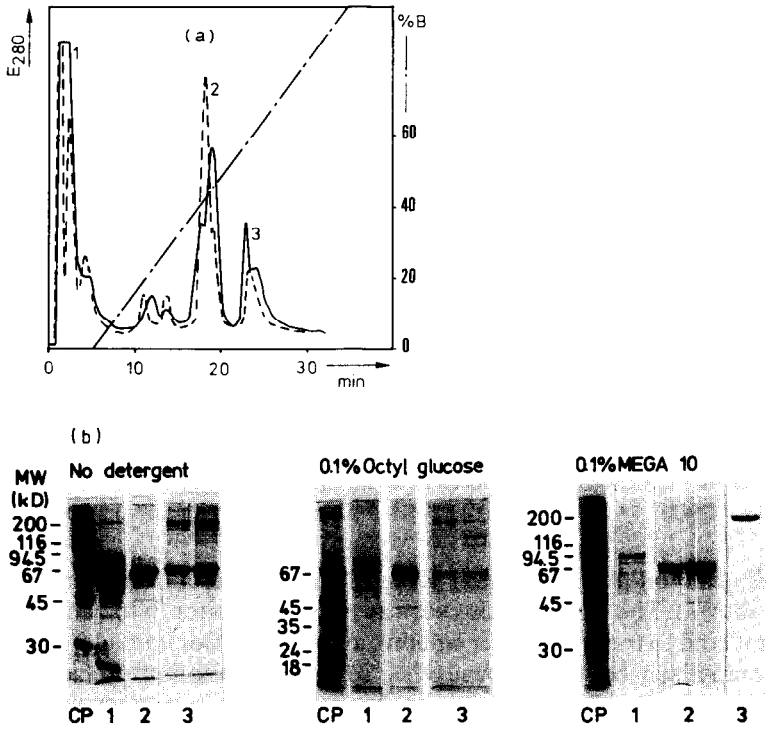

Fig. 8. Influence of detergents on the separation of serum proteins on a silica-based crown ether column. (a) The chromatogram, conditions as in Fig. 6c; (———) without detergent; (– – –) with 0.1% MEGA-10 in both buffers. (b) SDS-PAGE, showing the peaks in (a), with and without two different detergents. CP = Complex protein mixture.

In Fig. 9 another experiment is shown, involving the separation of a complex protein mixture. From a liver plasma membrane extract, 60 mg of protein were applied to an 120 mm × 8.0 mm crown ether column with silica gel as the support, leading to an enrichment of the enzyme dipeptidylpeptidase IV. The separation was carried out in the presence of 0.1% Triton X-100 (reduced). The recovery of the enzymatic activity was 80–95%; the enrichment in one step was 20- to 30-fold. During separation with a gradient of 0–1 *M* NaCl in the presence of Triton X-100, 80% of the proteins were recovered, and another 18% were obtained by elution with a 1% aqueous Triton X-100 solution (arrow in Fig. 9).

The following results, which are shown in this report, suggest that interactions other than ion-exchange mechanisms are also involved:

Mono- and short oligonucleotides are not bound by the crown ether sorbent, see Fig. 5. Apparently several phosphate groups are required for retention, see the model in Fig. 4

Without potassium ion loading, none of the investigated substances was bound to the column

Comparisons carried out between crown ether sorbents and anion exchangers have shown a clearly different behaviour in the separation of standard proteins as well as serum proteins, *cf.*, ref. 13.

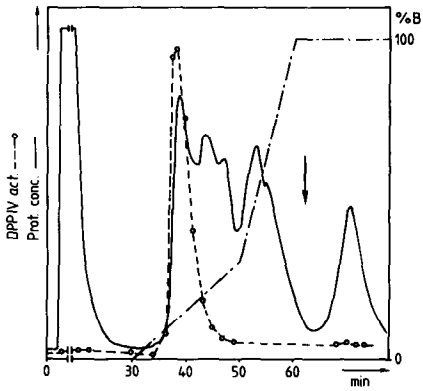

Fig. 9. Separation of membrane proteins on a 120 mm × 8.0 mm silica-based crown ether column. A protein (60 mg), dissolved in 20 ml of eluent A, was applied to the column. Separation was carried out in the presence of 0.1% Triton X-100 (reduced). The protein content (———) and dipeptidyl peptidase (DPP) IV enzymatic activity (– – –) were determined. In the non-bound fraction and up to 100% eluent B, 47.6 mg of protein (80%) were recovered. The remaining 10.8 mg of protein (18%) were recovered by an injection of 5 ml of 1% Triton X-100 in eluent B (arrow in the chromatogram). Chromatographic conditions: flow-rate, 1 ml/min; pressure, 1.0 MPa; room temperature. The gradient is shown. For other conditions see Fig. 6c and Experimental.

These results also show a similarity between crown ether sorbents and other sorbents used for the separation of biopolymers, such as hydroxyapatite and triazine dyes. All these substances are regarded as pseudo-affinity ligands, which can bind a variety of biopolymers. The nature of the underlying separation mechanism, however, is not yet fully understood[14].

Both a silica-based and a synthetic support column have shown no impairment in performance after being used for 150 separations. The application of non-ionic and zwitterionic detergents has not affected the column life. However, the use of the denaturing agents urea and guanidine hydrochloride (guanidinium) must be avoided, as they impair columns performance considerably and irreversibly, even after only two to three separations. This is probably due to the blocking of crown ethers by the formation of complexes with these substances[4]. This, in turn, prevents the loading of the column with potassium ions. SDS can form an insoluble precipitate with sodium, and cannot be used for separation for this reason.

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