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# Immobilized residue-specific endoproteinases for protein sequencing

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**Abstract**

Before proteins can be sequenced, the peptide chain has to be cut into small fragments of less than about 50 amino acids using residue-specific endoproteinases. These enzymes can be immobilized in a highly active form. Using immobilized endoproteinases for protein sequencing results in a series of advantages: (1) the high enzyme activity in the column results in short reaction times; (2) the protein fragments are easily eluted from the column whilst the endoproteinase is completely retained on the column; the protein fragments are clean yielding in low sequencing background; (3) the protein sample to be sequenced is free of exogenous enzymes; (4) endoproteinase self-digestion is prevented by immobilization; therefore, the sample solution does not contain any endoproteinase fragments; (5) enzymes are especially stable when immobilized. Columns with immobilized endoproteinases can be applied repeatedly and stored for many months.

## 1. Introduction

Proteins (polypeptides) consist of one or more chains of amino acids. The sequence of the amino acids in a chain (the primary structure) contains the basic information about the protein. In order to characterize a protein, knowledge of the primary structure is often needed. This can be obtained by sequencing the gene (DNA sequencing). In eucaryotes, however, due to the presence of introns DNA and protein sequence may not be directly related. The most reliable way to obtain the primary peptide sequence is therefore by protein sequencing. This cannot normally be directly achieved since most proteins are too long and/or may be N-terminally blocked [1]. Thus the chain must first be cut at defined sites.

Proteins (polypeptides) can be sequenced using automatic analyzers. Modern instruments can analyze very small amounts of protein (*ca.* 10 pmol). In most cases the protein is bound to a membrane and then chemically degraded step by step. The degraded amino acids are then detected and identified chromatographically. Each degrading cycle leaves a small percentage of molecules untreated. They are degraded out-of-phase in the following steps and thus contaminate the next steps. With increasing cycle numbers the background increases and finally prevents the identification of the degraded amino acids. Therefore, the number of degrading cycles still allowing for safe amino acid identification is restricted to between 20 and 50.

The length of the polypeptide chains to be

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sequenced is thus limited. If the chain is too long to be sequenced directly (longer then *ca.* 50 amino acids), it has to be cut at certain defined places into pieces suitable for the process. For this purpose site-specific peptide hydrolases [2], endoproteinases, are used. These endoproteinases recognize a specific amino acid and cut the peptide chain either before or after this site. Although several of these are known, only a few can be refined to the appropriate high degree of purity. Such site-specific endoproteinases are listed in Table 1. Further endoproteinases exist the recognition sites of which consist of more than one amino acid [like for example endoproteinase Xa and Igase which recognize the amino acid sequences Ile–Glu–Gly–Arg and Pro–Pro–Y–Pro (with Y being Thr, Ser or Ala), respectively]. These longer recognition sites, however, are rare and thus these endoproteinases are used less frequent for protein processing for sequencing. The endoproteinases cut the polypeptide chain to be analyzed at the sequence given above, if this is present. Thus, defined fragments are obtained and then purified [by *e.g.* HPLC or sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)] followed by sequencing in automatic instruments (for example, gas-phase sequencing).

There are serious difficulties with this method. Since the endoproteinase also consists of an amino acid chain, the chain to be sequenced is contaminated with the peptide chain of the endoproteinase. To keep this to a minimum, very small amounts of the endoproteinase are used (ratio endoproteinase:substrate *ca.* 1:25 to 1:100, w/w). This leads to very long reaction times, often several days long. During this time, secondary activities in the enzyme, or sample, can develop to a significant extent. Thus, the endoproteinase and the sample must both be of a very high degree of purity, a labour-intensive and therefore expensive procedure. Proteinases, added at concentrations under 1:100, may not cut the substrate chain completely, even when the appropriate sequence is present [1]. Another difficulty is that endoproteinase chains are often their own substrate; thus self-digestion occurs. The resulting polypeptide can be identified by additional experiments but is not always easily distinguished from fragments of the sample chain. As a consequence, the endoproteinase, and not the sample, may be sequenced.

The use of immobilized endoproteinases solves these problems. Our data show that immobilized endoproteinases can specifically digest peptides effectively in short reaction times. Columns packed with the immobilized enzymes hardly bleed-out and can be washed clean. Unspecific binding is not observed.

Table 1
Sequence-specific endoproteinases recognizing a single amino acid with recognition and cutting site ($\theta$)

| Endoproteinase | Recognition and cutting site[a] |
|---|---|
| Trypsin | Lys–X and Arg–X |
| Endoproteinase Glu-C | Glu–X |
| Endoproteinase Lys-C | Lys–X |
| Endoproteinase Asp-N | X–Asp |
| *Further endoproteinases of limited purity are* | |
| Endoproteinase Arg-C | Arg–X |
| Endoproteinase Pro-C | Pro–X |

[a] X represents any amino acid.

## 2. Experimental

### 2.1. Immobilization chemistry

The diisocyanate immobilization chemistry of Biebricher and Luce [3] was used. Briefly, the matrix surface is activated with 2,4-toluylene diisocyanate. The free isocyanate group is coupled to 2-butane-1,4-diol, which again reacts with 2,4-toluylene diisocyanate, providing an active isocyanate group for coupling to free primary amines. This chemistry is extremely stable in aqueous solution between pH 5 and 10. The long stiff linkers (Fig. 1) are chemically designed in such a way that they separate the

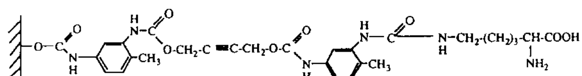

Fig. 1. Molecular structure of a diisocyanate spacer arm.

enzymes from the matrix surface, eliminating steric hindrance.

## 2.2. Matrix

Two types of matrix are used. The first, designated F7m, consists of intertwined polyvinyl chains (Fractogel, Merck) and has very large pores. The second, designated G3m, is a modified dextran gel (G10, Pharmacia) and has very few small pores. Both these matrices display the elution behaviour of gel filtration media. With F7m, molecules with a molecular mass ($M_r$) greater than $10^7$, with G3m with $M_r > 650$, elute in the void volume.

## 2.3. Proteolysis conditions

N-Tosyl-N-phenylalaninchlor-methyl-keton (TPCK)-treated trypsin (from bovine pancreas; Sigma; purity: 12 200 units/mg) was immobilized either on matrix F7m (12 200 units trypsin on 200 $\mu$l F7m corresponding to 50 mg dry matrix F7m) or matrix G3m (300 units trypsin on 200 $\mu$l G3m corresponding to 125 mg dry matrix G3m). The immobilized TPCK-trypsin was packed into a Mobicol column (MoBiTec, Göttingen, Germany; see Fig. 2) and incubated with either 30 $\mu$g (G3m) or 250 $\mu$g (F7m) insulin $B_{ox}$ (bovine insulin, chain B, oxidized; Sigma; $M_r$ 3495.9) in either 55 $\mu$l (G3m) or 90 $\mu$l (F7m), 3 m*M* phosphate buffer, pH 8.0, for the appropriate length of time (see captions to figures), wherupon the sample was, for matrix G3m, spun out of the Mobicol reaction column or, for matrix F7m, eluted with an additional 410 $\mu$l phosphate buffer. Dilutions of the eluate were taken for HPLC analysis.

Endoproteinase Glu-C (*Staphylococcus aureus* protease; Worthington; purity: 900 units/mg) was immobilized on F7m (900 units endoproteinase Glu-C on 200 $\mu$l F7m corresponding to 50 mg dry matrix F7m) or G3m (22 units endoproteinase Glu-C on 200 $\mu$l G3m corresponding to 125 mg dry matrix G3m). The immobilized endoproteinase Glu-C was packed into a Mobicol column and assayed with insulin $B_{ox}$ as described for trypsin, with the single exception that the buffer was 25 m*M* ammonium carbonate, pH 7.8.

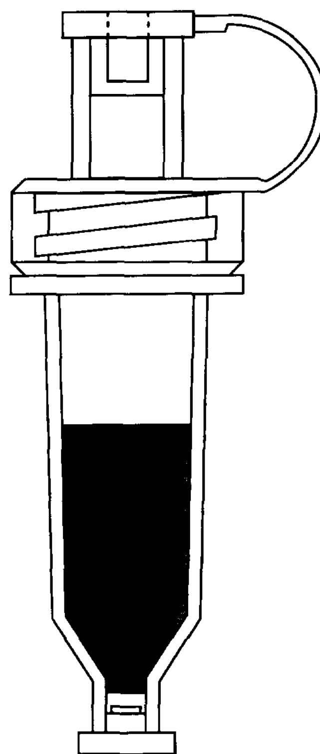

Fig. 2. Compact reaction column with immobilized endoproteinase (grey) between filters (black, with 90 $\mu$m pore size). The column (Mobicol) fits into a normal 1.5-ml tube and can be centrifuged in a bench-top centrifuge. The screw-on top has a Luer-lock adaptor for syringes or a connection to tubing. The outlet fits into Luer-lock adaptors and has a removable plug closing the outlet. Very small (10 $\mu$l) as well as very large (up to several liters) volumes can be handled easily.

Endoproteinase Lys-C (from *Lysobacter enzymogenes;* Boehringer Mannheim; purity: 150 units/mg) was immobilized on matrix G3m (1 unit endoproteinase Lys-C on 200 $\mu$l G3m corresponding to 125 mg dry matrix G3m). The immobilized endoproteinase Lys-C was packed into a Mobicol column and incubated with 30 $\mu$g melittin (from bee venom, 26 amino acids; Sigma) in 90 $\mu$l 50 m*M* Tris buffer, pH 8.5, containing 1 m*M* EDTA. After the appropriate reaction time (see captions to figures), the solution was recovered by centrifugation and analysed by HPLC.

Proteinase K (from *Tritirachum album;* Merck; purity: 30 mAnson units/mg) was immobilized on matrix G3m (0.75 mAnson units proteinase K on 200 $\mu$l G3m corresponding to 125 mg dry matrix G3m). The immobilized proteinase K was packed into a Mobicol column and treated with 50 $\mu$l 10 m*M* Tris buffer, pH 8.0, containing 2 m*M* EDTA. After 1 min, the column was spun dry and the eluate incubated for 26 h at 37°C with 20 $\mu$g bovine serum albumin (BSA). Eluate and substrate solutions were analysed by SDS-PAGE.

Alkaline phosphatase (from calf intestine; Biogenzia; purity: 4130 units/mg) was immobilized with three different chemical linkers: diisocyanate to F7m (Fractogel, Merck), CNBr to agarose (Pharmacia) and epoxy to vinyl acetate (VA, Riedel de Häen). An amount of 1000 units of alkaline phosphatase was applied to 200 $\mu$l swollen chemically modified matrix material of the three different kinds according to the conditions suggested by the manufacturers. This material was packed into Mobicol columns which then have equal volumes. The amount of immobilized protein in the column relative to the amount offered for immobilization was determined by measuring the remaining amount of enzyme in the supernatant after immobilization. The activity of the immobilized enzyme on the three columns was measured. A 100-$\mu$l volume of substrate solution containing 5 m*M* *p*-nitrophenylphosphate was pipetted onto the columns. After 20 s the columns were washed with 5 ml 1.5 *M* NaCl, 50 m*M* Tris pH 8.0. The absorbance was determined at 405 nm. For these three matrices the bleeding-out was determined. A 200-$\mu$l volume of buffer was applied to the columns for 30 min and eluted. These 200 $\mu$l were added to 400 $\mu$l of 5 m*M* *p*-nitrophenylphosphate in reaction buffer and incubated for 10 min. The reactivity was measured at 405 nm.

Digestions were carried out at room temperature except where otherwise stated.

### 2.4. Peptide fragment detection

Fragments were analysed on a Nucleosil 300-5 $C_4$ (Biometra, Göttingen, Germany) reversed-phase HPLC column. Oligopeptides were separated on a gradient from buffer A [0.1% (v/v) trifluoroacetate in water] to buffer B [0.1% trifluoroacetate in 50% (v/v) acetonitrile]. For insulin fragments, the program was 15 min 2% to 40% followed by 5 min 40% to 100%. For melittin fragments, 30 min 2% to 90%, followed by 2 min 90% to 100%. This HPLC system (column and gradients) allows maximal separation of substrate from products. The HPLC system is not optimized to separate the oligopeptide products. In all cases the flow-rate was 0.5 ml/min and peptide detection at 215 nm.

### 2.5. DNA methods

A 4-$\mu$g amount of plasmid pBR322 superhelical DNA (prepared from *Escherichia coli* K12 and purified by us from a CsCl gradient), in 50 $\mu$l 10 m*M* Tris buffer, pH 8.0, containing 2 m*M* EDTA, was loaded onto a column containing proteinase K immobilized on matrix G3m. After 2 min incubation, the plasmid solution was eluted by gravitation. The eluted plasmids were as biologically active in transformation as untreated plasmids. The column was washed repeatedly in steps of 100 $\mu$l buffer (6 times) and subsequently in steps of 1.5 ml (6 times). The eluate was transformed into competent $Ca^{2+}$-treated *E. coli* JM109 cells. At early elution steps, only portions of the eluate were transformed, in order to limit the number of colonies to manageable numbers. The colony numbers obtained on $Amp^+$ plates (agar medium plates containing ampicillin) were corrected for dilution steps and plotted *versus* the elution volume.

## 3. Results

In order to take full advantage of immobilization, it must first be shown that the proteinases stay on the matrix and do not bleed out. We thus took an amount of one immobilized, very active, non-specific proteinase (proteinase K), which can digest between 250 and 500 $\mu$g BSA per min, washed the matrix, and assayed the eluate. Fig. 3 shows that the eluate contained so little proteinase K that even after 26 h incubation at

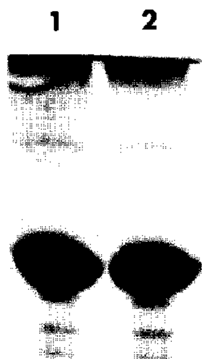


Fig. 3. No proteinase activity in the eluate of a proteinase K column. Eluate from a proteinase K column was incubated with 20 $\mu$g BSA for 26 h at 37°C in reaction buffer (10 m*M* Tris–HCl at pH 8.0, 2 m*M* EDTA). A 5-$\mu$g (25% of the reaction solution) sample was then loaded onto an SDS gel and separated electrophoretically. Lanes: 1 = BSA control; 2 = sample after incubation. No degradation can be detected. The same proteinase K column digests completely the 25-fold amount of BSA in 1 min.

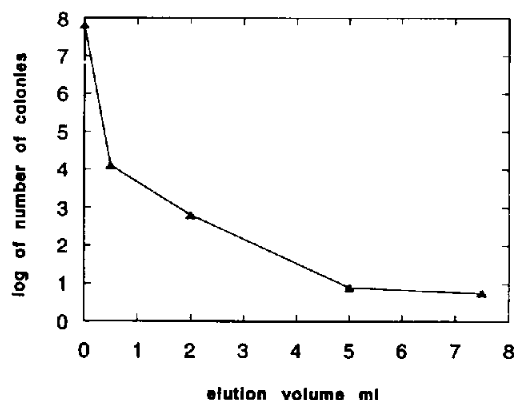


Fig. 4. Column contamination. Elution of covalently closed circular plasmids from a 200-$\mu$l immobilized proteinase K (matrix G3m) column. For experimental procedure, see Experimental. After 7.6 ml washing buffer the limit of detection ($10^4$ molecules) was reached. Since $3 \cdot 10^{11}$ molecules were applied, this means that a dilution factor down to $3 \cdot 10^{-8}$ had been reached.

37°C, no protease activity could be detected in the eluate. This is a sensitive assay since the first washing step contains the highest amount of washed-off proteinase (see Fig. 4). Further washing steps or washing with higher volumes reduces the concentration of the washed-off protein and reduces the sensitivity of the assay.

Using another even more sensitive test the bleeding-out rate could be determined. Alkaline phosphatase degrades *p*-nitrophenylphosphate to *p*-nitrophenol which has a yellow colour. The reaction can thus be detected easily, specifically and extremely sensitively at a wavelength of 405 nm. We, therefore, measured the enzyme activity in the eluate of a column containing immobilized alkaline phosphatase. We compared the bleeding-out of alkaline phosphatase immobilized by "our" diisocyanate linker on Fractogel with that of the enzyme immobilized by CNBr to agarose and epoxy to VA. Alkaline phosphatase (1000 units) was applied to 200 $\mu$l swollen chemically modified matrix material of the three different kinds. This material was packed into Mobicol columns which then have equal volumes. The amount of immobilized protein found in the column relative to the amount offered for immobilization was: 100% for the CNBr and the diisocyanate linkage, and 50% for the VA–epoxy. Thus, the same volume amount of VA–epoxy matrix binds only half the amount of protein compared to the other two matrices although the same amount of protein is offered for immobilization. We then measured the activity of the immobilized enzyme on the three columns relative to the diisocyanate linkage (100%): CNBr 75%, VA–epoxy 33%. Relative to the amount of enzyme immobilized on the 200 $\mu$l matrix these values are shifted to 75% for CNBr and 66% for VA–epoxy since the VA–epoxy matrix immobilized only half the amount of enzyme. Thus, the specific activity of the immobilized enzyme is considerably less for the CNBr and VA–epoxy compared to the diisocyanate linkage. For these three matrices the bleeding-out was determined. The low elution concentration of the diisocyanate/Fractogel

system ($1.9 \cdot 10^{-5}$, using the immobilization chemistry of Biebricher and Luce [3]) is *ca.* 20 times less than that for CNBr and about 10 times less than for VA–epoxy immobilization chemistries (see Table 2).

In order to re-use columns containing immobilized enzymes, one must be sure that after washing they are really clean, *i.e.*, that the fragments of the previous sample have been washed out of the column. The washing of the column is so effective that the elution is well beyond the range of chemical detection (for example in gels, HPLC or UV spectroscopy). Therefore, we tested the non-specific adsorption of two classes of biomolecules to the columns with biological methods. We loaded an enzyme (alkaline phosphatase) on a column containing an immobilized enzyme which leaves the loaded enzyme intact (RNase A) and measured the eluted phosphatase activity. Very small amounts of phosphatase activity (a few $\mu$units corresponding to a few nanograms of enzyme) can be measured rather easily due to the high extinction coefficient of the product. In parallel, covalently closed circular DNA plasmids were applied to columns containing immobilized enzymes which leave nucleic acids intact (in this case, proteinase K). The columns were washed and the eluate transformed into *E. coli* cells. Using this technique we were able to detect the effectiveness of the column washing down to about $10^4$ DNA molecules (*i.e.*, $10^{-19}$ mol). As Fig. 4 shows, the columns were essentially free of contamination after washing with 7.6 ml buffer. Similar data we obtained for the alkaline phosphatase. Thus after use, the columns were washed with 10 ml washing buffer. NaCl (1 *M*) was added to the washing buffer to reduce unspecific protein binding to the column.

Table 2
Protein elution behaviour using different linker chemistries

| | Activity |
|---|---|
| VA–Epoxy | $17.0 \cdot 10^{-5}$ |
| CNBr | $35.0 \cdot 10^{-5}$ |
| Diisocyanate | $1.9 \cdot 10^{-5}$ |

Activity of alkaline phosphatase in the eluate relative to the amount of enzyme immobilized on the Mobicol column.

Three endoproteinases typically used in protein sequencing (trypsin, endoproteinases Glu-C and Lys-C) were immobilized and tested for their ability to cleave model polypeptides. Larger proteins were digested by immobilized endoproteinases and analyzed by PAGE. However, PAGE does not give quantitative information on very small product peptides and on the disappearance of the substrate peak. Furthermore, unspecific digestion might result in a weak background smear which is difficult to evaluate quantitatively. We therefore carried out a more quantitative analysis using HPLC. In these experiments the substrate peak can be compared with the sum of all product peaks. The disadvantage of using HPLC, however, is that shorter peptides have to be used as substrates so that substrates and products can be well separated.

The digestion of insulin and melittin is especially easy to follow using HPLC and was therefore used here. The amino acid sequence of these substrates, together with the specific cleavage sites, is given in Fig. 5. The substrates were loaded on the columns containing immobilized endoproteinases. The eluates from the columns were analyzed by HPLC (see Experimental). After substrate passage through the column, the substrate peak decreases and lower-$M_r$ product peaks appear (see Figs. 6–10). During the HPLC gradient run, the oligopeptides of different size elute at different gradient buffer concentrations.

Sequence of insulin $B_{OX}$

FVNQHLCGSHLVEALYLVCGERGFFYTPKA

Sequence of melittin

GIGAVLKVLTTGLPALISWIKRKRQQ

Fig. 5. Sequence of insulin $B_{ox}$ and of melittin. Endoproteinase Glu-C cuts after E, endoproteinase Lys-C after K, and trypsin after R and K.

The separation of small chains of similar length having differences of only a few amino acids, is, however, difficult using HPLC and beyond the range of the system used here. Single amino acids and dipeptides are not detectable in our system. Nevertheless, the detected product fragments of insulin and melittin are of the expected size [4].

The short-chain polypeptide, insulin $B_{ox}$, was cut with trypsin. Using the beads with small pores (G3m), 50% of the insulin (originally 30 $\mu$g) was digested in 30 min (Fig. 6). Using the beads with large pores (F7m), 250 $\mu$g insulin were digested in less than 1 min (Fig. 7). Similar results were obtained using endoproteinase Glu-C. Here, on small-pore (G3m) matrix, 60% of the 30 $\mu$g insulin $B_{ox}$ was digested in 30 min (Fig. 8) whereas, on large-pore (F7m) matrix, 90% of 250 $\mu$g insulin was digested in 5 min (Fig. 9). The three product peaks of 13, 8 and 9 amino acids are hardly resolved in Fig. 9. A digest intermediate can be seen in Figs. 8 and 9 between the peaks of substrate and final products, which disappears in Fig. 9 after 10 min reaction time. Immobilized endoproteinase Lys-

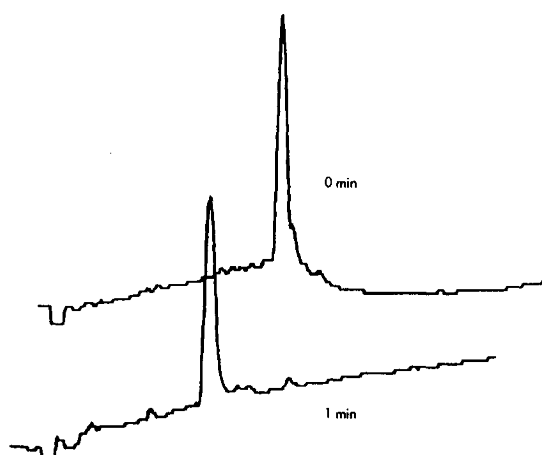


Fig. 7. The digestion of insulin $B_{ox}$ by trypsin immobilized on matrix F7m. The HPLC peak of insulin was reduced with reaction time in the column (substrate solution: top profile; eluate after 1 min reaction time: lower profile). The HPLC trace shows that all 250 $\mu$g insulin were digested in under 1 min.

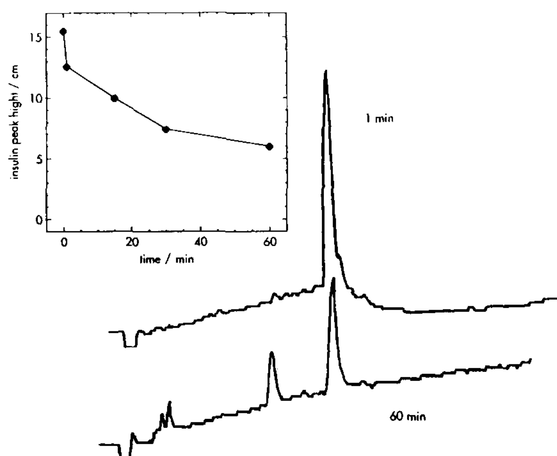


Fig. 6. The digestion of insulin $B_{ox}$ by trypsin immobilized on matrix G3m. For experimental procedure, see Experimental. The HPLC peak of insulin (main figure) was reduced with reaction time in the column and fragment peaks appear (eluate after 1 min reaction time: top profile; eluate after 60 min reaction time: lower profile). The decrease of the substrate peak with time is plotted in the insert. After 30 min, 50% (15 $\mu$g) of the insulin had been digested.

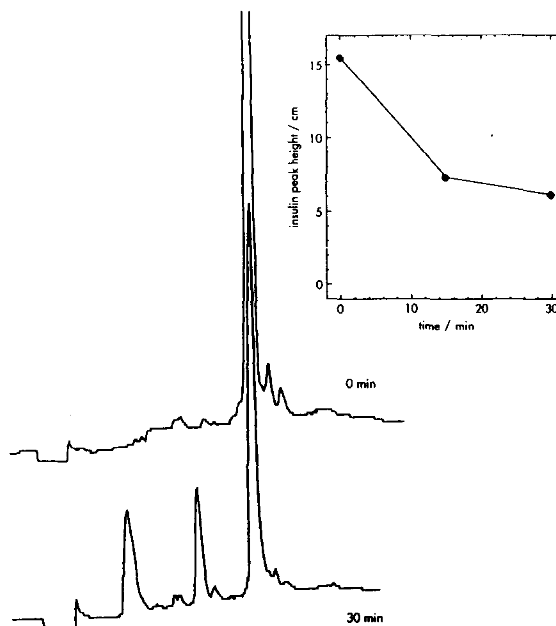


Fig. 8. The digestion of insulin $B_{ox}$ by endoproteinase Glu-C immobilized on matrix G3m. The HPLC peak of insulin (main figure) was reduced with reaction time in the column and fragment peaks appear [substrate solution: top profile; eluate after 30 min reaction time: lower profile (reaction intermediates are detected)]. The decrease of the substrate peak with time is plotted in the insert. After 30 min, 60% (18 $\mu$g) of the insulin had been digested.

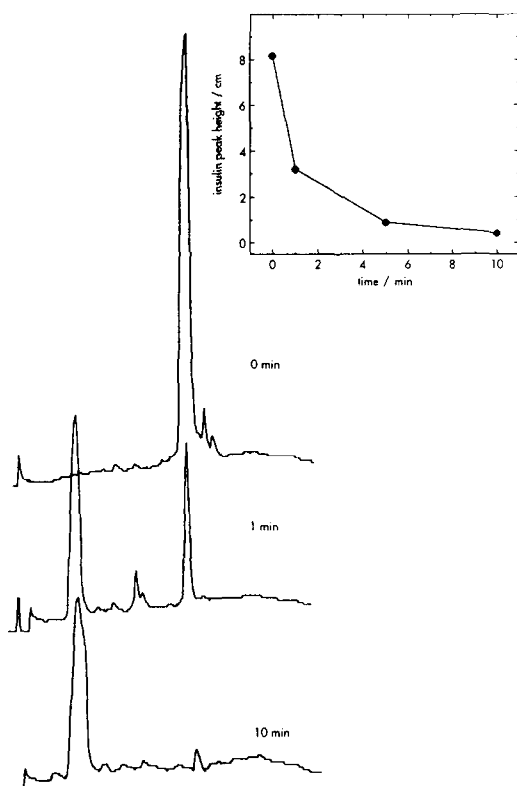


Fig. 9. The digestion of insulin $B_{ox}$ by endoproteinase Glu-C immobilized on matrix F7m. The HPLC peak of insulin (main figure) was reduced with reaction time in the column and fragment peaks appear. Substrate solution: top profile; eluate after 1 min reaction time: middle profile (reaction intermediates are detected); eluate after 10 min reaction time: lower profile. The three product peaks are not resolved. The decrease of the substrate peak with time is plotted in the insert. After 5 min, 90% (225 μg) of the insulin had been digested.

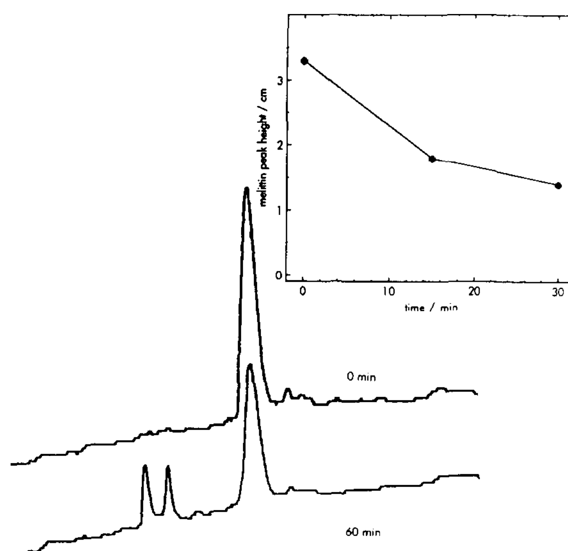


Fig. 10. The digestion of melittin by endoproteinase Lys-C immobilized on matrix G3m. The HPLC peak of melittin (main figure) was reduced with reaction time in the column and fragment peaks appear (substrate solution: top profile; eluate after 60 min reaction time: lower profile). The decrease of the substrate peak with time is plotted in the insert. After 30 min, 44% (18 μg) of the melittin had been digested.

-C, on small-pore (G3m) beads, could digest 60% of 30 μg melittin in 30 min (Fig. 10).

Obviously, the immobilized enzyme columns containing 200 μl F7m matrix are much more effective than the columns with 200 μl G3m matrix. However, the F7m matrix binds about 40 times the amount of enzyme compared to matrix G3m while in these experiments only 5–10-fold more substrate is used with the F7m than with the G3m gel. Per immobilized enzyme both gels show rather similar activities (within a factor of 2).

We did not detect any peak in the HPLC when no substrate was loaded (HPLC diagram not shown) nor could endoproteinase activity be observed when the column elute was incubated with substrate (data not shown).

## 4. Discussion

We show here that (endo)proteinases can be immobilized on inert carrier matrices in such a way that they not only remain in a highly active state, but also that they do not bleed out to detectable levels. The columns can be washed clean for repeated use.

The choice of matrix (F7m or G3m) depends on the volumes to be used. When using a *small volume* (*e.g.* 10 μl), carrier beads (called G3m) are used, which have such small pores that biopolymers cannot enter (excluded are molecules with $M_r > 650$). Thus, the endoproteinase molecules are immobilized only on the surface of the beads. Neither substrate nor digestion fragments can enter the beads. Therefore, the reac-

tion takes place in the space between the beads where the endoproteinase, substrate and product are present. The full volume (the same as the added volume) can then be completely removed by centrifugation. The columns used in this study (Mobicols, see Fig. 2) are centrifugable and are compatible with standard laboratory equipment (centrifuge vials and Luer-lock plastic ware).

Where the *volume of substrate is large* (at least larger than the dead volume of a column, *ca.* 80 $\mu$l), then a different type of matrix should be applied consisting of beads having very large pores (called F7m). Thus, the endoproteinases and the substrate can enter the bead pores. The available surface area (including the surface inside the beads) is so large that very large amounts of endoproteinase can be immobilized. The activity immobilized on such columns is around 40 times that of columns with small-pore beads (as above). As a result of the high activity, very short reaction times are sufficient for complete digestion. Due to these large pores, however, the product (protein fragments) cannot be completely centrifuged out of the column. In this case the product fragments have to be washed out of the column. The resulting pooled eluate then has a volume of about 500 $\mu$l more than the substrate sample solution.

Polypeptides can also be digested at specific sites non-enzymatically. In the 1960s, chemical methods were developed to cleave proteins. The CNBr method [5] cleaves polypeptides specifically after methionine. Methionine is, however, an infrequent amino acid. Cleavage is further complicated due to some of the methionine residues (up to 20%) being oxidized in any one sample [6]. During the 1970s therefore, chemical techniques were refined to include the cleavage after Trp by a variety of methods (see, *e.g.* refs. 7 and 8). Still, however, the fragment length remained too long for widespread automatic analysis. Eventually, in the 1980s, further biological hydrolysis was introduced using other site-specific peptide hydrolases [2] which are now used most frequently.

Such endoproteinases can be tightly bound to an insoluble matrix in a highly active form and packed in ready-to-use centrifugable columns. The combination of residue specificity, high activity, immobilization and the special form of the spin-column offer a series of advantages. The high activity in the column means short reaction times. Fragments of the substrate protein are easily recovered whilst the endoproteinase is completely retained in the columns. The protein digest is pure since immobilized endoproteinases are incapable of self-digestion. Immobilized endoproteinases are especially stable, thus the columns can be stored for many months and used repeatedly. The columns can be centrifuged, resulting in fast, controlled elution behaviour with negligible sample dilution.

## 5. References


[1] H. Charbonneau, in P.T. Matsudaira (Editor), *A practical Guide to Protein and Peptide Purification for Microsequencing*, Academic Press, San Diego, CA, 1989, pp. 17–32.

[2] P. Jekel, W. Weijer and J. Beintema, *Anal. Biochem.*, 134 (1983) 347–352.

[3] C. Biebricher and R. Luce, *US Pat.*, 4 177 038 (1979).

[4] S. Fischer, U. Brunner, E. Galaboff, U. Geuss, M. Schäffer and G. B. Kresse, *Biochemica*, Boehringer Mannheim, Mannheim, 1990.

[5] E. Gross, *Methods Enzymol.*, 11 (1967) 238–242.

[6] W.A. Schroeder, J.B. Shelton and J.R. Shelton, *Arch. Biochem. Biophys.*, 130 (1969) 551–561.

[7] I. Schechter, A. Patchornike and Y. Burstein, *Biochemistry*, 15 (1976) 5071–5073.

[8] W.C. Mahoney and M.A. Hermodson, *Biochemistry*, 18 (1979) 3810–3813.