Journal of Chromatography A, 660 (1994) 137–145
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2956

Perfusible and non-perfusible supports with monoclonal antibodies for bioaffinity chromatography of *Escherichia coli* penicillin amidase within its pH stability range

V. Kasche*, N. Gottschlich, Å. Lindberg, C. Niebuhr-Redder and J. Schmieding

AB Biotechnologie II, TU Hamburg-Harburg, D-21071 Hamburg (Germany)

ABSTRACT

Several monoclonal antibodies (mABs) have been prepared and immobilized for the biospecific isolation of penicillin amidase (PA) from *Escherichia coli* (EC 3.5.1.11), an enzyme without S–S bridges and a pH stability range of 4–9. During the immobilization the fluorescence emission maxima of the mABs were found to change from 336 nm to *ca.* 350 nm. Only one of these mABs was found to be suitable for preparative bioaffinity chromatography of PA within the pH stability range. This mAB was immobilized on different spherical supports (Eupergit C 250 L and Sepharose) and one perfusible support (Knauer Quick Disc) and used for analytical and preparative bioaffinity chromatography. Under isocratic conditions the plate height for the perfusible biospecific adsorbent was found to be an order of magnitude lower than for the other supports. The different forms of this proteolytically processed bacterial enzyme could not be separated, however, by the biospecific adsorbents. At the mAB density used in the adsorbents (10–30 μM), less than 30% of the theoretical binding capacity of the immobilized mABs could be used to adsorb the enzyme.

INTRODUCTION

Immobilized monoclonal antibodies (mABs) and inhibitors against enzymes, antigens and other antibody-binding proteins are suitable adsorbents for bioaffinity chromatography [1]. With these the enzyme or mAB can be quantitatively isolated from homogenates. The adsorbed proteins can generally be desorbed using H^+ as acid–base equilibria control its interaction with the immobilized ligate [2]. For enzymes without S–S bridges and oligomeric enzymes the pH range within which they retain their biological function is limited. Outside this range such enzymes rapidly denature owing to partial unfolding of dissociation caused by charge–charge repulsions. The denatured proteins can be renatured in a time-consuming procedure. For the isolation of these enzymes only those mABs or inhibitors that allow the desorption within the pH range where the enzyme is stable can be used for biospecific affinity chromatography. This was investigated to select mABs against *Escherichia coli* penicillin amidase (PA) that are suitable for the biospecific isolation of this enzyme from homogenates. This bacterial enzyme consists of two polypeptide chains ($M_r \approx 88\,000$) formed by proteolytic processing of a single polypeptide pro-enzyme ($M_r \approx 90\,000$). It has no S–S bridges and denatures rapidly (half-life <1 h) at pH <4 and pH >9. Several active enzyme forms that differ in the composition of the smaller peptide chain have been observed [3]. When their dissociation constant for the interaction with the biospecific support differs, they have different capacity factors. Then these and the plate height determine the possible resolution of these different enzyme forms by bioaffinity chromatography. The plate height for different biospecific supports varying in particle and pore size, includ-

* Corresponding author.

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(93)E1062-5

ing one perfusible support, was therefore determined.

For preparative purposes, the binding capacity of the immobilized mABs should be large. It is frequently observed, however, that at high mAB densities only a fraction of the immobilized molecules retain their function to interact biospecifically with soluble ligands. This may be due to steric hindrance when the ligand is so large that its binding to one ligate molecule sterically hinders the simultaneous binding of ligands by the nearest neighbour ligates [4–6]. This indicates the existence of an optimum ligate concentration for biospecific affinity chromatography that depends on the size of ligand. Another possibility is that some mABs lose their binding function owing to partial unfolding on immobilization. This was studied by direct determination of the fluorescence spectra of the immobilized mABs using a specially designed cuvette with a fibre-optic light guide [7–9].

EXPERIMENTAL

Materials and methods

The following supports were used for immobilization: cyanogen bromide-activated Sepharose 4B (average particle radius 40 μm) was obtained from Pharmacia (Uppsala, Sweden) and Eupergit C 250 L (average particle radius 100 μm) and Knauer Quick-Disc [10] were kindly provided by Röhm (Darmstadt, Germany) and Knauer Säulentechnik (Berlin, Germany), respectively. Crude PA (EC 3.5.1.11) preparations were a gift from Röhm. The PA forms with isoelectric points (p*I*) of 7.0 ($PA_{7.0}$) and 6.7 ($PA_{6.7}$) were prepared as described previously [3]. All other chemicals were of analytical-reagent grade.

Monoclonal antibody production and purification

Hybridoma cell lines that produced mABs against $PA_{7.0}$ were isolated from mouse (Balb/c) spleen cells and myeloma (PAI/0) cells using a standard immunization and fusion protocol. Different hybridoma cell lines, which secrete mABs (IgG1) against *E. coli* PA, were used throughout this study. Cell line IV F 19 was grown in continuous culture in a membrane dialysis bioreactor as described previously [11]. The media for all cell lines was a 1:1 mixture of Iscove's MDM and Ham's F12 (Gibco, Paisley, UK) supplemented with 3% foetal calf serum (FCS), 2 mmol/l L-glutamine from Gibco and 2 g/l $NaHCO_3$. The other cell lines were grown in a roller bottle unit (Tecnomara, Fernwald, Germany). Cells and debris were removed from the supernatant by centrifugation with a Contifuge (Heraeus, Osterode, Germany) and the spent medium was concentrated [Heraeus cross-flow or Amicon (Beverly, MA, USA); M_r cut-off 10 000] and filtered with a 0.2-μm filter. If necessary, the pH was adjusted to 6–7 and this medium was directly applied to a protein G column [protein G Sepharose Fast Flow (Pharmacia), 70 mm × 10 mm]. The column was washed with 50 m*M* phosphate buffer (pH 6.5) and the bound antibodies were eluted with 0.2 *M* glycine–HCl (pH 2.7). The pH of the eluted antibodies was immediately adjusted to 7. For comparison of fluorescence spectra, one part of the antibodies was purified by 50% $(NH_4)_2SO_4$ precipitation according to ref. 12. The precipitate was dissolved in phosphate-buffered saline (PBS), dialysed against PBS and the antibody-containing solution was concentrated to 2–5 mg/ml with Centricon or Centriprep concentrators (Amicon). Further purification was achieved by size-exclusion chromatography on Superdex 200 in a HiLoad 16/60 column (Pharmacia). The purity of antibodies was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF).

Determination of monoclonal antibodies

Mouse IgG contents in the media were determined with a Mouse-IgG-ELISA kit (Boehringer, Mannheim, Germany) or with an antigen-specific enzyme-linked immunosorbent assay (ELISA). For pure antibodies the antibody content was determined at 280 nm using a molar absorptivity of 13.5 for a 1% solution with a UV spectrophotometer [13].

Electrophoretic methods

IEF was carried out with Ampholine-PAG plates (pH 3.5–9.5) on a Pharmacia Multiphor II apparatus according to the instructions of the manufacturer.

SDS-PAGE was performed on SDS gels according to the method of Laemmli [14] in a MINI-Gel system from Bio-Rad (Richmond, CA, USA).

Immobilization

The immobilization of the antibodies on the solid supports was carried out according to the instructions of the manufacturer with the following change. For the coupling reaction, 0.2 *M* phosphate buffer (pH 7.0) was used, as this has been found to decrease the heterogeneity in the dissociation constants and to increase the resolving power of biospecific supports when the ligate is bound via the ε-amino group of lysine side-chains [2]. Remaining binding sites were blocked overnight with 1 *M* ethanolamine (pH 8.0).

Determination of enzyme activity

Penicillin amidase activity was determined with 2-nitro-5-phenylacetaminobenzoic acid (NIPAB) as substrate as described previously [3].

Determination of dissociation constants and static capacity

For the determination of dissociation constants, equal amounts of antibodies coupled to the solid supports were incubated with various amounts of purified $PA_{7.0}$ at different pH values using phosphate buffers (ionic strength, $I = 0.2$ *M*). The time course of the binding of the enzyme to the immobilized mABs was determined from the enzyme activity outside the particles as a function of time. The adsorption was virtually completed within 1 h as the amount adsorbed between 1 and 24 h of equilibration was found to be negligible. The equilibrium K_{diss} value and the static capacity could therefore be determined after 1 h of equilibration. The suspensions were then filtered and the concentration of unbound PA was determined from the enzyme activity in the filtrate. The amount of bound PA was determined from the mass balance.

Analytical and preparative bioaffinity chromatography

An LKB–Pharmacia (Pharmacia, Uppsala, Sweden) HPLC solvent delivery system (Model 2150 pump, Model 2152 controller) and a Knauer (Berlin, Germany) Typ 87.00 UV–Vis detector were used for these experiments. Analytical affinity chromatography was performed under isocratic conditions with buffers (phosphate, $I = 0.2$ *M*) in the pH range 4–5 in small columns (radius 5 mm) packed with *ca.* 2 ml of biospecific support, except for the cylindrical Knauer Quick Disc (radius 25 mm, height 4 mm), which was used in a specially designed holder [10]. Samples (50 μl) of purified $PA_{7.0}$ and $PA_{6.7}$ (*ca.* 1 mg/ml) were applied to the columns and eluted under isocratic conditions at various flow-rates. When the active enzyme was eluted the plate height was determined from its peak in the elution curve.

For the preparative isolation of PA from homogenates and crude enzyme preparations, a 20 mm × 14 mm column packed with IV F 19 mAB (4.9 mg/ml wet support) was used. For the adsorption of the enzyme from a 0.5-ml sample of crude enzyme (20 mg/ml, total protein content *ca.* 80 mg/ml) the column was equilibrated and eluted (flow-rate 0.5 ml/min) with buffer of pH 7.0 (phosphate, $I = 0.2$ *M*). When the first peak had been eluted the pH of the elution buffer was changed to 4.0 to desorb the active enzyme. When the enzyme had been eluted, the column was regenerated with the buffer of pH 7.0.

Determination of fluorescence spectra of free and immobilized proteins

The fluorescence spectra of free and immobilized enzymes were measured with a Shimadzu (Kyoto, Japan) RF-540 spectrofluorimeter. For the determination of the fluorescence spectra of immobilized proteins a specially designed cuvette and fibre-optic device as described previously [9,15] were used (excitation slit width 10 nm, emission slit width 5 nm, excitation at 280 nm). A small flow-through cuvette (15 μl) was packed with unreacted supports in phosphate buffer and the fluorescence spectrum was determined. In a second step, ligate-coupled support was packed

into the same cuvette and the fluorescence was measured as the difference spectrum between the coupled and uncoupled support.

RESULTS AND DISCUSSION

Isolation and characterization of mABs against penicillin amidase

Hybridoma cells from different stable clones that produced mABs against PA were cultivated in a specially designed bioreactor or in roller flasks [11]. The medium from these cultures was concentrated by ultrafiltration. The mABs in these solutions were purified either by Protein G affinity chromatography or a combination of salt precipitation and gel exclusion chromatography. The yield of mABs isolated was >95%. The purities of the mABs derived from IEF patterns are shown in Fig. 1A. The IEF patterns for the same mAB purified by different methods were identical, as shown in Fig. 1B. The mAB from hybridoma clones that produced most antibody and a mAB with a high and a low isoelectric point were selected for further experiments.

All mABs were heterogeneous with respect to IEF patterns. For the IV F 19 mAB this pattern was not changed during long-term fermentation or using cells that after frequent recultivations had changed their mean chromosome number from 66 to 61.

Immobilization of mABs: determination of fluorescence spectra and dissociation constants

The immobilization yields and the wavelengths of the fluorescence emission maxima for free and bound mABs using different supports are given in Table I. Fluorescence spectra of free and immobilized antibody are shown in Fig. 2. During immobilization, the wavelength of maximum fluorescence emission (λ_{max}) was found to increase more than has been observed previously for the globular enzymes α-chymotrypsin, β-trypsin and PA, where λ_{max} remained virtually unchanged on immobilization [7,15]. The fluorescence emission of proteins at 330–340 nm is due to the tryptophan residues. Their fluorescence emission maximum increases when they

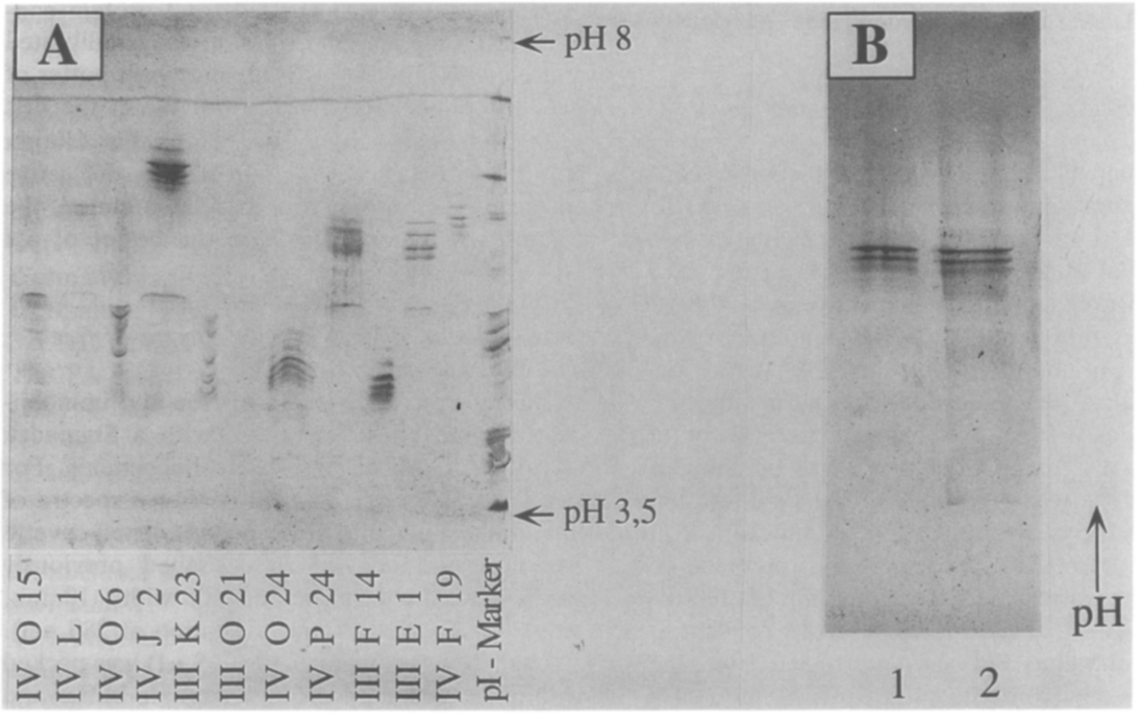

Fig. 1. Isoelectric focusing patterns of different mABs against *E. coli* penicillin amidase. (A) Monoclonal antibodies from different clones isolated by protein G chromatography. (B) mAB IV F 19 isolated by protein G chromatography (lane 1) and by a combination of salt precipitation and gel exclusion chromatography (lane 2).

TABLE I

WAVELENGTH OF MAXIMUM FLUORESCENCE EMISSION, λ_{max} (UNCORRECTED AT pH 7.0, 25°C), BINDING YIELD (IN % OF mABs ADDED TO THE ACTIVATED SUPPORT) AND mAB CONTENT IN THE SUPPORTS DETERMINED FROM THE MASS BALANCE

Support	Property	Monoclonal antibody			
		III E 1	III F 14	IV F 19	IV J 2
Sepharose	λ_{max} (nm)	336	335	336	n.d.[a]
	Binding yield (%)	47	68	>90	>90
	[mAB] (mg/ml)	2.9	3.9	4.9	4.7
Eupergit C 250 L	λ_{max} (nm)	355	350	352	n.d.[a]
	Binding yield (%)	84	76	60	–
	[mAB] (mg/ml)	4.2	4.5	3.0	–
Knauer Quick-Disc	λ_{max} (nm)	348	n.d.[a]	350	n.d.[a]
	Binding yield (%)	–	–	90	–
	[mAB] (mg/ml)	–	–	1.5	–

[a] Not determined.

are transferred from non-polar (alcohol) to a more polar (water) surrounding. When proteins unfold, some tryptophan residues become more exposed to water and λ_{max} increases. This has been used to study the stabilization of β-trypsin against thermal unfolding on immobilization [7]. The data for the mABs indicate a partial unfolding due to the immobilization procedure. The function to bind PA was, however, retained, as follows from the determination of K_{diss} as a function of pH (Fig. 3). From Scatchard plots the fraction of the immobilized antibodies that can bind the enzyme could be determined and the results are given in Table II. The experimental error in K_{diss} (±30%) depends on the error in the activity determinations (±5%) and can be calculated as described previously [16,17]. From these data and Fig. 3, it follows that K_{diss} differs significantly for the different antibodies with high

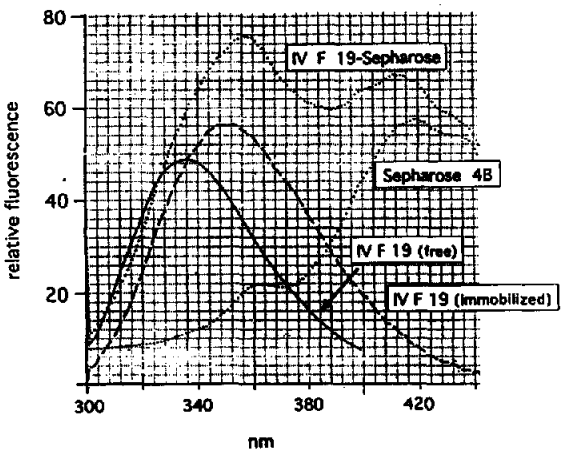

Fig. 2. Fluorescence spectra of free and immobilized mAB IV F 19 (excitation at 280 nm, excitation slit width 10 nm and emission slit width 5 nm) in phosphate buffer of pH 7.0 (I = 0.2 M) at 25°C. No change in the fluorescence emission maximum of the immobilized mABs was observed after 20 adsorption–desorption cycles.

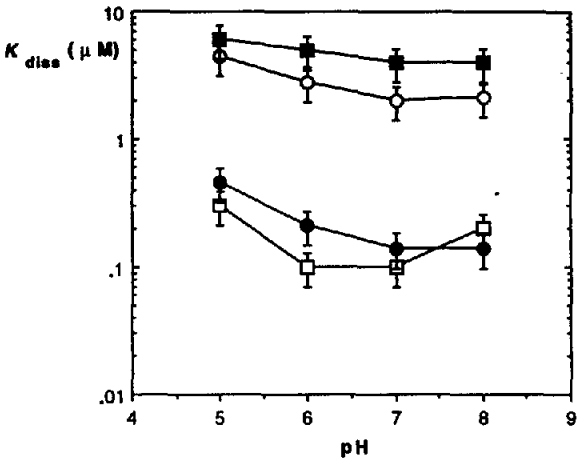

Fig. 3. pH dependence of the dissociation constant for the interaction of different mABs with penicillin amidase $(PA)_{7.0}$ at 25°C. The ionic strength in the buffers was 0.2 M. ■ = IV J 2; ○ = III E 1; ● = IV F 19; □ = III F 14.

TABLE II

FRACTION OF mABs USED FOR ADSORPTION FROM CAPACITY DETERMINATIONS USING EQUILIBRIUM BINDING AND CHROMATOGRAPHIC DATA WITH DIFFERENT SUPPORTS AND ANTIBODY DENSITIES

Antibody	Support	mAB immobilized (μM/l wet support)	Fraction of mABs used for adsorption in % of theoretical value (2 mol antigen/mol mAB)	
			From equilibrium data	From chromatography
IV F 19	Sepharose	10	>90	
		32	20	17 (0.006)[b]
	Knauer Quick-Disc	10	n.d.[a]	5 (0.01)[b]
III E 1	Sepharose	15	60	
III F 14	Sepharose	11	80	
IV J 2	Sepharose	30	30	

[a] Not determined.
[b] Interstitial flow-rate (cm/s) in parentheses.

and low isoelectric points, whereas the fraction of immobilized antibodies at similar mAB densities in the support that can bind the enzyme is equal, within experimental error.

Only the mABs whose K_{diss} have a marked pH dependence can be used for the desorption of adsorbed enzyme by changing the pH within the pH stability range of the enzyme (4–9). For quantitative adsorption, the distribution coefficient that to a first approximation equals n/K_{diss} should exceed 10. From Table II and Fig. 3, it follows that this applies only for the two mAB with the lowest K_{diss} value.

Analytical bioaffinity chromatography and plate height determinations

Samples of pure $PA_{7.0}$ or $PA_{6.7}$ were separated with the different biospecific supports under isocratic conditions. Of the four mABs tested, only IV F 19 was found to be suitable for the preparative isolation of PA within its pH stability range (4–9). The enzyme adsorbed to III F 14 could not be desorbed within this range. The other mABs had too large K_{diss} values to allow a good separation from the impurities under isocratic conditions. The elution volumes for $PA_{7.0}$ and $PA_{6.7}$ for the supports with the same mAB were equal, within experimental error, indicating a small difference in their K_{diss} values. For all supports where the active enzyme could be desorbed, more than 90% of the applied protein and enzyme activity was recovered in the eluates. From the elution curves of the separations of $PA_{7.0}$ the plate heights of the different biospecific adsorbents were determined under isocratic conditions in the pH range 4–5. Their dependences on the interstitial flow-rate are shown in Fig. 4, where they are compared with published data for other biospecific adsorbents [2,17,18]. The new data for the low-pressure, wide-pore supports Sepharose and Eupergit C 250 L are within the experimental error. They are approximately an order of magnitude better than older data obtained with Sepharose [17]. This may be due to improved methods of preparing this support. The lowest plate heights were observed for the perfusible support Knauer Quick-Disc. They are similar to the data published for other perfusible supports [19,20]. The plate heights lie on the part of the Van Deemter curve that is mainly influenced by the diffusion (coefficient and/or distance) from the mobile phase to the binding sites of these supports. The data in Fig. 4 can be explained by the larger pore

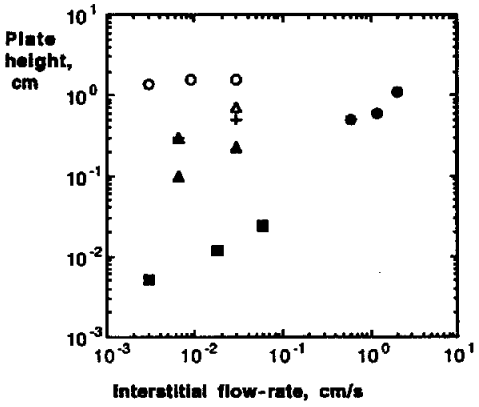

Fig. 4. Plate height as a function of interstitial flow-rate in isocratic analytical biospecific affinity chromatograph of PA using different supports and monoclonal antibodies. ○ = STI–Sepharose; ● = STI–Lichrospher. Supports with monoclonal antibodies IV F 19 and III E 1: △ = IV F19–Sepharose; + = IV F 19–Eupergit 250; ■ = IV F 19–Knauer Quick-Disc; ▲ = III E 1–Eupergit 250. The Knauer support is a macroporous perfusible disc (radius 50 mm, height 4 mm) [10]. They are compared with published data for other ligate (STI soybean trypsin inhibitor)–ligand (α-chymotrypsin, M_r = 25 000) systems immobilized on Sepharose and Lichrosphere (radius 10 μm) [17,18].

sizes or smaller diffusion distances compared with the non-perfusible supports of similar particle size.

Preparative isolation of PA

In Fig. 5 the isolation of PA from a crude enzyme preparation with IV F 19–Sepharose is shown. There was virtually no change in the amount isolated between the first and tenth isolations, indicating that the biospecific adsorbent has a high stability. The same was also observed for the mABs immobilized on the perfusible support Knauer Quick-Disc. The isolated PA is a mixture of different PA forms formed by proteolytic processing, as shown by the IEF immunoblot and zymogram in Fig. 5. They could not be separated by the biospecific adsorbents used here. That different enzyme forms along a processing chain or modified enzymes with similar K_{diss} values can be separated by bioaffinity chromatography has been shown previously for α-chymotrypsin and α- and β-trypsin [2,21]. The resolution in these separations depends on the plate height and differences in K_{diss}. These and the data from the analytical chromatography indicate that for the PA forms the difference in K_{diss} and the plate height do not allow their separation with the biospecific adsorbent used. The dissociation rate constant k_{diss} and the resolution are expected to increase when a less specific interaction, such as in ion-exchange chromatography, is used to separate the enzyme forms with different p*I*. This applies also for PA. The different forms, especially the dominating forms with p*I* 7.0 and 6.7 ($PA_{7.0}$ and $PA_{6.7}$) can be separated by ion-exchange chromatography [3]. $PA_{7.0}$ form can also be processed to $PA_{6.7}$, either autocatalytically or by immobilized α-chymotrypsin [3].

The operational capacities of the supports were determined from breakthrough curves at enzyme concentrations $>K_{diss}$. The results are shown in Table II. The fraction of immobilized mABs that can bind PA was found to depend on their concentration (Table II). In the equilibrium binding experiments this is expected to decrease when the binding of one ligand sterically hinders the binding to adjacent immobilized mABs. The supports used here have a surface area of *ca.* 10 m^2/ml. At ligate densities of 6 μM (ca. 1 mg/ml) and 30 μM (*ca.* 5 mg/ml) the average distance between adjacent ligate molecules is about 50 and 20 nm, respectively. For molecules of the size studied here (radius *ca.* 5 nm), a decrease in the fraction of immobilized ligates that can bind ligand molecules is expected to occur in this concentration range. Similar conclusions have been derived in other studies [4–6]. This also gives an upper limit for the static capacity for preparative supports with biospecific supports with a surface area of 10 m^2/ml for molecules of the size studied here; it is of the order of 1 mg/ml. In the dynamic adsorption step in preparative chromatography, this may be decreased owing to steric hindrance for diffusion in the pores that reduces the rate of adsorption. This can be also be studied directly with the fluorescence measuring device for adsorbents used here for the determination of the fluorescence spectra of immobilized mABs [9,15]. For the perfusible support Knauer Quick-Disc a much smaller fraction of the immobilized mAB could be used to adsorb PA than for Sepharose. This may be

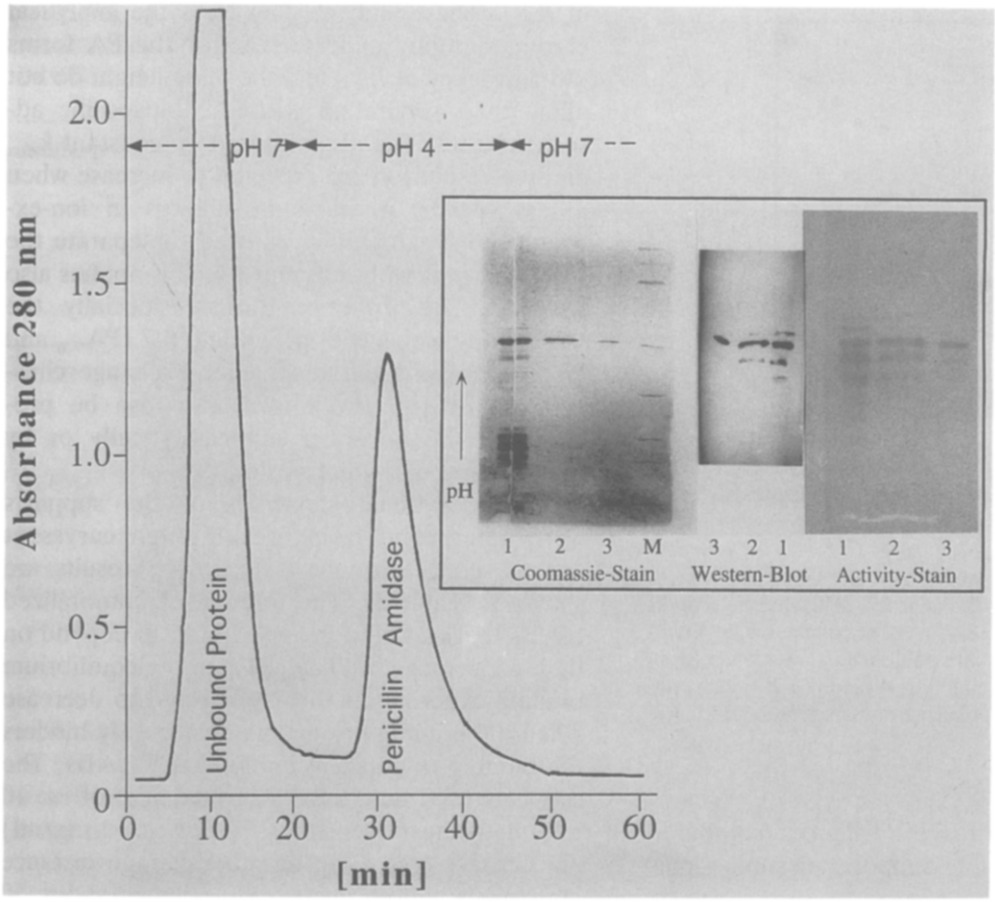

Fig. 5. Preparative PA isolation with the mAB IV F 19 immobilized on Sepharose. Elution curve for a 0.5-ml sample of crude enzyme (*ca.* 20 mg PA/ml; total protein content *ca.* 80 mg/ml). When the impurities had been eluted the pH of the buffer was changed to 4.0 to desorb the adsorbed enzyme. No enzyme activity was observed in the first peak of these diagrams. Inset: isoelectric focusing pattern as protein stain (Coomassie Brilliant Blue), activity stain (M = marker proteins) and Western blot using IV F 19 mAB for (1) crude enzyme, (2) the adsorbed peak in the above elution diagram and (3) $PA_{7.0}$ purified by ion-exchange chromatography.

partly because the liquid flow is not distributed over the whole support [10].

CONCLUSIONS

Of four mABs against PA from *E. coli*, an enzyme without S–S bridges and a pH stability range of 4–9, only one (IV F 19) could be used for the preparative biospecific isolation of the enzyme from homogenates. Owing to steric hindrance, the maximum capacity of the biospecific adsorbents was found to be *ca.* 1 mg/ml support for PA (M_r 88 000).

In contrast to globular enzymes, the fluorescence emission maximum was found to change from 336 to ≥350 nm when the mABs were immobilized in different supports. This indicates partial unfolding of the protein. In contrast to the enzymes, this does not lead to a loss of the biological function, *i.e.*, to binding of the antigen.

The plate heights for the biospecific adsorbents with immobilized mABs were an order of magnitude lower for the perfusible support Knauer Quick-Disc than for the non-perfusible spherical porous supports (Eupergit C 250 L and Sepharose).

ACKNOWLEDGEMENTS

This work was supported by the Bundesministerium für Forschung und Technologie (0319348A) and partly performed within the Graduiertenkolleg Biotechnologie funded by DFG (Ka 505/5-1).

REFERENCES

1 I.M. Chaiken, M. Wilchek and I. Parikh, *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983.
2 V. Kasche and B. Galunsky, in T. Gribneau and J. Visser (Editors), *Affinity Chromatography IV*, Elsevier, Amsterdam, 1982, pp. 93–110.
3 V. Kasche, U. Haufler, D. Markowsky, S. Melnyk and A. Zeich, *Ann. N.Y. Acad. Sci.*, 501 (1987) 97–102.
4 G. Fleminger, E. Hadas, T. Wolf and B. Solomon, *Appl. Biochem. Biophys.*, 23 (1990) 123–137.
5 M. Nachmann, *J. Chromatogr.*, 597 (1992) 167–172.
6 W. Schramm and S.H. Paek, *Anal. Biochem.*, 205 (1992) 47–56.
7 D. Gabel and V. Kasche, *Acta Chem. Scand.*, 27 (1973) 1971–1981.
8 P. Oroszlan, R. Blanco, X.-M. Lu, D. Yarmush and B.L. Karger, *J. Chromatogr.*, 500 (1990) 481–493.
9 V. Kasche, E. Renken, G. Schietke, R. Ulrich, R. Bücke, H. Gnewuch and K. Jansen, in S. Scheller and R. Schmid (Editors), *Biosensors, Fundamentals, Technologies and Applications*, VCH, Weinheim, 1992, pp. 262–274.
10 H. Abouh-Rebyeh, F. Körber, K. Schubert-Rehberg, J. Reusch and D.J. Josic, *J. Chromatogr.*, 566 (1991) 341–350.
11 A. Bohmann, R. Pörtner, J. Schmieding, V. Kasche and H. Märkl, *Cytotechnology*, 9 (1992) 51–57.
12 E. Harlow, E. and D. Lane, *Antibodies—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.
13 J.R. Little and H. Donahue, in C.A. Williams and M.A. Chase (Editors), *Methods in Immunology and Immunochemistry*, Vol. 2, Academic Press, New York, 1967, p. 343.
14 U.K. Laemmli, *Nature*, 227 (1970) 680.
15 E. Renken, *Thesis*, Technische Universität Hamburg-Harburg, Hamburg, 1993.
16 D.A. Deranleau, *J. Am. Chem. Soc.*, 91 (1969) 4044–4049.
17 V. Kasche, *Acta Univ. Ups.*, 2 (1971) 1–135.
18 V. Kasche, K. Buchholz and B. Galunsky, *J. Chromatogr.*, 216 (1981) 169–174.
19 N.B. Afeyan, S.P. Fulton and F.E. Regnier, *LC·GC Int.*, 4, No. 12 (1991) 14–19.
20 K. Mosbach, personal communication, 1992.
21 V. Kasche and I. Büchtmann, *Hoppe Seyler's Z. Physiol. Chem.*, 362 (1981) 1007–1012.