*Journal of Solid-Phase Biochemistry, Vol. 3, No. 4, 1978*

# APPLICATION OF THE FCP-ACTIVATION PROCEDURE TO THE SYNTHESIS OF A BIOSPECIFIC ADSORBENT FOR TRYPSIN[1]

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Accepted November 21, 1978

The synthesis of a biospecific adsorbent for trypsin was chosen as a model to investigate the applicability of FCP activation in affinity chromatography. *p*-Aminobenzamidine was chosen as a ligand, directly suitable for immobilization. The nonspecific binding properties of the first series of synthesized agarose derivatives were obviated either by FCP activation of the ligand instead of the matrix, or by modifying the initial FCP-activation procedure. The adsorbents prepared in this way, however, demonstrated no selectivity between trypsin and chymotrypsin. The introduction of $\varepsilon$-aminocaproic acid as a spacer was ineffectual. These problems were solved by the application of glycylglycine as a spacer. The final affinity matrices had a degree of substitution of approximately 4 μmol of ligand per gram gel (100 μmol ligand per gram dry adsorbent). The specific activity of a current trypsin preparation was increased by 58% in a single cycle. The biospecificity of these adsorbents was demonstrated.

## INTRODUCTION

The development of a procedure for activation of polysaccharides with 2,4,6-trifluoro-5-chloropyrimidine (FCP) as an alternative to the currently used CNBr activation (1) in the synthesis of affinity adsorbents has been described in previous publications (2–4). The ligand–polymer conjugates, prepared by this procedure, demonstrate a highly improved stability with respect to hydrolytic ligand detachment. In addition, in the case of agarose (Sepharose 4B), the matrix itself showed an improved thermal stability, due to partial cross-linking by FCP.

The synthesis of a biospecific adsorbent for trypsin was chosen as a model to investigate the applicability of the FCP-activation procedure to ligand immobilization in affinity chromatography.

[1]This paper has been dedicated to Prof. Dr. E. Havinga on the occasion of his seventieth birthday and his retirement from the chair of Organic Chemistry at the University of Leiden, The Netherlands.

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0146-0641/78/1200-0271$03.00/0 

*Biospecific Adsorbents for Trypsin and Chymotrypsin*

Several affinity matrices for trypsin, based on high- or low-molecular-weight inhibitors as ligands and with or without the application of spacers, have been described in the literature. Feinstein (5) used chicken ovomucoid, a protein from egg white capable of inhibiting trypsin but without effect on chymotrypsin, coupled to CNBr-activated Sepharose for the purification of trypsin. The adsorbent bound trypsin, but not chymotrypsin, at pH 8.1 and the enzyme was finally eluted at pH 2.0. Trypsin and chymotrypsin were both bound by immobilized turkey ovomucoid and soybean trypsin inhibitor at pH 8.1, and were both again released at pH 2.0 (6). Robinson et al. (7) also reported the use of chicken ovomucoid covalently coupled to agarose. They were able to separate $\alpha$- and $\beta$-trypsin by the application of a pH gradient (4.50–2.75) during the elution procedure; chymotrypsin again was not retarded by this column. Light and Liepnieks (8) activated bovine trypsinogen with enterokinase in the presence of an excess of Sepharose-bound soybean trypsin inhibitor. Elution of the agarose with a pH gradient yielded two separate peaks for $\alpha$- and $\beta$-trypsin. Immobilized soybean trypsin inhibitor was used by Porath and Kristiansen (9) for the separation of trypsin from chymotrypsin, in spite of the affinity of this ligand for both enzymes. They found a different pH dependence for the binding of trypsin and chymotrypsin to the inhibitor. Chymotrypsin was eluted between pH 5.5 and 3.5, whereas trypsin was released in the pH interval 3.3–2.5; specific elution with tryptamine and benzamidine, respectively, at constant pH and ionic strength was also performed. Johnson and Travis (10) used a similar pH-gradient elution during the purification of human trypsin and chymotrypsin on Sepharose-bound Trasylol [kallikrein-trypsin inhibitor from bovine lung, identical with the bovine pancreatic trypsin inhibitor described by Kunitz and Northrop (11)].

These high-molecular-weight polypeptide inhibitors are very stable to denaturation and to the action of proteolytic enzymes because of their compact tertiary structure, resulting from a high content of disulphide bridges. They only rarely demonstrate, however, an exclusive specificity for trypsin (12). Furthermore, only few of the naturally occurring inhibitors have been well-identified chemically, and the point(s) of attachment to the support are uncertain due to the random coupling. The affinity matrix will, therefore, have a heterogeneous character. The heterogeneity of the ligand material itself, resulting in rather poorly reproducible separations with immobilized ligands from different batches, presents an additional problem (13).

A different approach has been described by Kasai and Ishii (14,15) and Yokosawa et al. (16). They prepared an affinity matrix for trypsin by the

immobilization of a mixture of oligopeptides (mainly dipeptides and tripeptides) containing L-arginine as the carboxyl terminus. The mixture was obtained by tryptic digestion of protamine, a highly basic protein from fish sperm. Trypsin was bound by the adsorbent in the range pH 7.3–5.0; chymotrypsin was only slightly retarded. Trypsin was released by elution with 5 mM HCl or 10 mM benzamidine (pH 7.3–5.0). One of the components of the protamine digest, viz. Gly-Gly-Arg, has also been used as a synthetic ligand (17–19). However, a decreased affinity, compared to the immobilized oligopeptide mixture, was found. Van Muijen (19) observed no affinity at all. The ligand Gly-Ala-Arg was found to yield better results (20).

Low-molecular-weight synthetic inhibitors of trypsin must combine a hydrophobic region with a positively charged group (12,21). A great number of compounds which meet these structural requirements has been developed. Benzamidine, *p*-aminobenzamidine, and phenyl-guanidine were found to be highly potent, competitive inhibitors of trypsin (22), having inhibition constants ($K_i$) of 18.4, 8.25, and 72.5 μM, respectively. Several other benzamidines appeared equally useful (23–25).

Markwardt et al. (25) and Mares-Guia et al. (26) investigated a number of benzamidine derivatives and observed a decreasing inhibitory capacity in going from electron-donating to electron-withdrawing *p*-substituents. Mares-Guia et al. (26) reported a 27-fold decrease for *p*-nitrobenzamidine compared with the *p*-amino compound ($K_i$: 227 and 8.25 μM, respectively). This phenomenon was ascribed to an enzyme-inhibitor interaction of the dipole–dipole type; correlations with acid–base properties, charge densities on the amidinium function, or charge-transfer properties were ruled out on the basis of experimental results.

The inhibitors, *p*- and *m*-aminobenzamidine, are directly suitable for immobilization through the free, aromatic amino function. Several workers have reported the use of these inhibitors as ligands during the affinity chromatographic purification of trypsin; polymethylene chains of varying lengths were applied as spacers. The structures of the different affinity matrices are represented in Fig. 1. Jameson and Elmore (28), using *p*-(*p'*-aminophenoxypropoxy)-benzamidine as a ligand, were able to separate α- and β-trypsin. Trypsin-like proteases have also been isolated by means of immobilized benzamidine derivatives; for example, acrosin (29), thrombin (24,27), and kallikrein (30).

The sample of trypsin was applied to the column, which had been previously equilibrated with an appropriate buffer solution, pH 8.0 or 5.5; the same buffer was used to elute the impurities. Release of the purified trypsin was generally performed by the application of a more acidic buffer (pH 2.0–2.5). The biospecificity of the affinity matrices was demonstrated by the unretarded passage of chymotrypsin and chymotrypsinogen at the pH of

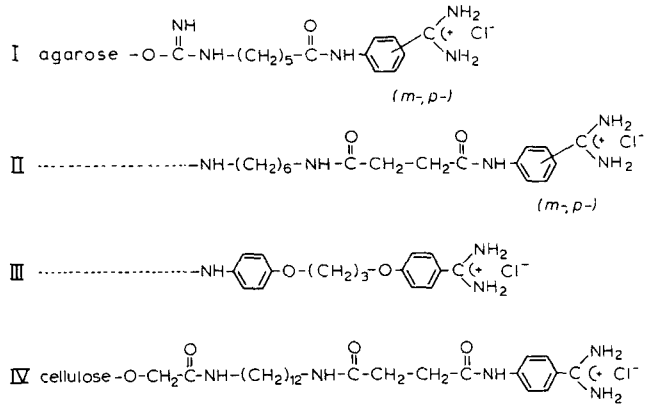



FIG. 1. Affinity matrices for trypsin. I, II, Hixson and Nishikawa (24,27); III, Jameson and Elmore (28); IV, Brümmer (31).

the starting buffer, and by the release of trypsin after the addition of 10 mM benzamidine to the starting buffer (for references, see Fig. 1).

Another method for the preparation of chymotrypsin-free trypsin is the use of a matrix which specifically binds chymotrypsin, but leaves trypsin unretarded. Cuatrecasas et al. (32) have described the use of the enantiomeric substrate analogue D-tryptophan methyl ester as a ligand in combination with $\varepsilon$-aminocaproic acid as a spacer. 4-Phenylbutylamine, coupled directly to CNBr-Sepharose, has been used by Stevenson and Landman (33). Control experiments demonstrated the nonretarded passage of chymotrypsinogen, trypsin, and diisopropyl fluorophosphate-treated chymotrypsin. Tomlinson et al. (34), however, observed a "marked variation" in specificity with both of the above affinity matrices. The biospecificity of Sepharose-*N*-$\varepsilon$-aminocaproyl-D-Trp-OMe has also been questioned by Sharma and Hopkins (35). They observed that acetylated chymotrypsin, in spite of its fully retained enzymatic activity, was not bound by the affinity support at pH 8; this is in contrast to the native enzyme.

Turková et al. (36) have described the specific adsorption of chymotrypsin by hydroxyalkyl methacrylate gels, substituted with *N*-benzyloxycarbonylglycyl-D-leucine or *N*-benzyloxycarbonylglycyl-D-phenylalanine, and with 1,6-diaminohexane as a spacer. Trypsin was not adsorbed to this gel, whereas chymotrypsin was not bound by the gel, substituted with the spacer only. *N*-benzyloxycarbonyl-D, L-phenylalanyl-triethylenetetramine-Sepharose was applied to the separation of chymotrypsin, subtilisin, pepsin, and neutral metalloendopeptidases; these enzymes possessed a different affinity, depending upon the pH of the

medium (37). Chymotrypsinogen, trypsinogen, trypsin, and the formerly mentioned alkali-denatured enzymes were all found to pass through the adsorbent column.

The successive use of a chymotrypsin-specific adsorbent (4-phenylbutylamine-Sepharose) and a trypsin-specific adsorbent (*p*-aminobenzamidine-cellulose), for the preparation of highly purified trypsin has been reported by Jany et al. (38).

## EXPERIMENTAL SECTION

### *Materials*

Sepharose 4B was purchased from Pharmacia, and was checked by microscopic investigation before use (2,39). 2,4,6-Trifluoro-5-chloropyrimidine (FCP) was a gift from Bayer AG, Leverkusen, W. Germany. *p*-Aminobenzamidine dihydrochloride was synthesized according to Shaw and Woolley (40), but became commercially available during the investigations (Merck AG, Darmstadt, W. Germany). All other chemicals used were of analytical-grade quality (Merck), except 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, which was obtained from Aldrich-Europe (Janssen Pharmaceutica, Beerse, Belgium). Affi-Gel 10 was obtained from Bio-Rad Laboratories (Richmond, Calif., U.S.A.).

The enzymes trypsin (from bovine pancreas), chymotrypsin $A_4$ ($\alpha$-chymotrypsin; from bovine pancreas), and pepsin (from porcine gastric mucosa) were products of Boehringer Mannheim GmbH (Mannheim, W. Germany). Human serum albumin and cytochrome *c* were obtained from Behringwerke AG (Marburg-Lahn, W. Germany) and Sigma (St. Louis, Mo., U.S.A.), respectively.

All the chromatographic procedures were performed in a cold room (2–4°C). The column eluates were monitored with LKB Uvicord I or III instruments.

The following buffers were used as eluents: pH 10.0 (0.1 M ethanolamine/HCl), pH 8.0 (0.1 M Tris/HCl), pH 5.5/5.4/4.0 (0.1 M acetic acid/sodium acetate), pH 2.0 (0.1 M glycine/HCl); all buffers contained in addition 0.1 M NaCl and 0.001 M $CaCl_2$.

### *Methods*

The relevant experimental details of the preparation of various adsorbents which appeared unsuitable for the biospecific adsorption of trypsin are briefly mentioned in the following section, and will not be discussed further.

*FCP Activation of Sepharose 4B (Adsorbents IX, X).* FCP activation was performed according to the procedure, previously described as method C (2,4), and starting with approximately 25 g of standard dry Sepharose 4B (washed with 250 ml of 0.27 M $Na_2CO_3$) and 1 g of FCP.

Elemental analyses:

| | | | |
|---|---|---|---|
| FCP-Sepharose (for IX) | %N 0.55 | %F 0.4 | %Cl 0.6 |
| FCP-Sepharose (for X) | %N 0.6 | %F 0.5 | %Cl 0.55 |

*FCP-Sepharose/Gly-Gly/*p*-Aminobenzamidine (Adsorbents IX, X).* (IX) FCP-activated Sepharose 4B (sucked dry by vacuum, 4 g) was suspended in a solution of glycylglycine (0.79 g) in buffer, pH 10.6 (0.5 M $Na_2CO_3/NaHCO_3$; 5 ml) and the suspension was rotated for 27 h at room temperature. The product was filtered off, washed with double-distilled water (250 ml), and suspended in a solution of *p*-aminobenzamidine dihydrochloride (0.62 g, 3 mmol) in water; the pH of the suspension was adjusted to 5.0 and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.57 g, 3 mmol) was then added. The reaction was allowed to proceed for 2 h at room temperature and at pH 5. The product was then filtered off, washed with double-distilled water, aqueous NaCl (0.5 M, 100 ml) and again with double-distilled water (250 ml). The agarose derivative was stored, suspended in a 0.05% aqueous sodium azide solution, at 4°C.

(X) FCP-activated Sepharose 4B (sucked dry by vacuum, 11 g) was reacted with glycylglycine (0.74 g) and then with *p*-aminobenzamidine dihydrochloride (1.25 g, 6 mmol; carbodiimide: 1.15 g, 6 mmol), as described for (IX). The nitrogen contents of the Gly-Gly-substituted support and of the final affinity adsorbent were determined by elemental analysis and found to be 0.8% and 1.3%, respectively. Calculations yielded the following empirical formulas: $(ag)_1(FCP)_{0.06}(GlyGly)_{0.03}$ and $(ag)_1(FCP)_{0.06}(GlyGly)_{0.03}$(*p*-aminobenzamidine · $HCl)_{0.035}$ or 108 μmol of ligand/g of dry affinity gel.

*Determination of Enzyme Activities.* Tryptic and chymotryptic activities were determined with the corresponding "Biochemica Test Combination" of Boehringer (*N*-benzoyl-arginine-*p*-nitroanilide as substrate for trypsin and *N*-carboxy-propionyl-phenylalanine-*p*-nitroanilide as substrate for chymotrypsin).

## STRATEGIES, RESULTS, AND DISCUSSION

In view of the literature data, *p*-aminobenzamidine was used as a ligand in the preparation of trypsin-specific adsorbents. The structures and ligand

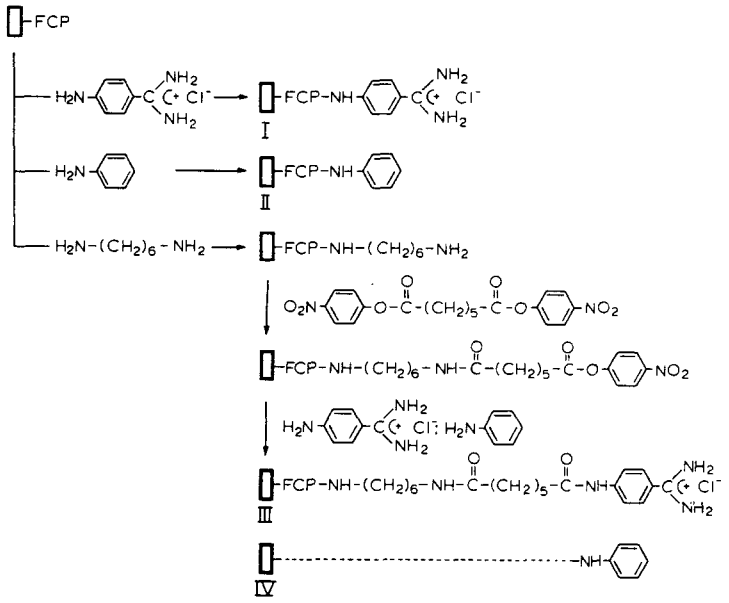


FIG. 2. First series of *p*-aminobenzamidine/FCP-Sepharose derivatives; ligand contents: 86 (I), 87(II), 3.9 (III), and 2.2 (IV) μmol per g wet gel.

contents of the first series of agarose derivatives are presented in Fig. 2. Sepharose 4B was activated with FCP according to method A (2–4) and then coupled with *p*-aminobenzamidine or aniline at pH 7.5; the latter compound was coupled in order to obtain a control matrix. FCP-Sepharose was also substituted with hexamethylenediamine, and the resulting product was reacted successively with the di-*p*-nitrophenyl ester of pimelic acid, and *p*-aminobenzamidine or aniline, to introduce a long spacer arm between the matrix and the ligands. All matrices were reacted with an excess of ethanolamine, after the ligand coupling, to eliminate residual reactive fluorine. The ligand contents were calculated from spectrophotometric determinations of the free ligand, remaining in solution after the coupling (I, II), or of the liberated *p*-nitrophenol (III, IV).

The chromatographic performance of adsorbent III, with respect to trypsin, chymotrypsin, and human serum albumin is depicted in Fig. 3. The proteins were applied to a column, which had been previously equilibrated with acetate buffer, pH 5.5. Following the elution of nonadsorbed material the eluent was changed to HCl/glycine buffer, pH 2.0, but only very small amounts of protein were released. Comparison with the elution pattern of the same amount of trypsin applied to an unsubstituted Sepharose column

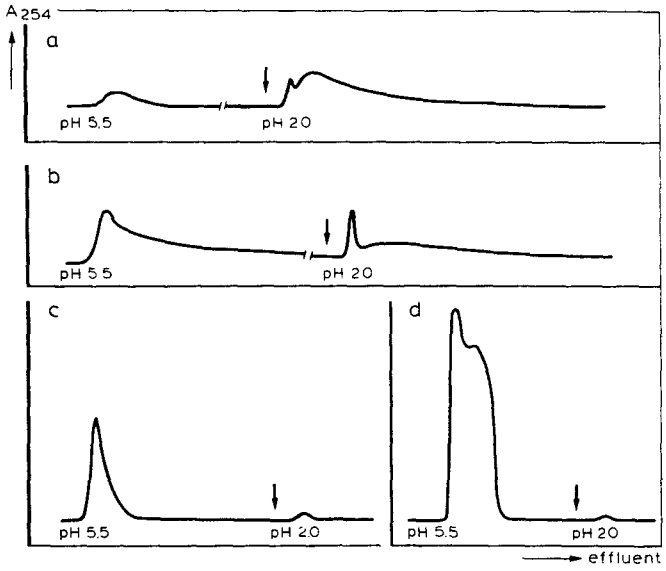



FIG. 3. Elution profiles of (a) trypsin, (b) chymotrypsin, (c) human serum albumin, on adsorbent III; (d) trypsin, on unsubstituted Sepharose 4B. Bed volumes were 1.6 ml, and the flow rate was 20 ml/h. Samples (10 mg) of each protein were applied to fresh columns.

revealed that almost all of the enzyme had remained adsorbed to the affinity support III, in spite of the change in elution conditions. The matrices I, II, and IV demonstrated a similar behavior, as did a sample of the corresponding FCP-Sepharose after deliberate, alkaline hydrolysis. Also, the proteins could not be eluted by an aqueous 1 M KCl/25% ethylene glycol solution, or by aqueous 7 M urea.

The applicability of the ligand was reinvestigated by using Affi-Gel 10 as the reactive, immobilizing support material (Fig. 4). The elution patterns of trypsin, chymotrypsin, and human serum albumin are represented in Fig. 5. The profiles were as expected; part of the applied trypsin was adsorbed at

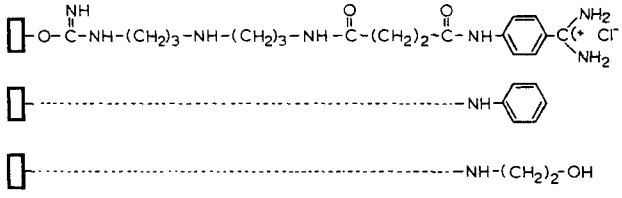



FIG. 4. Adsorbents based on Affi-Gel 10 [Bio-Rad; Cuatrecasas and Parikh (41)].

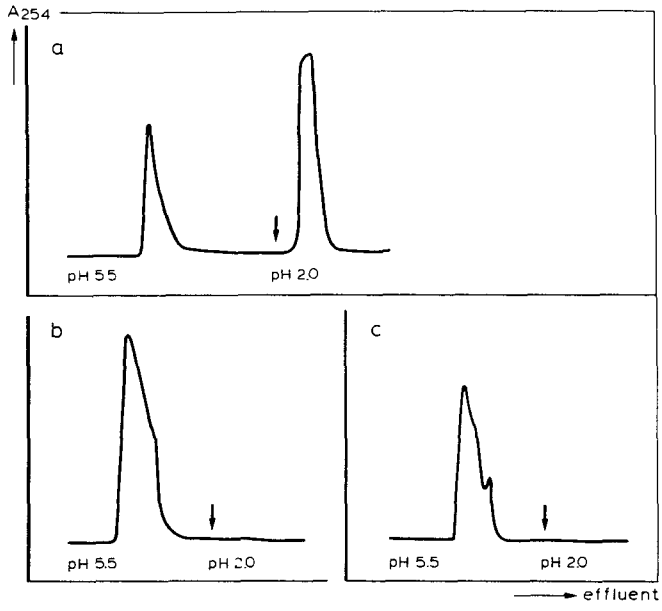



FIG. 5. Elution profiles of (a) trypsin, (b) chymotrypsin, (c) human serum albumin, on Affi-Gel/*p*-aminobenzamidine. Bed volumes were 1.6 ml, and the flow rate was 20 ml/h. Samples (10 mg) of each protein were applied to fresh columns.

pH 5.5, and subsequently released at pH 2.0, whereas chymotrypsin and albumin were not retarded by the adsorbent. The aniline-substituted matrix did not bind albumin, whereas only a small fraction of the trypsin sample was bound; chymotrypsin was considerably retarded (Fig. 6). These results are understandable in view of the described properties of the enzymes (12,21,42,43). The ethanolamine-treated matrix retarded none of the applied proteins.

Returning to the FCP-based gels, we wondered what type of nonspecific bonding was involved that could not be reversed by salt, polarity-reducing agents, or denaturing agents. Small columns of deliberately hydrolyzed FCP-Sepharose were equilibrated with buffer (pH 5.5) and the chromatographic behavior of proteins with different isoelectric points was investigated (Fig. 7). The basic proteins trypsin and cytochrome *c* were released only at a pH value near or above the isoelectric point; the highly acidic protein pepsin was also found to pass unretarded at a pH value above its isoelectric pH. These phenomena, in combination with the apparently acidic character of hydrolyzed FCP-Sepharose (2–4), indicate

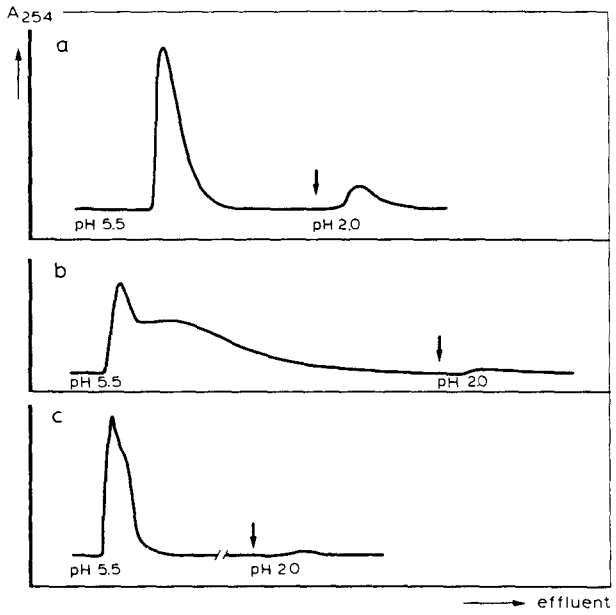



FIG. 6. Elution profiles of (a) trypsin, (b) chymotrypsin, (c) human serum albumin on Affi-Gel/aniline. Conditions as in Fig. 5.

that ionic effects play a role.[3] The behavior of albumin and the inability of salt alone to affect the binding of the proteins, however, remain to be explained. The latter may be understood in view of "salting-in/salting-out" phenomena (46,47).

The presence of an excess of hydroxy-chloropyrimidinyl residues in the final affinity matrix was avoided by inverting the coupling procedure. The ligand was activated with FCP, and subsequently coupled to Sepharose 4B (adsorbent V, Fig. 8). The adsorbent was tested with trypsin, and demonstrated the expected behavior: the enzyme was bound at pH 5.5 and released at pH 2.0 (Fig. 9). The initial binding was improved by changing the pH of the starting buffer to 8.0, which is nearer to the optimum pH of catalysis (12,15). Nonspecific side effects were again investigated by means of cytochrome *c*, which had already been found to be a suitable test protein due to its similar isoelectric point as compared with trypsin, and also its clear coloration. This protein did not bind to the column, indicating the absence of

[3]Lang et al. reported during the preparation of this communication a similar phenomenon in the desorption of $\alpha$-chymotrypsin from an affinity matrix prepared by coupling D-tryptophan methyl ester to $\varepsilon$-aminocaproic acid substituted, cyanuric chloride-activated agarose. [Lang, T., Suckling, C. J., and Wood, H. C. S. (1977) J. Chem. Soc. Perkin I: 2189].

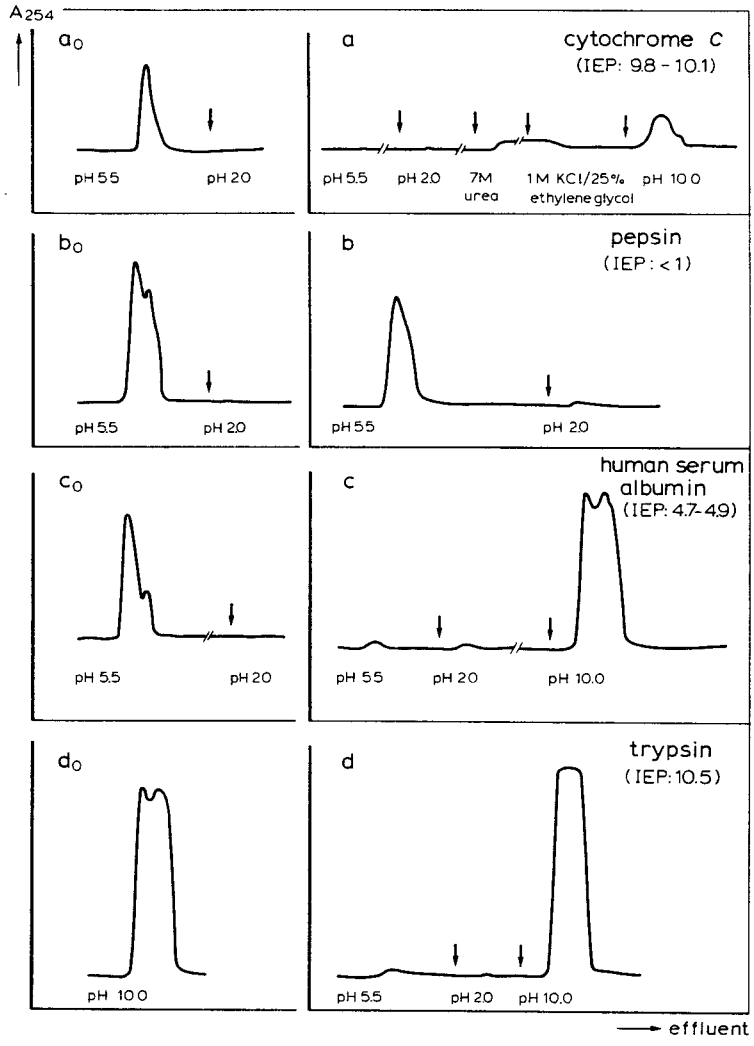
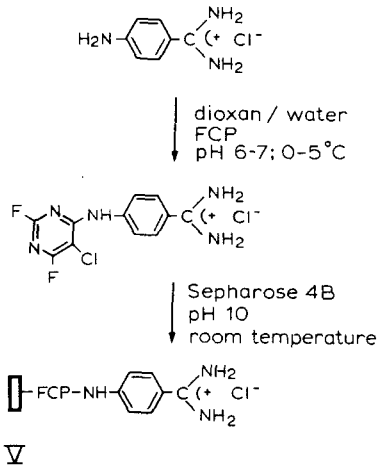



FIG. 7. Elution profiles of proteins with different isoelectric points on hydrolyzed FCP-Sepharose 4B (a–d) and on unsubstituted Sepharose 4B ($a_p$–$d_0$). Bed volumes were 1.6 ml, and the flow rate was 20 ml/h. Samples of the enzymes and of albumin (10 mg) and of cytochrome *c* (1 mg) were applied to fresh columns. The values of the isoelectric points (IEP) are taken from Refs. 44 and 45.




V

FIG. 8. FCP derivatization of *p*-aminobenzamidine [cf. (48) and Bayer, British Patent, 1,169,254 (5.11.1969)], and subsequent coupling to Sepharose 4B.

nonspecific effects observed with the previous adsorbents. Chymotrypsin, however, was also bound to the affinity matrix at pH 8 and again released at pH 2.0.

The lack of selectivity of adsorbent V between trypsin and chymotrypsin may be caused by the accumulation of two aromatic ring systems. Therefore, $\varepsilon$-aminocaproic acid was applied as a spacer between the ligand and the pyrimidinyl residue. Three different strategies were used in the preparation of the final affinity matrix (adsorbents VI–VIII, Fig. 10a). $\varepsilon$-Aminocaproyl-*p*-aminobenzamidine hydrochloride was synthesized according to Fig. 10b; FCP activation of the spacer–ligand conjugate was performed as described for *p*-aminobenzamidine (Fig. 8). FCP-activated Sepharose 4B was prepared according to method C (2–4) to reduce the number of hydroxy-chloropyrimidinyl residues in the product. All three matrices exhibited behavior toward trypsin and chymotrypsin that was similar to that described for adsorbent V. Both enzymes were bound at pH 8.0 and released at pH 2.0, although chymotrypsin appeared to be eluted at a slightly higher pH value than was trypsin (Fig. 11).

The $\varepsilon$-aminocaproyl spacer was replaced by the dipeptide glycylglycine. Sepharose 4B was activated with FCP, according to method C (2–4), and then coupled with Gly-Gly. *p*-Aminobenzamidine was then covalently bound by carbodiimide coupling (adsorbent IX, Fig. 12). Chromatography of trypsin and chymotrypsin on this adsorbent yielded the elution profiles represented in Fig. 13a,b. Trypsin was bound at pH 8.0 and released at

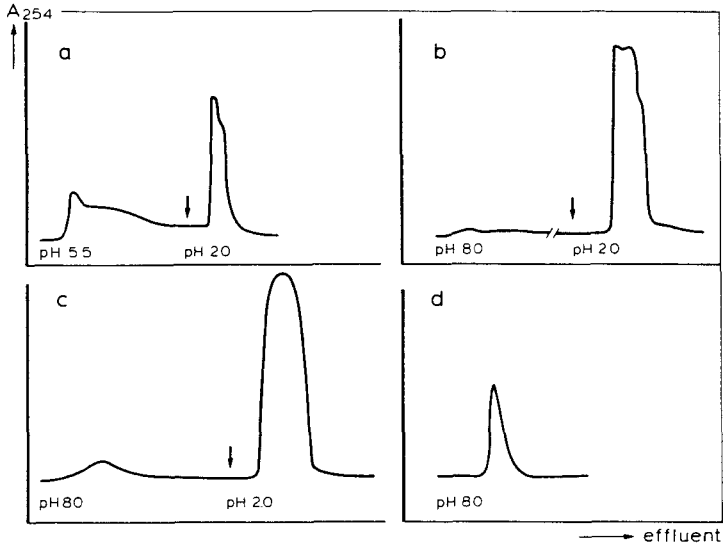



FIG. 9. Elution profiles of (a) and (b) trypsin, (c) chymotrypsin, (d) cytochrome *c*, on adsorbent V. Bed volumes were 1.6 ml, and the flow rate was 20 ml/h. Samples of the enzymes (10 mg) and of cytochrome *c* (1 mg) were applied to fresh columns.

pH 2.0, whereas chymotrypsin passed through the column with slight retardation. The reproducibility of the results was proven, and the system was then investigated more thoroughly by the following experiments:

1. The affinity column was eluted with buffer (pH 10) after a complete chromatographic cycle with trypsin. The adsorbent did not release any additional protein, indicating the absence of nonspecific effects observed with previous adsorbents (I–IV). Performing the FCP activation according to method C instead of A effectively diminishes the simultaneous introduction of hydroxy-chloropyrimidinyl residues.
2. In the case of trypsin, the material eluted at pH 8.0 was subjected to a new chromatographic cycle; the elution profile did not contain any peak at pH 2.0, which indicated that the column had not been "overloaded" with enzyme.
3. Trypsin, bound at pH 8.0, was also released by eluting the column with a benzamidine solution (15 mM, pH 8.0); a subsequent change of the eluent to buffer pH 8.0 (without benzamidine), and then to buffer pH 2.0, no longer yielded a peak. This result confirmed the biospecificity of the adsorbent, which had already been demonstrated by the lack of affinity toward chymotrypsin.

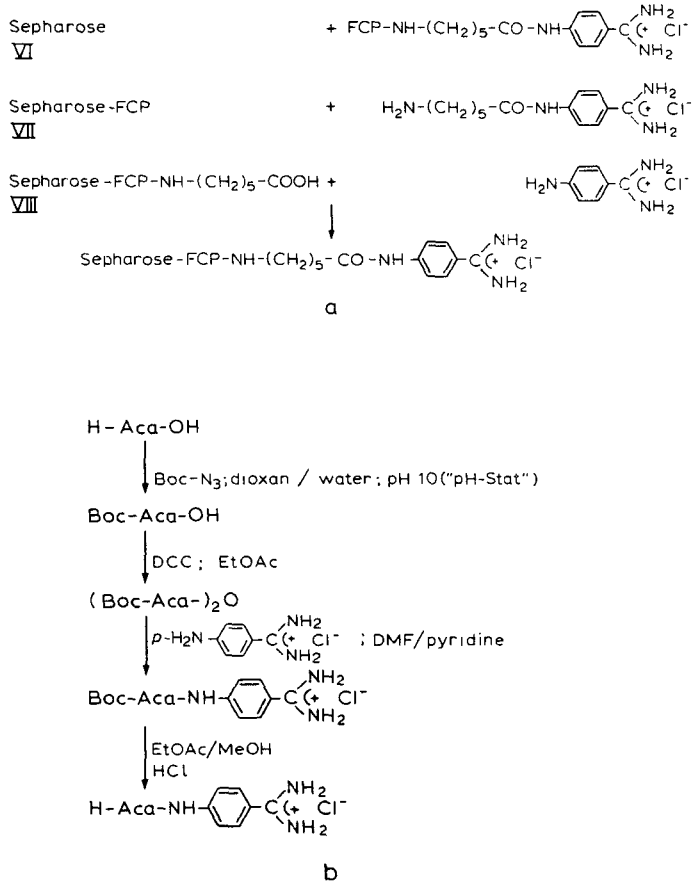



FIG. 10. (a) Introduction of $\varepsilon$-aminocaproic acid as a spacer. (b) Synthesis of $\varepsilon$-aminocaproyl-*p*-aminobenzamidine hydrochloride.

4. The pH of the starting buffer could be decreased to 5.4 without any effect on the elution profile of trypsin; chymotrypsin, however, was eluted as a considerably sharper peak than with buffer at pH 8.0 (cf. Fig. 13d). Operation in a pH range near 5 considerably stabilizes trypsin against autodigestion (49).
5. Determination of the tryptic and chymotryptic activity of the pooled fractions of the nonadsorbed material demonstrated the absence of these two enzymes.
6. Bovine serum albumin was not retarded by the adsorbent at pH 5.4, and subsequent elution with buffers at pH 2.0 and at pH 10.0 did not release

additional material, indicating the absence of nonspecific protein binding.

7. FCP-Sepharose, which was coupled with the spacer only, merely demonstrated ion-exchange properties.

Quantitative data with respect to the purification of trypsin were obtained with adsorbent X, which was prepared as described for IX (degree of substitution, determined by elemental analysis: 108 μmol of *p*-amino-benzamidine per g of dry gel, or approximately 4.3 μmol per g wet gel). The elution profiles are presented in Fig. 13c,d; peaks A and B represent 1.5 and 2.0 mg of protein, respectively, as determined by the Lowry method (50), which means a recovery of 87.5%. The enzymatic activities of the original, commercial trypsin sample and of the protein eluted as peaks A and B were determined with *N*-benzoylarginine-*p*-nitroanilide as substrate. The following values were found: 1.2 U/mg (original trypsin), 0.06 U/mg (A), and 1.9 U/mg (B). One chromatographic cycle resulted in an increase in activity of 58% with a total recovery of activity of 81%. The capacity of the adsorbents IX and X was approximately 10 mg of commercial trypsin per g wet gel.

The degree of purification obtained is at least comparable with the literature data; values of 20–40% have been reported for commercial trypsin (24,27,28,31). Trypsin-binding capacities of 1–3 mg per ml gel and ligand contents of 0.9 μmol per ml gel (0.1 g of CNBr per ml gel) and

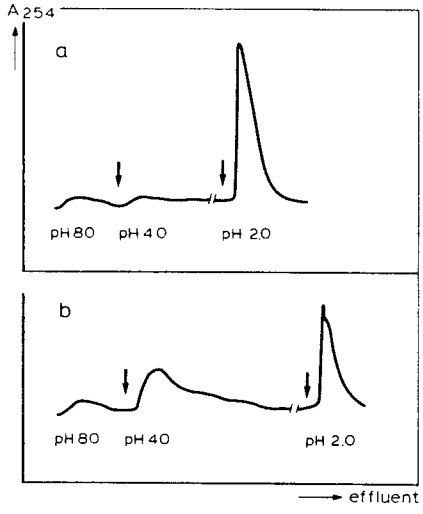
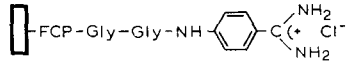



FIG. 11. Elution profiles of (a) trypsin (5 mg), and (b) chymotrypsin (5 mg), on adsorbents VI–VIII. Bed volumes were 0.5 ml, and the flow rate was 20 ml/h.




IX

FIG. 12. Adsorbent IX.

12–30 μmol per ml gel (0.2 g of CNBr per ml gel) have been obtained (24,27,28).

The apparent dependence of the selectivity of the adsorbent on its ligand content was remarkable. Adsorbents IX and X, based on FCP-activated Sepharose with the composition $(ag)_1(FCP)_{0.06}$, resulting in a final ligand content of 108 μmol per g dry gel, demonstrated the expected difference in affinity toward trypsin and chymotrypsin. An affinity matrix, based on FCP-Sepharose: $(ag)_1(FCP)_{0.59}$, bound both enzymes at pH 8.0 and released them at pH 2.0!

In conclusion, it is clear that FCP activation can be used successfully as a coupling method for the preparation of biospecific adsorbents. The high degree of substitution which can be obtained may be advantageous in the application of ligands with a low affinity; a potential decrease in selectivity, due to a high ligand content, should be considered carefully.

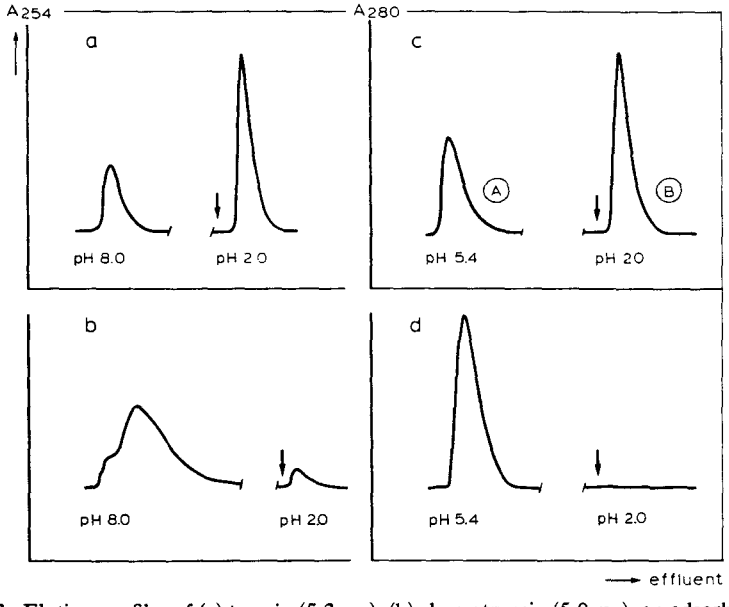



FIG. 13. Elution profiles of (a) trypsin (5.3 mg), (b) chymotrypsin (5.0 mg), on adsorbent IX; (c) trypsin (4.0 mg), (d) chymotrypsin (2.4 mg), on adsorbent X. Bed volumes were 2.7 ml (a, b) and 2.6 ml (c, d), and the flow rate was 20 ml/h.

The hydrophobic, aromatic character of the pyrimidinyl moiety, potentially disadvantageous, did not present a problem at a low degree of activation (comparable with the values obtained with CNBr), and in combination with a rather rigid and hydrophilic spacer such as glycylglycine. The choice of an optimal spacer remains an empirical factor, as is apparent from the described results. An additional example was found in experiments with FCP-Sepharose and Sepharose-FCP-Gly-Gly, both coupled with 4-phenylbutylamine, which has not been described further. Chymotrypsin was irreversibly bound by the former adsorbent, whereas the latter exhibited no affinity whatsoever.

## ACKNOWLEDGMENTS

The investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

We thank Dr. D. Hildebrand and Dr. K. Neufang (Bayer AG, Leverkusen) for their interest and ready cooperation in providing samples of FCP.

Elemental analyses were carried out by the Elemental Analytical Section of the Institute for Organic Chemistry TNO (Utrecht) under supervision of Mr. W. J. Buis.

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