Journal of Chromatography, 317 (1984) 193–200
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 504

COMPARISON OF LOW- AND HIGH-PRESSURE AFFINITY CHROMATOGRAPHY FOR THE PURIFICATION OF SERINE AND SULFHYDRYL ESTERASES

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SUMMARY

A series of affinity chromatography packings for the purification of serine and sulfhydryl esterases (acetylcholinesterase, alkaline phosphatase, urokinase and papain) have been synthesized using commercially available agarose, glass and acrylate parent matrices. Two ligands were coupled to the matrices by utilizing carbodiimide or reaction with active groups already present on the matrix: the quaternary ammonium compound trimethyl(*p*-aminophenyl)ammonium chloride and the serine esterase inhibitor analog *p*-aminobenzamidine. It was found that enzyme purification on the agarose- or acrylate-based packings was most successful, resulting in as much as fifty-fold purification over starting material. The pressure stability of the acrylate packing allowed purification by high-pressure affinity chromatography and decreased purification times as much as six-fold in compoarison to agarose columns.

INTRODUCTION

Esterases and proteases represent a ubiquitous group of hydrolytic enzymes found throughout the plant and animal kingdoms. This group includes serine esterases, such as acetylcholinesterase (AChE), trypsin, chymotrypsin and alkaline phosphatase; and sulfhydryl (cysteine) esterases, such as papain, ficin and bromelain. These enzymes are of considerable importance not only for mechanistic and molecular studies, but also for industrial and commercial applications. Thus, new methodologies for the rapid, cost-effective purification of these enzymes could result in significant expansion of their use in research and industry.

Affinity chromatography has been applied to the purification of many serine and sulfhydryl esterases, including acetylcholinesterase, trypsin, chymotrypsin, urokinase and papain[1–6]. Nearly all of these methods, however, utilize standard agarose columns as the affinity matrix thus preventing the use of pressure to decrease purification time. Recent studies have now shown that pressure-stable affinity columns can be used for enzyme purification[7–10]. Thus, we undertook studies to compare the purification of serine and sulfhydryl esterases by both low-pressure and high-pressure affinity chromatography (LPAC and HPAC, respectively). As our affinity ligands,

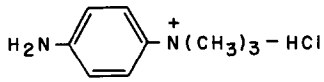
0021-9673/84/$03.00

TABLE I

AFFINITY SUPPORTS USED AND METHOD OF LIGAND ATTACHMENT

Support	*Supplier*	*Bead composition*	*Coupling reaction*
AH-Sepharose 4B	Pharmacia-PL Biochemicals (Piscataway, NJ, U.S.A.)	60–140 μm, Agarose (4%)	React 15 g of washed gel in 100 ml water (pH 5) with 500 mg ligand and 1000 mg carbodiimide for 24 h at 25°C
Reacti-Gel 6X	Pierce Chemical (Rockford, IL, U.S.A.)	40–210 μm, Agarose (6%)	React 25 ml of washed gel in 100 ml 1 *M* sodium carbonate pH 10 with 800 mg ligand for 18–24 h at 25°C. Wash and block remaining active groups by reaction in 100 ml of 0.5 *M* ethanolamine for 3 h at 25°C
ACA-Separon HEMA E_{max}	Laboratory Instruments Works (Prague, Czechoslovakia)	100–200 μm, acrylate copolymers	React 15 g of beads in 100 ml water (pH 5) containing 800 mg ligand and 1500 mg carbodiimide for 24 h at 25°C
CDI-Glycophase	Pierce Chemical	74–125 μm, hydrophillic controlled pore glass	React 15 g of beads with 800 mg ligand in 100 ml of 0.1 *M* sodium borate–boric acid (pH 10) as per instructions for Reacti-Gel 6X

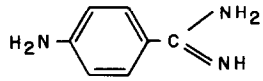
we chose to use the quaternary ammonium compound trimethyl(*p*-aminophenyl)ammonium chloride (*p*-TAPA), which is known to bind to AChE[1,2], and the serine esterase inhibitor analogue *p*-aminobenzamidine (*p*-ABZ), which has been used in the purification of trypsin and urokinase[5] (Fig. 1). Our goal was to synthesize and use packing materials containing these ligands that would be capable of enzyme purification with activity retention in a single step, requiring less than 60 min. This goal was achieved, as detailed in this report.



TRIMETHYL (p-AMINOPHENYL) AMMONIUM CHLORIDE (p-TAPA)

$\lambda_{MAX} = 242$

E = 486



p-AMINOBENZAMIDINE (p-ABZ)

$\lambda_{MAX} = 289$

E = 741

Fig. 1. Structures of the ligands covalently attached to the matrices illustrated in Fig. 2 to produce affinity chromatography packings for the purification of serine and sulfhydryl esterases.

MATERIALS AND METHODS

Apparatus

All HPAC studies were carried out on a Micromeritics Model 701 HPLC apparatus, equipped with binary gradient elution and a variable UV–visible detector. The column effluent was routed to a fraction collector to allow analysis of separated proteins for enzymatic activity. All HPAC columns were 250 × 8 mm stainless steel.

Materials

Acetylcholinesterase was extracted from the excised electric organs of freshly killed *Electrophorus electricus* according to standard methods[1,2,11]. The final extract from this procedure, in 20 m*M* NaH_2PO_4–Na_2HPO_4 (pH 7) containing 1 *M* sodium chloride and 1% Triton X-100, was stored at 4°C and used directly for affinity purification. Trypsin (10,200 U/mg protein, from bovine pancreas), alkaline phosphatase (7 U/mg solid, from bovine intestinal mucosa), papain (21 U/mg protein, from papaya latex), acetylthiocholine, 5,5′-dithiobis(2-nitrobenzoic acid), *p*-nitrophenylphosphate (*p*-NPP) N-α-benzoyl-L-arginine ethyl ester (BAEE), *p*-ABZ and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (carbodiimide) were purchased from Sigma (St. Louis, MO, U.S.A.). Urokinase (*ca.* 1000 U/mg protein from human urine) was purchased from U.S. Biochemical (Cleveland, OH, U.S.A.). The affinity base matrices and their sources are listed in Table I. *p*-TAPA was synthesized as

reported previously[12]. All other reagents and solvents used were analytical reagent grade or better.

Preparation of packing materials

The matrices used and the method for ligand coupling to the matrices are detailed in Table I. The structures of the reactive portions of these packing materials are illustrated in Fig. 2. The structures of the two ligands used, *p*-TAPA and *p*-ABZ are illustrated in Fig. 1. All the affinity chromatography packings were washed twice after ligand coupling and ethanolamine blocking with 100 ml volumes of water, 0.2 *M* sodium acetate pH 4.5, 1% (w/v) aqueous sodium chloride, 0.1 *M* sodium hydrogencarbonate pH 9, 1% sodium chloride and water. The washed affinity packings for LPAC were stored in 10 m*M* NaH_2PO_4–Na_2HPO_4 (pH 7.2) containing 0.02% sodium azide. The washed affinity packings for HPAC were dehydrated by washing four times with 50-ml volumes of methanol and acetone and then dried *in vacuo* at 30°C.

The amount of ligand bound to a matrix was determined from its concentration in the reaction mixture before and after coupling by using the $E^{1\%}_{1\,cm}$ value of the ligand (Fig. 1). Aliquots of the agarose packings were dried to constant weight *in vacuo* at 80°C for such determinations. Trypsin binding to the affinity chromatography packings, was determined by reacting 100–200 mg of packing material with 5 ml of trypsin (1 mg/ml) in 10 m*M* NaH_2PO_4–Na_2HPO_4 (pH 7) for 30 min, centrifuging at 1000 *g* for 10 min and determining the absorbance of the supernatant at 280 nm. The difference between the latter value and the absorbance of the original (pre-reaction) trypsin solution at 280 nm was used to calculate the mg trypsin bound per g packing.

Chromatographic procedures

LPAC packing materials in 10 m*M* NaH_2PO_4–Na_2HPO_4 (pH 7.2) (run buffer) were packed into 9 mm (I.D.) glass columns to a height of 250 mm. Enzyme samples were applied and the columns were eluted (*ca.* 0.4–0.8 ml/min) with the run buffer until all non-adsorbed protein was eluted (*ca.* 80–100 ml eluate). At that time, elution

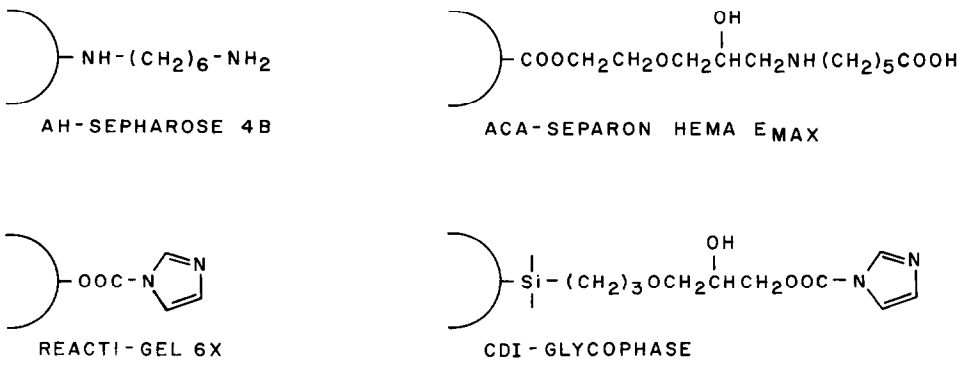


Fig. 2. Structures of the reactive portions of the chromatographic packings used in these studies. The ligands were covalently coupled to these reactive sites either directly or using carbodiimide coupling.

with run buffer containing 1 *M* sodium chloride was begun and continued until purified enzyme was desorbed from the column (*ca.* 40–60 ml eluate).

HPAC columns (250 × 8 mm) were dry-packed with packing material, wetted at 1–2 ml/min with water and then equilibrated in run buffer. Enzyme samples were injected and the column was eluted with run buffer (1 ml/min) until all non-adsorbed protein was eluted (*ca.* 20–30 min). At that time, run buffer containing 1 *M* sodium chloride was used to desorb purified enzyme (*ca.* 10–15 min).

Assays

Protein amounts were determined by the standard Lowry method[13]. AChE activity (1 unit = 1 μmol acetylthiocholine hydrolyzed/ml/min) by the method of Ellman *et al.*[14]. Alkaline phosphatase (1 unit = 1 μmol *p*-NPP hydrolyzed/ml/min) activity was determined from the increase in absorbance at 405 nm resulting from the hydrolysis of *p*-NPP (1 mg/ml in 0.85 *M* diethanolamine–hydrogen chloride (pH 9.8), containing 0.5 m*M* magnesium chloride). Papain activity (1 unit = 1 μmol BAEE hydrolyzed/ml/min) was determined from the acid produced during enzyme hydrolysis of BAEE (58 m*M* in a solution containing 10 m*M* EDTA, 60 m*M* mercaptoethanol, and 50 m*M* cysteine). After 5 min, the amount of 0.01 *N* sodium hydroxide required to neutralize enzyme-produced acidity was used to calculate enzyme activity. Urokinase activity (in CTA units) was determined from an increase in absorbance at 405 nm due to the enzymatic hydrolysis and release of *p*-nitroaniline from the synthetic peptide carbobenzoxy-L-γ-glutamyl(α-*tert.*-butoxy)-glycyl-arginine-*p*-nitroanilide using a commercial assay kit (American Diagnostica, Greenwich, CT, U.S.A.).

RESULTS AND DISCUSSION

Preparation of affinity chromatography packings

The successful linkage of ligand to a matrix was determined by both direct and indirect methods, as reported in Table II. By direct quantitation of ligand bound using spectroscopic analysis, it was found that from 10–50 μmol of ligand bound per

TABLE II

LIGAND CONTENT AND TRYPSIN BINDING TO THE AFFINITY CHROMATOGRAPHY PACKINGS

All values are the mean of at least two determinations where no single value deviated from the mean by more than ±9.3%. The values for the agarose packings were normalized to dry weight by using values of 0.23 g/ml and 0.19 g/ml for the Sepharose and Reacti-Gel packings respectively.

Support	*Amount of ligand bound (μmol/g)*		*Amount of trypsin bound (mg/g)*	
	p-TAPA	*p-ABZ*	*p-TAPA packing*	*p-ABZ packing*
AH-Sepharose 4B	13	31	2.9	8.4
Reacti-Gel	30	21	4.3	5.4
ACA-Separon HEMA E_{max}	41	46	6.2	10.7
CDI-Glycophase	35	10	4.5	1.3

g of matrix. This range of ligand concentration proved ideal for affinity purification of the enzymes studied. Since such ligand concentrations are far in excess of the molar amounts of enzyme purified on the columns, direct comparisons are possible between columns with varying ligand concentrations within this range. Preliminary studies (not reported here) with packings containing 100–300 μmol of ligand per g of matrix resulted in poor purification, presumably due to the high ionic group content of the packing material[15].

Trypsin, another serine esterase, has been used to characterize affinity chromatography packings for serine esterase binding[5]. As shown in Table II, trypsin bound to all the matrices roughly in proportion to the ligand content of the matrix. The best trypsin-binding packings were the *p*-ABZ-Sepharose and *p*-ABZ-Separon HEMA packing materials.

Enzyme purification

A direct comparison was carried out of AChE purification on the affinity chromatography packings. As shown in Fig. 3, a variety of chromatographic elution patterns resulted. Notably, and most obviously, the LPAC runs took approximately 5–6 times longer to complete than the HPAC runs. Significant purification of AChE

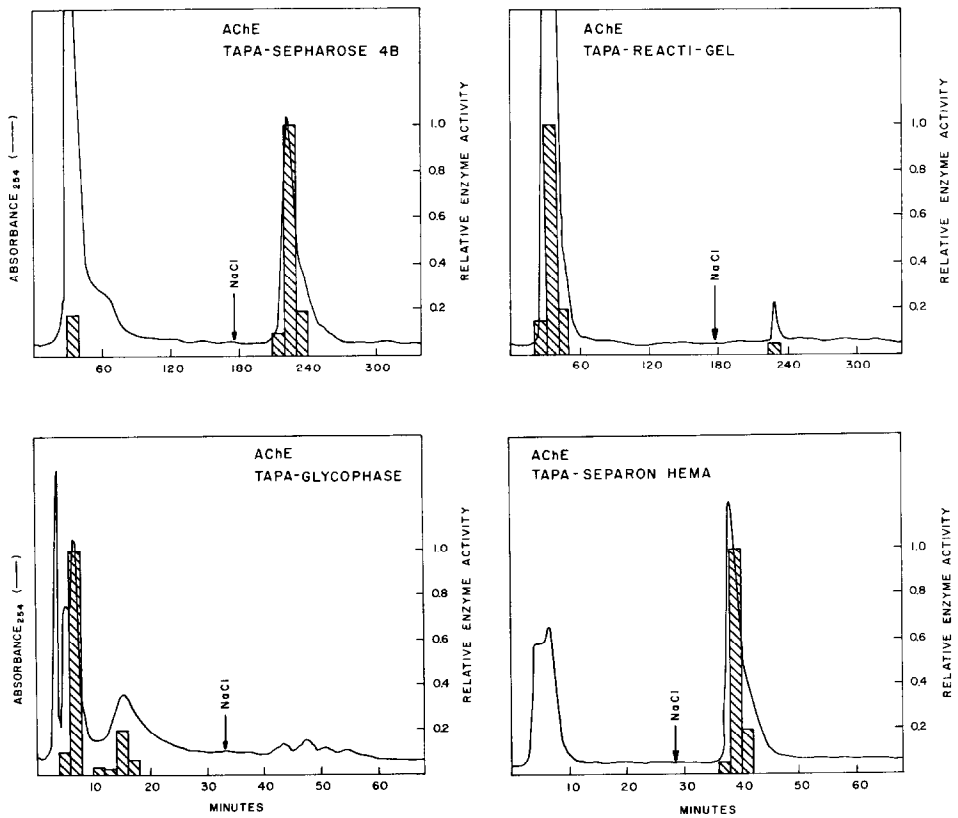


Fig. 3. Comparison of the column elution profiles for the affinity purification of eel acetylcholinesterase on the four *p*-TAPA affinity chromatography packings.

was accomplished only on the TAPA-Sepharose and TAPA-Separon HEMA columns. AChE was not retained by the TAPA-Reacti-Gel column, presumably due to the lack of a spacer arm between the matrix and the ligand (Fig. 2). AChE was also unretained by the TAPA-Glycophase column and, upon repeated analysis, showed apparent degradation on this glass bead-based column material. This was observed in the net loss of activity eluted from the column and the appearance of multiple peaks. Similar results were obtained for columns containing *p*-ABZ packings. We have also found that glycophase beads used for size-exclusion chromatography of AChE lead to its degradation[16]. Thus, this degradation appears to be matrix-related. As shown in Table III, the Sepharose- and Separon HEMA-based packings achieved *ca.* 40-fold enzyme purification. The specific activities of the non-adsorbed protein peak, which is eluted first from the columns, is reported to contrast the purity achieved by affinity adsorption. The percent protein recovery represents total protein recovered in all eluted fractions as compared to protein in the starting sample. This degree of purification by means of *p*-TAPA as an AChE affinity ligand is in agreement with the observation of Berman and Young[1]. Further, the Separon HEMA affinity columns achieved such purification in *ca.* 50 min.

The rapid purification achieved with the Separon HEMA affinity columns led us to test them for purification of other esterases. As shown in Table IV, such purification was achieved not only for AChE, but also for alkaline phosphatase, urokinase and papain. The consistent purification level achieved, with the exception of urokinase on the *p*-ABZ column, attests to the similarity of binding site between these enzymes.

TABLE III

PURIFICATION OF ACETYLCHOLINESTERASE ON THE AFFINITY CHROMATOGRAPHY PACKINGS

The matrices and ligands are described in Figs. 1 and 2. For each column run, 0.5 ml of eel extract, containing *ca.* 35 U of AChE (3.66 U/mg protein) was used. The non-adsorbed peak(s) was (were) eluted from the column with run buffer. The adsorbed peak(s) required 1 *M* sodium chloride in run buffer for desorption from the column.

Column	*Operating mode**	*Recovery (%)*		*Fold purification in eluted peak(s)*	
		Protein	*Activity*	*Non-adsorbed*	*Adsorbed*
TAPA-Sepharose	LP	61	97	0.4	46
TAPA-Reacti-Gel	LP	104	100	1.2	NA**
TAPA-Glycophase	HP	82	35	0.7***	NA
TAPA-Separon HEMA	HP	82	108	0.8	38
ABZ-Sepharose	LP	40	102	NA	38
ABZ-Reacti-Gel	LP	89	100	1	NA
ABZ-Glycophase	HP	96	51	0.8***	NA
ABZ-Separon HEMA	HP	92	105	0.8	40

* LP = atmospheric pressure; HP = high pressure (200–800 p.s.i.).
** No activity.
*** Multiple peaks.

TABLE IV

PURIFICATION OF SERINE AND SULFHYDRYL ESTERASES BY HPAC

Enzyme	*Initial enzyme activity (U/mg)*	*Recovery (%)*		*Fold purification in eluted peak(s)*	
		Protein	*Activity*	*Non-adsorbed*	*Adsorbed*
TAPA-Separon HEMA packings					
AChE	2.1	84	105	0.9	37
Alkaline phosphatase	6.0	84	92	1.2	49
Urokinase	23	92	113	0.7	32
Papain	3.1	95	99	2.9	35
ABZ-Separon HEMA packings					
AChE	2.1	90	107	0.9	41
Alkaline phosphatase	6.0	57	95	0.7	31
Urokinase	23	75	101	1	18
Papain	3.1	95	99	2	48

CONCLUSIONS

Our studies show that rapid HPAC can be used for the preparation of purified serine and sulfhydryl esterases. The rapid purification times and gentle conditions utilized in this method are especially suitable for proteins and other macromolecules. While these initial studies have not achieved the highest levels of purity attainable with these enzymes, our approach and methods are applicable to the synthesis and use of other, more specific affinity chromatography packings (*i.e.* those utilizing ligands specific for a single enzyme) which could produce extremely high specific activity enzymes from crude enzyme extracts in a single step. Such studies are underway in our laboratory.

REFERENCES

1 J. D. Berman and M. Young, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 395.
2 Y. Dudai and I. Silman, *Methods Enzymol.*, 34 (1974) 571.
3 A. H. Patel and R. M. Schultz, *Biochem. Biophys. Res. Commun.*, 104 (1982) 181.
4 M. Nishikata, K. Kasai and S. Ishi, *Biochim. Biophys. Acta*, 660 (1981) 256.
5 L. Holmbeng, B. Bladh and B. Astedt, *Biochim. Biophys. Acta*, 445 (1976) 215.
6 K. Brocklehurst, J. Carlsson, M. P. J. Kierstan and E. M. Crook, *Methods Enzymol.*, 34B (1974) 531.
7 J. Turková, K. Bláha, J. Horáček, J. Vajčner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
8 G. S. Bethell, J. S. Ayers, M. T. W. Hearn and W. S. Hancock, *J. Chromatogr.*, 219 (1981) 361.
9 R. R. Walters, *J. Chromatogr.*, 249 (1982) 19.
10 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 266 (1983) 151.
11 A. S. Brooks, G. E. Tiller and W. G. Struve, *Biochim. Biophys. Acta*, 615 (1980) 354.
12 R. F. Taylor, *J. Neurochem.*, 31 (1978) 1183.
13 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 G. L. Ellman, K. D. Courtney, V. Andres Jr. and R. M. Featherstone, *Biochem. Pharmac.*, 7 (1961) 88.
15 J. Schmidt and M. A. Raftery, *Biochemistry*, 12 (1973) 852.
16 R. F. Taylor, unpublished results.