Journal of Chromatography, 589 (1992) 101–108
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 718

Activation of matrices by 4,6-diphenylthieno[3,4-*d*]-1,3-dioxol-2-one-5,5-dioxide

High-performance liquid affinity chromatographic separations

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(First received April 9th, 1991; revised manuscript received August 26th, 1991)

ABSTRACT

High-performance liquid affinity chromatography requires activated matrices with specific properties. Standard matrices were modified and activated and then their characteristics were compared. A new activating method based on the use of 4,6-diphenylthieno[3,4-*d*]-1,3-dioxol-2-one-5,5-dioxide was developed. The coupling of ligands with these new supports was very fast. The hydrolysis/aminolysis ratio was higher than with N-hydroxysuccinimide-activated matrices.

INTRODUCTION

High-performance liquid affinity chromatography (HPLAC) is a recent field of affinity chromatography which combines the high selectivity of immobilized ligands with the reproducibility, sensitivity and speed of high-performance liquid chromatography. Various aspects of HPLAC have been surveyed [1–6]. From the data, it appears that the choice of activated matrices is still limited. Two factors are determining: the matrix and the activation.

An ideal matrix must be hydrophilic, macroporous, uncharged and mechanically, biochemically and chemically stable; the suitability for ligand attachment is also an important criterion. Two categories of supports are commonly used: inorganic and polymeric.

Porous silica is the most frequently used inorganic support because of its high porosity, particle size and mechanical stability. Unfortunately, this support is degraded at pH 8.5 and sometimes irreversible adsorption or denaturation of biomolecules occurs. Polymeric materials are generally constituted of polysaccharide, polyacrylamide or polystyrene backbones. These polymers are stable in the pH range 1–14 and have an acceptable hydrophilicity. However, their particle size is often too large and their mechanical stability is insufficient, especially above 2500 p.s.i. We consider that synthetic polymer matrices have a great future.

A considerable variety of activating methods have been described [7]. An ideal activated group must have a fast coupling time, a high coupling capacity of ligands, a physiological coupling pH, easy regeneration of the matrix without charged groups, stable attachment with a ligand, no activity lost during storage and spectral characteristics giving the possibility of measuring hydrolysis of the activated group and/or the coupling with a ligand.

Activation of hydroxylated matrices represents an attractive method because hydrolysis of the activated groups regenerates the original hydroxyl

0021-9673/92/$05.00

groups. In the various procedures for activation of hydroxyl groups, the most suitable reagents are 1,1′-carbodiimidazole [8–12] and particularly chloroformates [13–16] and tresyl chloride [17–20].

After a survey of the literature, we selected two epoxy-activated acrylic polymers, Eupergit C30N and HEMA-AFC BIO EH. These matrices were modified and then reactivated. First, we developed a new activation technique using 4,6-diphenylthieno[3,4-d]-1,3-dioxol-2-one-5,5-dioxide (TDO) esters. This technique produced matrices which bound proteins successfully and provided an original and efficient approach of HPLAC. TDO-activated matrices may be useful for the coupling of unstable protein ligands where short times are essential. In particular, they could be useful with pH-sensitive ligands and proteins.

In order to improve the efficiency of the new activation method, we compared the TDO-activated matrices with N-hydroxysuccinimide (NHS)-activated matrices.

EXPERIMENTAL

Materials

Eupergit C 30N was a gift from Röhm Pharma (Weiterstadt, Darmstadt, Germany) and HEMA-AFC BIO EH from Alltech (Deefield, IL, USA). N,N′-Disuccinimidyl carbonate (DSC) was obtained from Aldrich (Strasbourg, France) and 2-mercaptoethanol, triethylamine, pyridine, 4-dimethylaminopyridine, 4-nitrophenyl chloroformate and TDO from Merck (Darmstadt, Germany).

Concanavalin A, type V, horseradish peroxidase, types II, VI and crude, methyl α-D-glucopyranoside, horse heart cytochrome *c* type III, *p*-nitrophenyl *p*′-guanidinobenzoate and soybean trypsin inhibitor (STI) were obtained from Sigma (St. Louis, MO, USA). Trypsin was purchased from Worthington Biochemical (Freehold, NJ, USA).

Modification of Eupergit C 30N and HEMA-AFC BIO EH

The matrices, (2 g) were suspended in 25 ml of 0.2 *M* pyrophosphate buffer (pH 8.5)–1 *M* 2-mercaptoethanol and stirred at 20°C for 12 h. The modified matrices were successively washed on a sintered funnel with water and acetonitrile and finally dried under vacuum.

Activation with DSC

The experimental details were essentially those described previously by Wilchek and Miron [21], but we used acetonitrile instead of acetone to dissolve DSC; 1.2 mmol of DSC per gram of modified Eupergit C 30N were used. NHS-activated ester was determined by a quantitative spectrophotometric assay [22].

Activation with TDO

TDO (115 mg, 0.35 mmol) and modified Eupergit C 30N or modified HEMA-AFC BIO (500 mg) in dry dichloromethane (10 ml) were stirred at 20°C, then pyridine (28 mg, 0.35 mmol) was added dropwise. The mixture was tirred at 20°C for 1 h and sonicated twice for 3 min. After filtration on a sintered funnel, the activated matrix was successively washed with acetonitrile (20 ml), cold water (10 ml), cold saturated $NaHCO_3$ solution (25 ml), cold 20% citric acid solution (40 ml), cold water (40 ml) and acetonitrile (20 ml).

The matrix was dried under reduced pressure, then stored at −10°C. TDO-activated ester was determined spectrophotometrically at 405 nm by hydrolysis with 0.1 *M* NaOH and compared with TDO samples.

Cytochrome c coupling

Method A: bath procedure. A 1.2-ml volume of coupling buffer (0.2 *M* sodium phosphate, pH 7.4) containing cytochrome *c* (5 mg/ml) was added to 40 mg of NHS- or TDO-activated matrices. Coupling proceeded for 15–30 min at 20°C with stirring. The matrices were thoroughly washed with coupling buffer and 1 *M* sodium chloride solution.

Bound cytochrome *c* was determined spectrophotometrically at 550 nm after sodium dithionite reduction. Modified (but non-activated) Eupergit C 30N and HEMA-AFC BIO were compared under the same conditions to check ligand coupling.

Cytochrome *c* coupling on initial epoxy-activated Eupergit C 30N (epoxides >600 μmol/g) and HEMA-AFC BIO EH (epoxides >1400 μmol/g) was performed under the experimental conditions recommended by Röhm Pharma and Alltech, but coupling proceeded at pH 7.4 for 72 h and at pH 10 for 48 h, respectively, instead of 15 min under our conditions.

Method B: column procedure. A 2.5-ml volume of

coupling buffer (0.2 *M* sodium phosphate, pH 7.4) containing cytochrome *c* (2 mg/ml) was recirculated (0.6 ml/min) at 20°C through a Dupont guard column packed with 20 mg of NHS- or TDO-activated Eupergit C 30N. The column was connected with a circulation cuvette under magnetic stirring. As sodium dithionite reduced TDO-activated Eupergit C 30N, bound cytochrome *c* was determined directly by absorbance measurement at 530 nm (oxidized cytochrome *c*).

Hydrolysis of activated matrices

We used method B to study the rate of hydrolysis of NHS- and TDO-activated Eupergit C 30N in 0.2 *M* sodium phosphate buffer (pH 7.4) at 20°C.

Concanavalin A coupling

Concanavalin A (Con A) was coupled by method A. A 500-mg amount of NHS-, TDO- or epoxy-activated Eupergit C30 N was stirred at 20°C for 12 h (96 h for epoxy-activated Eupergit C 30N) with 3 ml of 0.2 *M* phosphate buffer–1 m*M* $MnCl_2$–1 m*M* $CaCl_2$ (pH 7.4) containing Con A (15 mg/ml). After washing with the coupling buffer and then 1 *M* NaCl, the activated groups in excess were quenched by means of 0.1 *M* ethanolamine in 0.2 *M* pyrophosphate buffer (pH 8.5). The matrices were slurry packed into glass columns (200 × 3 mm I.D. or 150 × 6 mm I.D.).

STI coupling

STI was coupled to modified and the TDO-activated matrices according to the procedure described above. The coupling was performed in 0.2 *M* phosphate buffer (pH 7.4) for 2 h.

Amino acid analysis

The hydrolyses were carried out at 105°C for 24 h. The analyses were performed with a Biotronik LC 2000 analyser equipped with a Dionex DC6A resin column (Durrum Chemical) and a Spectra-glo spectrofluorimeter (Gilson). Post-column detection was carried out by measuring the fluorescence intensity of isoindole derivatives obtained by the action of *o*-phthalaldehyde in the presence of 2-mercaptoethanol. The results were calculated with a Spectra-Physics SP4100 integrator.

Chromatographic procedure

Chromatography was carried out with a Gilson autoanalytic gradient system. The column effluents were monitored with a Beckman 165 variable-wavelength detector.

RESULTS AND DISCUSSION

The polymers Eupergit C 30N and HEMA-AFC BIO EH were selected to test their suitability for HPLAC, since these matrices have already been used for ligand attachment [23,24]. However, the coupling time of ligands to epoxy groups is a slow process; this characteristic is not favourable for a fast chromatographic technique. Moreover, the reaction requires high pH. The coupling capacity is low and charged groups are introduced after coupling [1,5,6,25]. Further, the estimated optimum length of a spacer is *ca.* 10 Å[1,26], and the Eupergit C 30N spacer (6 Å) is slightly short.

To overcome these disadvantages, we modified the spacers and the activated groups of Eupergit C 30N and HEMA-AFC BIO EH. Mercaptoethanol was used to lengthen the spacer (Fig. 1). The reaction was virtually quantitative and did not introduce charges. Moreover, hydrophilicity was preserved and the new diol was not a 1,2-diol. According to the literature [14] this last structure seems to possess a lower yield of activation.

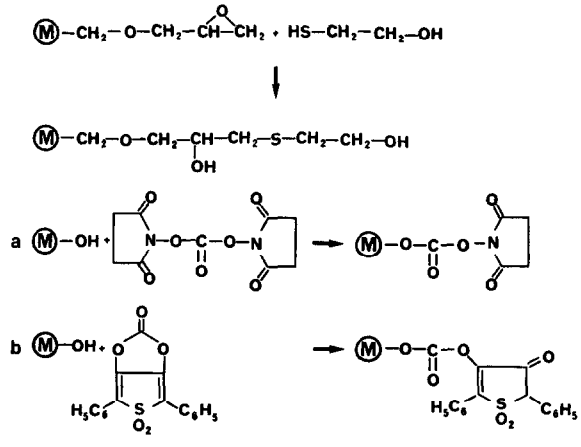

Fig. 1. Modification and activation of Eupergit C 30N.

Activation with NHS

To prepare the NHS–Eupergit C 30N we used DSC instead of hydroxysuccinimide chloroformate, an unstable reagent (Fig. 1a). The procedure is essentially the same as that described by Wilchek and Miron [21], with some modifications. The amount of activated groups was between 200 and 245 μmol/g of dry matrix determined by spectrophotometric assay at 260 nm [22]. In some experiments we used 4-nitrophenyl chloroformate to activate the matrix; higher activities were obtained, but unfortunately the matrix was less stable.

Activation with TDO

A new approach to activate the matrix was based on the use of TDO (Fig. 1b). This reagent is very efficient in the synthesis of amides and peptides [27,28]. TDO-activated matrices have not previously been used for affinity chromatography. Activation proceeded in one step and in less than 2 h. The amount of activated groups was between 240 and 250 μmol/g of dry Eupergit C 30N, determined by spectrophotometric assay at 405 nm. With HEMA-AFC BIO EH a smaller number of activated groups was observed (95 μmol/g).

Preliminary results showed that primary hydroxylated functions were preferentially activated. The dry TDO-activated matrices were yellow (orange at basic pH or in the presence of amines). The matrices, when subjected to hydrolysis or aminolysis, regenerated white powders and the released sulphone could be detected at 405 nm at alkaline pH.

The variations of the TDO absorption spectrum at different pH values are presented in Fig. 2; these spectra show two isosbestic points, at 312 and 381 nm. These colour changes are particularly advantageous to follow the evolution of an aminolysis (Fig. 3).

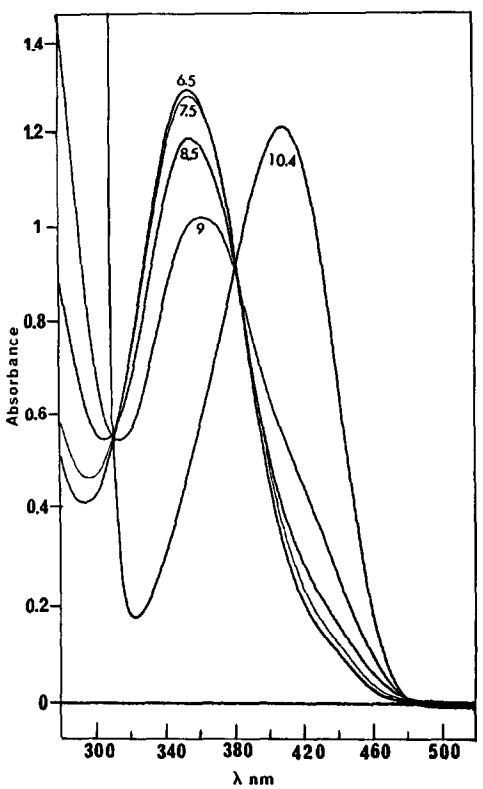

Fig. 2. TDO absorption spectrum at various pH values (6.5–10.4): 2 m*M* TDO, 0.2 *M* phosphate buffer and 0.1 *M* NaOH (pH 10.4, 20°C).

Comparison of the NHS and TDO-activated Eupergit C 30N

We compared hydrolysis and aminolysis of NHS- and TDO–Eupergit C 30N (Fig. 4). To study these reactions we used a flow technique; the solutions were recirculated through small columns containing the activated matrices. The rates of hydrolysis were measured in 0.1 *M* sodium phosphate (pH 7.4) at 260 nm for NHS and at 405 nm for TDO.

NHS–Eupergit C 30N had a half-time of about 29 min and TDO-Eupergit C 30N a half-time of about 20 min. The kinetics were very different and we observed that part of the material (15%) was rapidly released (NHS 0.5 min and TDO 1 min). This release might represent some particularly unstable carbonates.

To measure aminolysis, we followed the coupling of cytochrome *c* (2 mg/ml in 0.1 *M* sodium phosphate, pH 7.4); this ligand absorbs at 530 nm with-

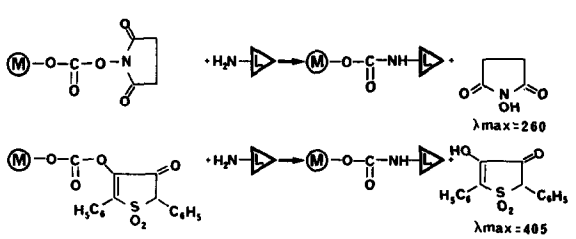

Fig. 3. Aminolysis of NHS- and TDO–Eupergit C 30N.

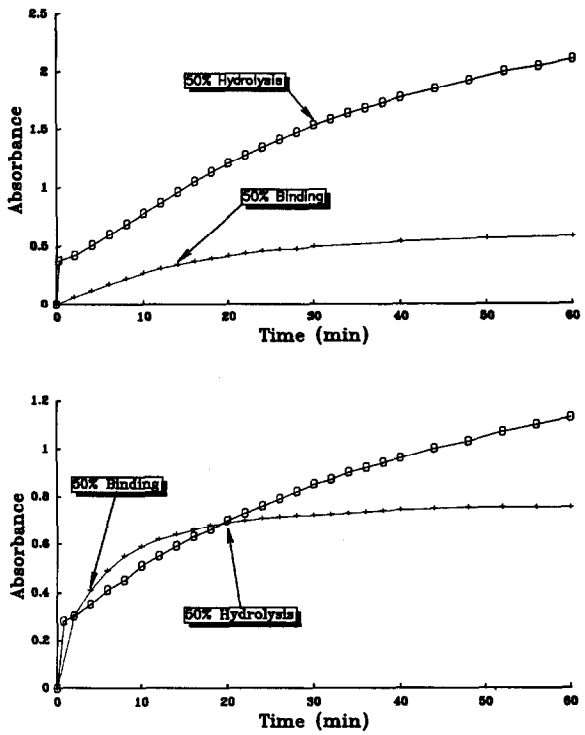

Fig. 4. Comparison of the hydrolysis ○, (measured at 260 nm for NHS and 405 nm for TDO) and cytochrome *c* binding +, for (top) NHS– and (bottom) TDO–Eupergit C 30N. For conditions, see Experimental.

out interference with NHS or TDO absorption. NHS–Eupergit C 30N coupled cytochrome *c* with a half-time of 13 min and TDO–Eupergit C 30N with a half-time of 3 min. It was also interesting to compare the hydrolysis/aminolysis ratios, which were 2.2 for the NHS matrix and 6.6 for the TDO matrix.

Hence the TDO matrices are particularly well adapted for the coupling of proteins.

Comparison of the coupling efficiency of the TDO, NHS and epoxy matrices

We also coupled cytochrome *c* (5 mg/ml) using method A at pH 7.4 for 15 min. With NHS–Eupergit C 30N (activity 245 μmol/g), 84 mg/g, *i.e.*, 6.8 μmol/g of cytochrome *c*, were bound. With TDO–Eupergit C 30N (activity 240 μmol/g), the binding of cytochrome *c* was higher at 140 mg/g, *i.e.*, 11.3 μmol/g.

These results showed the great efficiency of the TDO-activated matrices and their usefulness in coupling unstable ligands. TDO activation and coupling of ligands were lower with the HEMA-AFC BIO EH matrix (see Tables I and II).

When cytochrome *c* was coupled to epoxy–Eupergit C 30N (previous conditions but for 72 h), poor immobilization was found, 11 mg/g, *i.e.*, 0.89 μmol/g. With epoxy–HEMA-AFC BIO EH (pH 10, 48 h) only 2 mg/g, *i.e.*, 0.16 μmol/g, were bound. These observations were in agreement with the literature data concerning the epoxides [1,5,6,25].

In another experiment, the epoxy groups of the original Eupergit C 30N were hydrolysed into 1,2-diols, activated with TDO, then cytochrome *c* was coupled in the usual way. Activation was only 52 μmol/g and cytochrome *c* coupling 39 mg/g. This decrease in yields seems to be a general phenomenon observed with hydroxylated matrices having 1,2-diols [14,29]. The formation of a cyclic carbonate derivative has been invoked to explain this reactivity difference. All the coupling results are summarized in Table I.

TDO activation was also used successfully to prepare activated matrices from several diol silica matrices and some polymeric supports; this will be reported elsewhere.

TABLE I

COMPARISON OF THE ACTIVATED MATRICES AND COUPLING OF CYTOCHROME *c*

Dried activated matrix	Active groups (mmol/g matrix)	Bound cytochrome *c* (mg/g matrix)	Coupling time (pH 7.4, 20°C)
Epoxy–Eupergit C 30N	>600	11	72 h
Modified + NHS–Eupergit C 30N	245	84	15 min
Modified + TDO–Eupergit C 30N	240	140	15 min
Hydrolysed + TDO–Eupergit C 30N	52	39	30 min
Epoxy–HEMA-AFC BIO EH	>1400	2	48 h (pH 10)
Modified + TDO–HEMA-AFC BIO EH	95	36	30 min

TABLE II

COMPARISON OF THE COUPLING OF STI AND TRYPSIN CAPACITY ON MODIFIED MATRICES

Dried activated matrix	Active groups (μmol/g matrix)	Bound STI (mg/g matrix)	Coupling time (h)	Trypsin capacity (mg/g matrix)
Modified + TDO–Eupergit C 30N	246	57.2	2	38.2
Modified + TDO–HEMA-AFC BIO EH	95	13	2	10.6

Stability of TDO-activated matrices

No loss of activity was observed with a dry powder sample of activated TDO–Eupergit C 30N stored at −10°C for 6 months; these precautions were excessive.

Affinity chromatography

Illustrative of the use of TDO-activated matrices are the results obtained with the well known affinity chromatographic models, namely the purifications of peroxidase using a Con A-bound matrix and trypsin using an STI-bound matrix.

Affinity chromatography on concanavalin A–Eupergit C 30N

TDO coupling of ligands via their free amino acid groups yielded N-alkyl carbamates (Fig. 1b); it is well known that these linkages are stable [8–16].

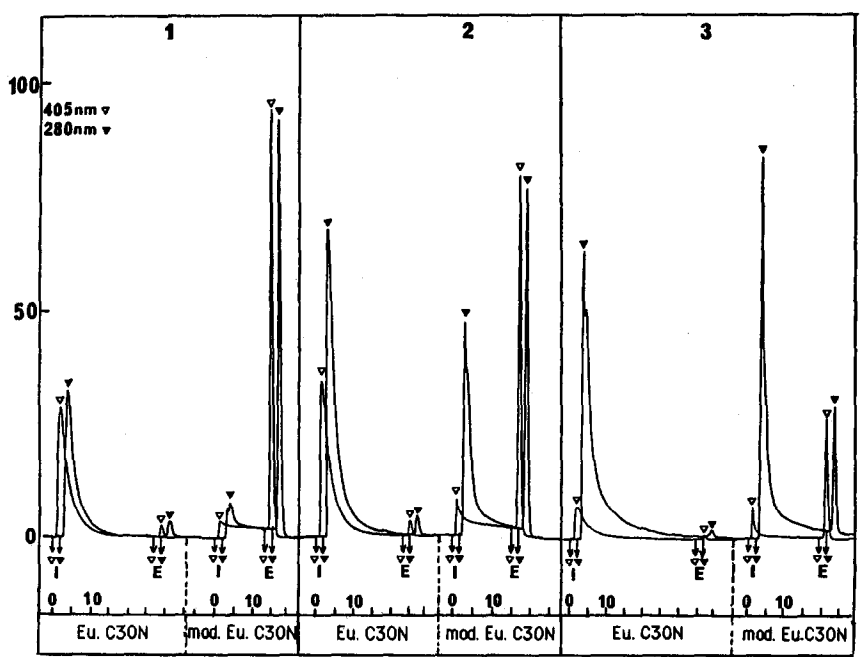

Fig. 5. Peroxidase purifications on Con A–Eupergit C 30N and Con A–modified Eupergit C 30N. (1) Peroxidase type VI; (2) peroxidase type II; (3) peroxidase crude. I = sample injection; E = peroxidase elution. Columns: 250 mm × 3 mm I.D. Conditions: at I, 250 μl of 2 mg/ml peroxidase were injected; mobile phase, 0.05 *M* sodium acetate–0.5 *M* NaCl–1 m*M* $CaCl_2$–1 m*M* $MnCl_2$ (pH 5.1); flow-rate, 0.5 ml/min; at E, 25 m*M* methyl α-D-glucopyranoside in the same mobile phase; flow-rate 1 ml/min. The detector response was calibrated in order to obtain the same amplitude at (▽) 405 and (▼) 280 nm. The 100% response corresponded to absorbances of 0.25 and 0.80 at 280 and 405 nm, respectively, using type VI peroxidase. To clarify the diagram, the records at 280 and 405 nm are arbitrarily shifted.

To demonstrate the efficiency of activated TDO matrices and illustrate their suitability for HPLAC, we purified horseradish peroxidase on a column containing immobilized Con A. Con A was coupled by method A to activated TDO–, NHS– and epoxy–Eupergit C 30N (see Experimental), then matrices with immobilized ligands were packed into columns. Con A–Eupergit C 30N columns were used to purify rapidly horseradish peroxidase. Immobilized Con A and purified peroxidase were not determined.

Horseradish peroxidase absorbs at 405 nm, a convenient property for discriminating peroxidase and contaminant proteins. The column effluent was monitored at 280 nm to obtain a measure of the total protein content and at 405 nm to detect the peroxidase. Three commercial grades of peroxidase were subjected to purifications (Fig. 5). Peroxidase samples were injected at a flow-rate of 0.5 ml/min; at higher flow-rates a small part of peroxidase was not immobilized, owing to the large particle size of Eupergit C 30N (30–80 μm) and its porosity (0.1–2.5 μm). On the other hand, the counter ligand methyl α-D-glucoside can be injected at 1 ml/min.

Fig. 5 clearly shows the low binding of peroxidase on a Con A–Eupergit column prepared from epoxy–Eupergit C 30N. Fig. 5. also illustrates the efficiency of the affinity column prepared from TDO–Eupergit.

The specificity of Con A–modified Eupergit C 30N for peroxidase is illustrated in Fig. 5 (from 1 to 3). As the amount of impurities increased, the ratio of the peaks at 280 and 405 nm corresponding to the eluted peroxidase remained constant. Hence the Con A–Eupergit C 30N and Con A–modified Eupergit C 30N did not undergo non-specific binding. Similar chromatograms were obsserved with NHS–Eupergit C 30N.

Affinity chromatography on STI–HEMA-AFC BIO EH and STI–Eupergit C 30N

STI (15 mg/ml) was coupled by method A to modified and TDO-activated matrices (see Experimental and Tables I and II). Then the matrices with immobilized ligand were packed into columns. Protein contents were determined by amino acid analysis.

In a typical procedure (Fig. 6), a sample of commercial trypsin (1 mg in 200 μl) was loaded onto an

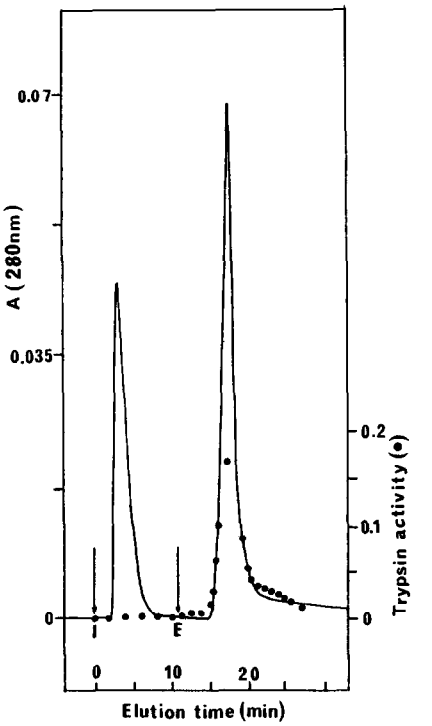

Fig. 6. Chromatography of bovine trypsin on an STI-modified HEMA-AFC BIO EH 1000 column (20 × 3 mm I.D.) previously equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.5)–0.5 *M* NaCl–0.02 *M* $CaCl_2$. At I, 200 μl of 5 mg/ml trypsin were injected (flow-rate 1 ml/min). At E, active trypsin desorption was performed with 12 m*M* HCl–0.5 *M* NaCl (flow-rate, 1 ml/min). The trypsin activity of eluted fractions (900 μl each) was measured by NPGB hydrolysis.

STI–HEMA-AFC BIO EH column (200 × 3 mm I.D.). Desorption of the bound active trypsin was carried out with 12 m*M* HCl containing 500 m*M* NaCl. The protein content was determined by measuring the absorbance at 280 nm. Active trypsin was evaluated by *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) hydrolysis [30]. This method titrates the active sites and therefore the molarity of active trypsin. Two peaks were obtained from STI affinity chromatography of trypsin (Fig. 6): the first (unbound fraction) had no trypsin activity and the second (bound fraction) contained 100% of the trypsin activity. The recovery of enzyme activity was 93% of the loaded trypsin.

To assess the adsorption capacity of STI-bound matrices, trypsin solutions were pumped through 33

× 6 mm I.D. columns for 30 min in a dynamic process. As summarized in Table II, the STI-bound Eupergit C 30N showed a higher capacity toward trypsin. On the other hand, the yield was better with STI-bound HEMA-AFC BIO EH (74%) than with STI-bound Eupergit C 30N (60%).

Controls measurements with deactivated matrices were performed to check non-specific interactions between matrices and ligands and proteins to be purified. Whatever our HPLAC conditions, non-specific binding was not observed in the control experiments.

CONCLUSIONS

A new coupling procedure for HPLAC on acrylic polymers was developed based on TDO matrices. The properties of the TDO matrices fitted the criteria required for HPLAC, *i.e.*, fast coupling in the physiological pH range and high capacity of protein binding and stability. No additional charges were generated by aminolysis or hydrolysis. The TDO absorbance at 405 nm of the activated groups was particularly useful for monitoring the matrix hydrolysis and the ligand binding. Moreover, the observed aminolysis/hydrolysis ratio showed the usefulness of the TDO method compared with NHS activation.

Epoxy-activated matrices required pH or temperature conditions that are incompatible with sensitive ligands. The TDO activation process could be extended to any hydroxylated matrix and consequently to the matrices used for size-exclusion chromatography without preliminary derivatization reactions.

ACKNOWLEDGEMENT

The authors are deeply grateful to Dr. G. Auger for amino acid analyses.

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