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Affinity of trypsin for amidine derivatives immobilized on dextran-coated silica supports

M. ELLOUALI, S. KHAMLICHI, J. JOZEFONVICZ and D. MULLER*
L.R.M., C.N.R.S.–URA 502, Université Paris-Nord, Avenue J. B. Clément, 93430 Villetaneuse (France)

ABSTRACT

The pancreas contains two very analogues enzymes: trypsin and chymotrypsin. These two enzymes are very similar in their physicochemical characteristics and are therefore quite difficult to separate by classical purification procedures. They constitute a good model for affinity chromatography. It was previously demonstrated that amidine derivatives are able to interact strongly and specifically with these serine proteases and are often used as ligand in affinity chromatography. To understand the trypsin interaction mechanism, we synthesized different amidines and immobilised them with or without spacer arm on silica beads previously coated by dextran substituted with a calculated amount of positively charged diethylaminoethyl functions, in order to minimize the non-specific interactions of silanol groups of the silica material. First the affinity constant and the adsorption capacity of these supports for trypsin were determined in batch procedures, then they were used in affinity chromatography. The effects of ionic strength, pH and competitive inhibitors on proteins desorption were also studied. Last, to demonstrate the importance of passivation, the chromatographic performances of dextran-coated silica phases and a commercial support grafted with the same amidine were compared.

INTRODUCTION

The purification of biomolecules and proteins of therapeutic or commercial interest generally involves a combination of different steps, in particular, precipitation steps using solvents, salts or pH and chromatographic procedures. Each step has a relatively low specificity. Affinity chromatography could be used on a large scale in order to remove the remaining impurities. As well as its technological interest, it is also a good tool for a better understanding of the interaction mechanism between proteins and the active phase.

Benzamidine derivatives are strong and specific serine protease inhibitors [1,2]. It is claimed that the amidine group mimics the cationic side-chain of arginine and lysine binding sites of the protein substrates. Previously, Hixon and Nishikawa [2] used *m*-aminobenzamidine linked via a monosuccinylated-1,6-diaminohexane spacer to cyanogen bromide (CNBr)–Sepharose to purify bovine trypsin. Jameson and Elmore [3] prepared affinity adsorbents for bovine trypsin by covalently coupling *p*-(*p'*-aminophenoxypropoxy)benzamidine to cellulose and agarose and used these supports to separate α- and β-trypsins. The trypsin interaction mechanism with these amidine derivatives is still unknown. Trypsin is obtained in crystalline form from beef

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pancreas by the method of Northrop and Kunitz [4]. Further work [5] has improved methods of preparation and has established that the transformation of trypsinogen into trypsin is a proteolytic process and may be accomplished either by autocatalysis or by enterokinase, or by the kinase from a mould of the genus *Penicillium*. The primary structure of bovine trypsin has not yet been completely established [6]. It is a single-chain structure of 226 amino acid residues, cross-linked by six disulphide bridges [7]. Many studies [8,9] have demonstrated that Ser 195, His 57 and Asp 102 residues are important in the trypsin catalytic site and represent a charge relay system in which the serine hydroxyl is acylated by the substrate. The binding site is presumably in the form of a crevice which binds the carbon side-chains or rings of substrates and inhibitors by hydrophobic interactions. The bottom of this crevice contains a negatively charged aspartic acid (Asp 189) which binds electrostatically to the positively charged groups of substrates or inhibitors [6,10].

In this paper, we describe the preparation of coated silica supports bearing differents types of trypsin inhibitors. First, affinity constants and binding capacities from adsorption isotherms of these supports for trypsin were determined using batch procedures. Then, the active supports were used in affinity chromatography to study the specific interactions between the amidine function and the trypsin in solution. Finally, in order to demonstrate the importance of passivation, the chromatographic performances of a dextran-coated silica phase and of a commercial support grafted with the same amidine were compared.

EXPERIMENTAL

Reagents

Silica beads provided by IBF Bio-technics (Villeneuve la Garenne, France) were in the range of 40–100 μm and the pore diameter was about 1250 Å. *p*-Aminobenzamidine (pABA) and L-arginine monohydrochloride were provided by Fluka (Buchs, Switzerland). Guanidium carbonate was purchased from Prolabo (Paris, France). 1,1′-Carbonyldiimidazole (CDI) used as coupling agent, was provided by Sigma (St. Louis, MO, USA). 1,4-Butanediol diglycidyl ether (BDGE) was provided by Polysciences (Warrington, PA, USA). Dextran T40 [weight-average molecular weight ($\bar{M}_w$) 42 000; number-average molecular weight $\bar{M}_n$ 24 700; $I = \bar{M}_w/\bar{M}_n = 1.70$] was obtained from Pharmacia-France (Bois d'Arcy, France).

The commercial support was Nugel Benzamidine (Si-pABA) obtained from Separation Industries Diagnostic Specialties (Metuchen, NJ, USA). The particle size was in the range 40–60 μm and the porosity of the support was 500 Å. The pABA was coupled to the activated support Nugel–polyhydroxyl via an hydrophic spacer arm of eight carbons [2].

N-Benzoyl-L-arginine-4-nitroanilid hydrochloride (L-BAPA) as trypsin substrate was purchased from Merck-Clevenot (Nogent-sur-Marne, France).

Bovine trypsin (type I), with a specific activity of 13.8 U BAEE per mg of protein [one unit of BAEE producing a ΔA_{253} of 0.001 per min at pH 7.6 at 25°C using N-benzoyl-L-arginine ethyl ester (BAEE) as substrate, reaction volume 3.2 ml] was obtained from Sigma.

Preparation of affinity supports

Passivated silica beads (SiD). Silica beads were coated with dextran polymers substituted with a calculated amount of DEAE to minimize the non-specific interactions due to silanol groups of the silica surface as previously described [11].

The percentage of dextran units substituted by diethylaminoethyl (DEAE) functions is 4%. Passivated silica beads were coupled with different types of amidine or guanidine as follows.

Coated silica functionalized by L-*arginine (SiD–Arg) (Fig. 1).* SiD (2 g) was suspended in 20 ml of 1,4-dioxane and mixed with 1 g of CDI. The gel suspension was gently shaked at room temperature for 3 h. The activated support was then washed successively with 200 ml of 1,4-dioxane and 200 ml of 0.1 *M* carbonate buffer (pH 10.5), and then resuspended in 20 ml of carbonate buffer containing 500 mg of L-arginine monohydrochloride. The mixture was gently stirred at room temperature for 48 h and the support obtained was filtered and washed successively with 200 ml of 0.1 *M* carbonate buffer, 0.1 *M* NaCl (pH 10.5) and 200 ml of 0.05 *M* phosphate buffer (pH 7.5). The excess of activated groups was neutralized by suspending the support in 0.1 *M* ethanolamine solution for 3 h. The final support was washed with 200 ml of phosphate buffer (pH 7.5), filtered and dried under vacuum at 40°C.

Coated silica functionalized by pABA (SiD–pABA) (Fig. 1). The coupling procedure was realised in conditions similar to SiD–Arg: 500 mg of pABA were left to react with the activated support.

Coated silica functionalized by pABA using a spacer arm (SiD–B–pABA) (Fig. 1). The synthesis of this support was described previously [12]. Briefly, 2 g of SiD were suspended in 20 ml of diethyl ether and 2 ml of BDGE were added. The suspension was gently stirred for 15 h at room temperature and the activated support was washed successively with 200 ml of diethyl ether, and 200 ml of 0.1 *M* carbonate buffer (pH 10.5). After filtration, it was suspended in 20 ml of 0.1 *M* carbonate buffer

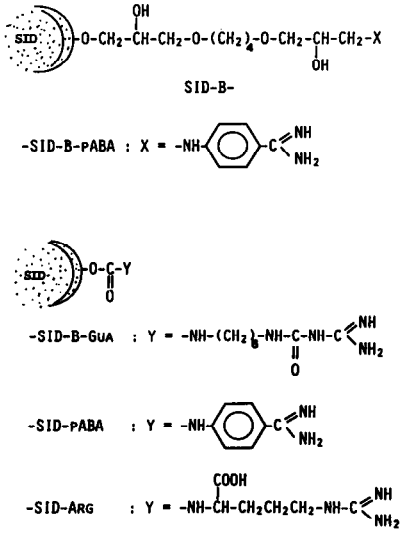

Fig. 1. Structures of the active supports.

(pH 10.5) containing 256 mg of pABA in solution. The mixture was stirred at room temperature for 48 h and the final support was washed and filtered according to the method used for the activated support SiD–Arg.

Coated silica functionalized by guanidine (SiD–B–Gua) (Fig. 1). The coupling reaction of guanidine to the SiD support was described previously [12]. The step of support activation was similar to that already described for the SiD–Arg support. The activated support was then suspended in 20 ml of 0.1 *M* carbonate buffer solution (pH 10.5) containing 1.5 g of hexamethyldiamine (HMD). The mixture was gently stirred at room temperature for 48 h and then, the support was washed successively with carbonate buffer and 1,4-dioxane. The amine functions of HMD fixed on the support were activated by CDI (1 g of CDI per 2 g of support) and the activated support was then suspended with 1 g of guanidinium carbonate dissolved in 20 ml of 0.1 *M* carbonate buffer (pH 10.5). The mixture was stirred at room temperature for 48 h. Finally, the support was washed with 0.05 *M* phosphate buffer and dried under vacuum at 60°C.

Characterization of the functionalized coated silica beads. For each support, the substitution rate of amidine derivatives was determined by acidimetric titration and by elemental analysis (Service Central d'Analyse, CNRS, Vernaison, France). The preparation technique of these derivatisable supports is easy in comparison with the coupling reactions previously reported [2,13] and provides silica supports with a minimum non-specific adsorption of proteins in high-performance liquid affinity chromatography (HPLAC). The passivation of the silica phases is observed by the determination of elution conditions of several proteins with different p*I* values at low ionic strength (phosphate buffer 0.05 *M*, pH 7.5, NaCl 0.15 *M*) in high-performance size-exclusion chromatography [14].

Adsorption isotherms procedure of trypsin

Adsorption experiments were performed at room temperature. Isotherms are generated from measurements of trypsin adsorption after 20 min incubation with the active support using the following procedure.

The trypsin concentration varied from 5 to 60 μg/ml; 100 μl of support suspension (20–100 mg/ml) were incubated with 500 μl of bovine trypsin solution at various concentrations in a polystyrene tube for 20 min. After decantating, the amount of residual trypsin in the supernatant was determined by taking 100 μl of this supernatant and adding 700 μl of 0.05 *M* phosphate buffer containing 0.1 *M* NaCl (pH 7.5) and 100 μl of the trypsin chromogenic substrate L-BAPA. After adding trypsin, the reaction was stopped after 10 min at 37°C by addition of 100 μl of pure acetic acid. The difference between the control and the remaining concentration of active trypsin in the supernatant corresponds to the amount of trypsin adsorbed. Concentrations of trypsin were determined by reading the absorbance at 405 nm. Isotherms were established and affinity constants were determined using a computer program on the basis of the Langmuir and Tempkin equations [15].

In order to determine the adsorption enthalpy of trypsin on these supports, we established adsorption isotherms at two supplementary temperatures, 37 and 4°C.

Chromatographic assay.

Chromatographic assays were performed on the two supports, SiD–pABA and

the commercial Si–pABA support grafted with the same amidine. These two supports were used in HPLAC according to the following procedure.

The column (12.5 cm × 0.4 cm I.D) was packed using a slurry method with a suspension of 1 g of resin in the eluent. The liquid chromatographic apparatus consists of a three-head (120°C) chromatographic pump (Merck LC 21B), connected to a Rheodyne 7126 injection valve (sample loop 100 μl). A Merck-IC 313 variable-wavelength UV–visible detector and the gradient system are connected to a Epson QX-10 computer. The chromatographic signal details are monitored, integrated and stored by the computer. All the equipment was provided by Merck-Clevenot. (Nogent-sur-Marne, France).

All eluents were prepared from high-purity water (ELGA, Villeurbanne, France), degassed and filtered through a Millipore HA 0.22-μm membrane (Velizy, France).

Trypsin (2 mg) was dissolved in 1 ml of 0.05 *M* phosphate buffer containing 0.1 *M* sodium chloride (pH 7.5) (buffer 1) and 100 μl of this solution were applied to a column of immobilized pABA support equilibrated with buffer 1. The column was washed with buffer 1 and adsorbed trypsin was eluted with 0.05 *M* phosphate buffer containing 0.1 *M* sodium chloride (pH 2) (buffer 2) or using buffer 1 containing arginine or guanidine as competitive inhibitors at varying concentrations. Assays were also performed using buffer 1 containing various concentrations of NaCl (buffer 3). The amount of trypsin desorbed was determined from the surface area of the peak given by the computer.

RESULTS AND DISCUSSION

Trypsin adsorption isotherms

In preliminary experiments it was ascertained that steady-state trypsin adsorption was achieved in about 10 min at room temperature. Therefore a time of 20 min was chosen for the determination of the isotherms, which are presented in Figs. 2 and 3. The affinity of these supports was studied by determining the affinity constants from Langmuir or Tempkin adsorption isotherms. The affinity constants of these supports are about $10^6\ M^{-1}$ (Table I). These values demonstrate a strong affinity of all these supports for bovine trypsin. SiD–pABA and SiD–B–pABA supports exhibit affinity constants in the same range. This indicates that the spacer arm has no real influence on the bovine trypsin adsorption process. SiD–Arg and SiD–B–Gua supports also show a similar affinity for trypsin. All the supports functionalized with amidine or guanidine groups possess similar affinity constant values. Among synthesized supports, SiD–pABA demonstrates the highest binding capacity, which is probably due to the suitable coupling reaction. The binding capacity of the commercial support is higher than that observed for the SiD–pABA support. This may be explained by the presence of non-specific trypsin adsorption on this commercial support or by a lower availability of the ligand on the SiD–pABA support. This demonstrates the importance of passivation of the inorganic material by the polysaccharide coating, which minimizes the non-specific adsorption of proteins. The other supports, SiD–B–pABA, SiD–Arg and SiD–B–Gua, show a lower binding capacity. This could be due to the lower amount of ligand coupled on these supports or to the fact that ligands fixed on these supports are not really available for interaction with the enzyme in solution.

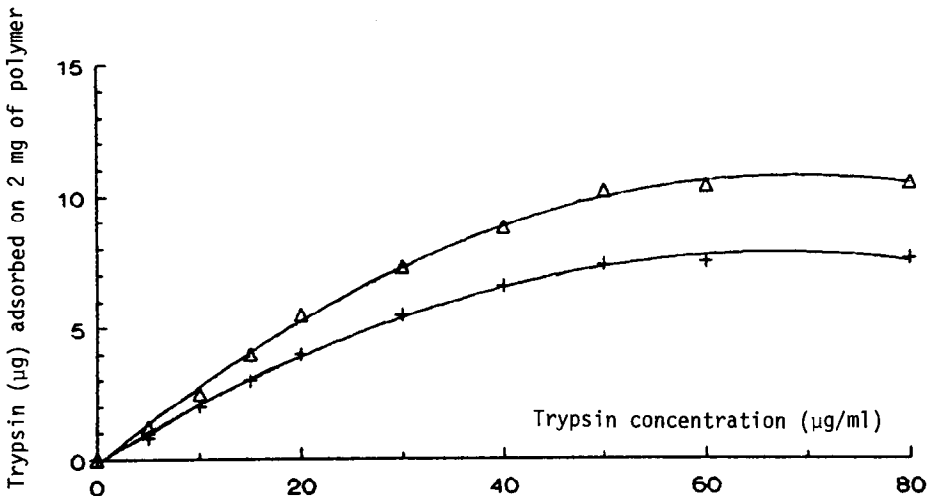

Fig. 2. Adsorption isotherms of bovine trypsin on SiD–pABA (+) and commercial support Si–pABA (△).

The affinity constants decrease with temperature (Table II). If we assume that trypsin does not present any structural changes which affect adsorption in the temperature range 4–37°C, we can estimate the trypsin adsorption enthalpy using the relation $\mathrm{dln}\ K_{Aff}/dT = \Delta H^0_{ads}/RT^2$ where K_{aff} is the affinity constant, ΔH^0_{ads} is the enthalpy of adsorption and T is the temperature. The overall binding process for each support is endothermic with ΔH^0_{ads} around 4.5 kcal/mol for all the supports except SiD–B–pABA, which presents a higher ΔH^0_{ads} value. This higher ΔH_{ads} value may be explained by the presence of a spacer arm which facilitates the access of the ligand to the binding site of the enzyme.

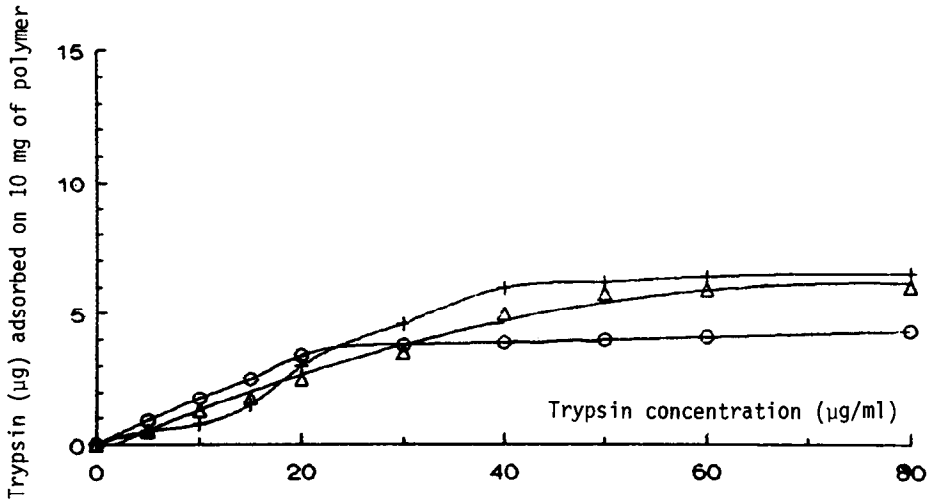

Fig. 3. Adsorption isotherms of bovine trypsin on SiD–B–pABA (+), SiD–Arg (△) and SiD–B–Gua (○).

TABLE I

AFFINITY CONSTANTS AND BINDING CAPACITIES OF PASSIVATED SILICA BEADS FUNCTIONALIZED BY AMIDINE DERIVATIVES TO BOVINE TRYPSIN

T = Tempkin isotherm; L = Langmuir isotherm.

Material	Isotherm	Affinity constant (M^{-1})	Binding capacity	
			μg/g	mol/g
Si–p-ABA	T	$1.5 \cdot 10^6$	810	$35 \cdot 10^{-9}$
SiD–pABA	T	$3.7 \cdot 10^6$	550	$23 \cdot 10^{-9}$
SiD–B–pABA	T	$2.6 \cdot 10^6$	73	$3.1 \cdot 10^{-9}$
SiD–Arg	T	$1.1 \cdot 10^6$	65	$2.8 \cdot 10^{-9}$
SiD–B–Gua	L	$1.2 \cdot 10^6$	40	$2.5 \cdot 10^{-9}$

HPLAC

The SiD–pABA support presents a high binding capacity for trypsin and can easily be used in affinity chromatography to study the trypsin adsorption process. The chromatographic performances of this support were compared to those of the commercial one. In order to understand the trypsin interaction mechanism with these supports, the enzyme was eluted with solutions of different physicochemical characteristics, and the influence of ionic strength, competitive effects and pH was studied.

Influence of arginine and guanidine. The elution of trypsin with arginine and guanidine in solution at different molarities is presented in Fig. 4. Trypsin desorption from the stationary phase is allowed by the competition between arginine or guanidine and the coupled amidine derivatives. As shown in this figure all the trypsin adsorbed on SiD–pABA is eluted with a 0.5 *M* arginine or guanidine solution. The same profile is obtained with these two substances. This indicates that probably the same type of interactions occur between trypsin and the two competing substances.

TABLE II

ENTHALPY OF ADSORPTION FOR TRYPSIN BINDING TO AMIDINE DERIVATIVES COUPLED ON PASSIVATED SILICA BEADS DETERMINED FROM ADSORPTION ISOTHERMS AT 4, 21 AND 37°C

Material	Temperature (°C)	Affinity constant (M^{-1})	Enthalpy of adsorption (kcal/mol)
Si–pABA	37	$1.7 \cdot 10^6$	4.5
	21	$1.5 \cdot 10^6$	
	4	$0.7 \cdot 10^6$	
SiD–pABA	37	$4.1 \cdot 10^6$	4.7
	21	$3.7 \cdot 10^6$	
	4	$1.8 \cdot 10^6$	
SiD–B–pABA	37	$4.1 \cdot 10^6$	7.7
	21	$2.6 \cdot 10^6$	
	4	$1 \cdot 10^6$	
SiD–Arg	37	$1.5 \cdot 10^6$	4.2
	21	$1.1 \cdot 10^6$	
	4	$0.5 \cdot 10^6$	

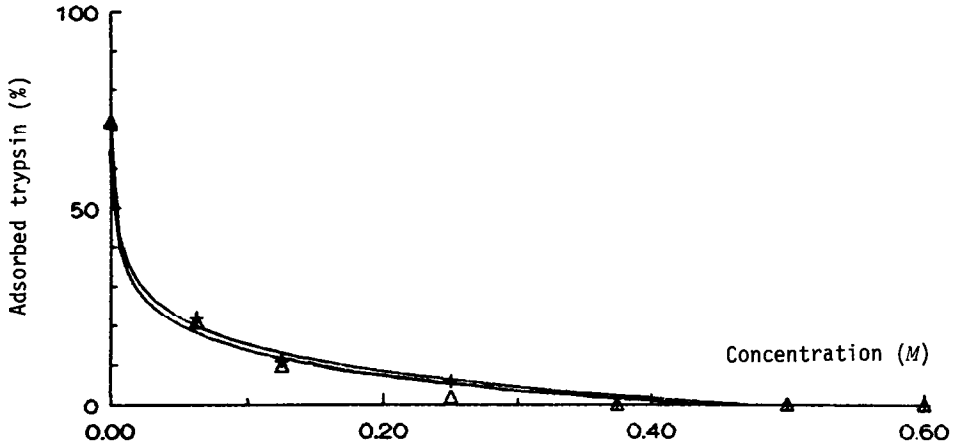

Fig. 4. Elution of bovine trypsin on SiD–pABA support by arginine (+) and guanidine (△) in HPLAC. Elution conditions: column, 12.5 × 0.4 cm; 0.05 *M* phosphate buffer, 0.1 *M* NaCl (pH 7.5) containing the competing substance at various concentrations; flow-rate, 1 ml/min.

Influence of ionic strength. Elutions of trypsin adsorbed on SiD–pABA and Si–pABA supports with solutions of sodium chloride at different molarities are presented in Fig. 5. Trypsin is adsorbed at low ionic strength and cannot be desorbed by increasing the ionic strength of the eluent. This observation is valid for these two supports. This result demonstrates that the interactions between trypsin and the supports functionalized with pABA are complex and are not only due to the ionic interactions taking place between the positively charged amidine function fixed on the support and the negatively charged carboxylic function of the aspartic acid 189 residue of the trypsin primary binding site. We must suppose the existence of different

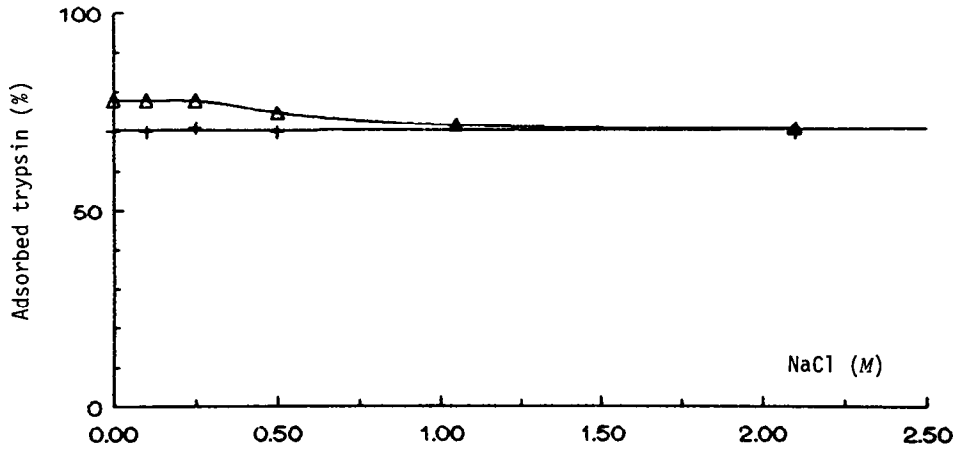

Fig. 5. HPLAC elution of bovine trypsin adsorbed on SiD–pABA (+) and Si–pABA (△) supports by sodium chloride. Elution conditions: column, 12.5 × 0.4 cm; 0.05 *M* phosphate buffer containing NACl at various concentrations (pH 7.5); flow-rate, 1 ml/min.

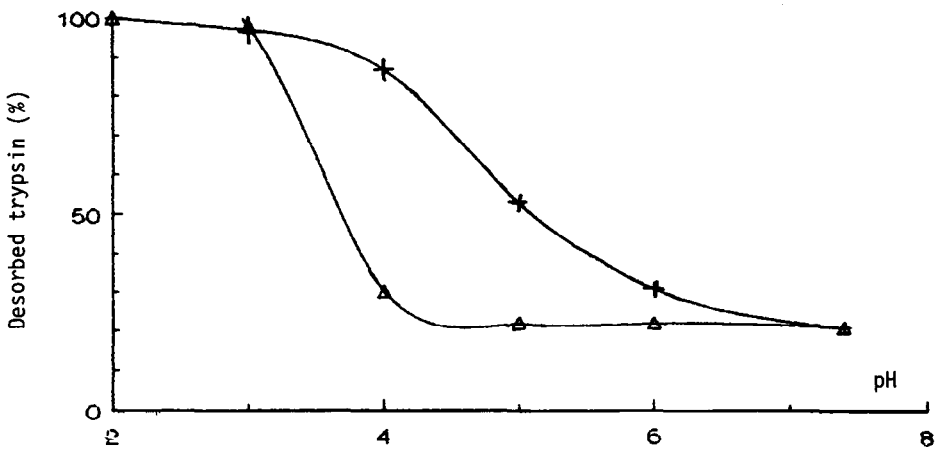

Fig. 6. Elution of bovine trypsin on SiD–pABA (+) and Si–pABA (△) supports by pH at 21°C in HPLAC. Elution conditions: column, 12.5 × 0.4 cm; 0.05 *M* phosphate buffer, 0.1 *M* NaCl; pH, from 7.5 to 2; flow-rate, 1 ml/min.

types of interactions, in particular hydrophobic interactions between the aromatic ring of pABA and the enzyme. At low salt concentrations on the commercial Si–pABA support, the amount of trypsin adsorbed is higher than in the case of SiD–pABA support. This may be due to the presence of non-specific interactions between the commercial support and the enzyme. Higher salt concentrations allowed the desorption of trypsin adsorbed in a non-specific manner. This result again demonstrates the importance of the passivation of the native silica obtained by coating with modified polysaccharides.

Influence of pH. The results of the chromatographic elution of trypsin with solutions at different pH values are presented in Fig. 6. The maximum adsorption of

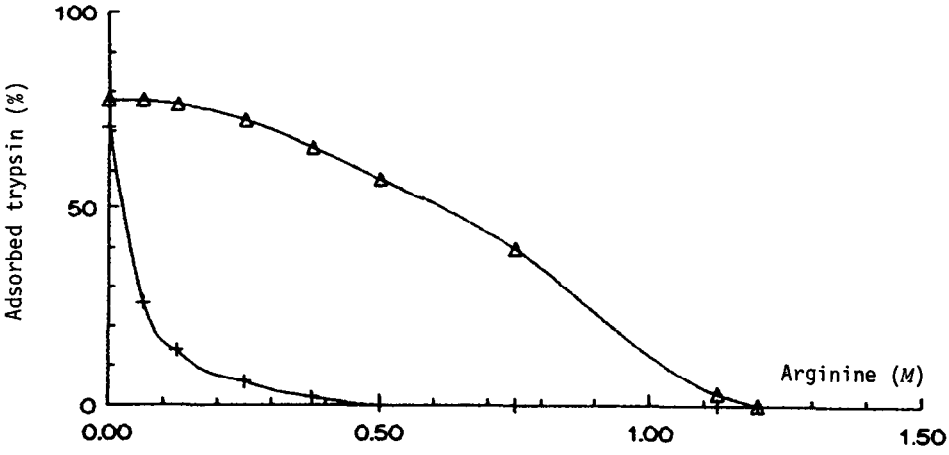

Fig. 7. HPLAC elution of bovine trypsin adsorbed on SiD–pABA (+) and Si–pABA (△) supports by arginine. The elution conditions are the same as in Fig. 4.

the enzyme is observed in the pH range 7–8. Desorption of the enzyme occurs when the pH of the eluent decreases. The breakdown of the interaction between trypsin and the support can be explained by the fact that at acidic pH the carboxylic function of the aspartic acid of the primary binding site, which possesses a pK_a of 2.3, is protonated and cannot interact with the amidine function. Desorption is obtained between pH 6 and 3 in the case of SiD–pABA support and between pH 4 and 3 in the commercial support. This result seems to indicate that the commercial support has a better resolution than SiD–pABA, probably related to the smaller granules and the better-defined porosity of the starting silica phase used for the commercial support.

Fig. 7 shows the trypsin elution by arginine in solution at different concentrations on the two supports. On the SiD–pABA support, trypsin elution is obtained by eluting the column with a 0.5 *M* arginine solution, but on the commercial Si–pABA support, this elution is obtained at higher arginine concentration (1.1 *M*). This indicates that the interactions taking place between trypsin and the ligand fixed on these two supports are stronger in the case of the commercial support but this result is inconsistent with the affinity constant values (Table I), which are the same for these two supports. The trypsin desorption differences on these two supports may be explained by the fact that the adsorbed trypsin is rapidly eluted by arginine according to a complex mechanism in the case of SiD–pABA and is slowly eluted from the commercial support. This observation demonstrates the improvement due to the better passivation obtained by polysaccharide coating of the silica beads. On this support, because of the passivation, the adsorption process is essentially the complex formation between the amidine residue and the binding site of the enzyme.

CONCLUSIONS

Amidine derivatives display a selective affinity for serine proteases, in particular for trypsin. These amidine derivatives are easily coupled on silica beads passivated with DEAE–dextran using conventional coupling reagents. Because of their mechanical properties, these phases are excellent supports for high-performance affinity chromatography. These active supports exhibit minimum non-specific ionic interactions with proteins in solution but a strong affinity for bovine trypsin. Affinity constant values are similar for all these supports. Among the synthesized supports, SiD–pABA presents a higher binding capacity in batch procedures and constitutes a good stationary phase in HPLAC for studying the interactions between trypsin and the specific site of the solid support. On this support, trypsin elutions obtained by competition with arginine and guanidine are similar, indicating that the same interactions probably occur between trypsin and these two competing substances. Comparison of the elution profiles of trypsin adsorbed on SiD–pABA or on the commercial Si–pABA support shows that in all cases the profiles are different, demonstrating that the trypsin interactions with pABA fixed on these two supports are different, complex, and depend on the nature of the support. On these two supports, the increase in ionic strength is unable to desorb the trypsin, indicating that the ionic interactions between trypsin and amidine do not prevail in this affinity reaction. The excellent resistance to hydrostatic pressure and high resolution of these two supports means that they can be used in HPLAC to separate and understand the serine protease interaction mechanism. Finally, the decrease in non-specific interactions obtained by coating the silica

beads with the modified polysaccharide enhances the importance of the specific affinity of amidine residues in the affinity process.

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