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High-performance affinity chromatography of proteins on non-porous polystyrene beads

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Abstract

Non-porous monodisperse polystyrene (PS) beads with an average diameter of 3.7 μm and a surface area of 1.56 m^2/g were prepared for the rapid analytical and micropreparative high-performance affinity chromatography of proteins. The PS was nitrated with acetic acid–sulfuric acid–nitric acid and reduced to yield hydrophilic amino groups by hydrogenation. Two affinity ligands, *p*-aminophenyl-β-D-glucopyranoside and *p*-aminobenzamidine, were immobilized on the amino-containing support via a cross-linker, hexamethylene diisocyanate. When the column (5 cm × 0.46 cm I.D.) packed with the resultant *p*-aminophenyl-β-D-glucopyranoside–PS was employed, the retained component of the concanavalin A (Con A) samples could be completely eluted in 3 min following a stepwise change of pH from 7.4 to 4.8. Thus, a calibration graph could be established from the peak area versus amount of Con A injected. A column (1 cm × 0.46 cm I.D.) packed with immobilized *p*-aminobenzamidine was also examined for protein chromatography. For a sample containing up to 200 μg of trypsin, the retained portion of proteins could be completely eluted in ca. 4.5 min. In summary, the prepared non-porous PS-based affinity adsorbent was found to be very efficient for analytical and micropreparative applications.

1. Introduction

Non-porous adsorbents of small particle diameter have been gaining in interest in recent years for protein chromatography using high-performance liquid chromatography (HPLC). Non-porous polystyrene-based adsorbents have been employed for the reversed-phased chromatography of proteins [1] and anion-exchange chromatography of proteins [2] and other small molecules [3–5]. Polystyrene (PS) or cross-linked poly(styrene–divinybenzene) can be used directly as an adsorbent for reversed-phase chromatography. In addition, it can be either surface sulfonated to give negative sulfonic groups or surface coated with polyethyleneimine for applications in ion chromatography. Only a few studies on the application of non-porous PS in affinity chromatography of proteins have been reported. Cross-linked poly(styrene–divinybenzene) microbeads have been coupled with dinitrophenylamino acids for the affinity chromatography of immunoglobulin E [6]. In a recent paper [7], PS microbeads were coated with poly(vinyl alcohol) and coupled with the reactive dye Cibacron Blue F3G-A for albumin adsorption. PS is inexpensive, readily available in different particle sizes and possesses mechanical

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rigidity. The great advantage of a PS-based over a silica-based support is its stability, covering a wide range of pH values. In this paper, we report a procedure for surface modification and ligand immobilization on microsized non-porous PS. PS was converted into aromatic amino-containing PS by nitration and successive reduction (hydrogenation). This amino-containing PS was ready for immobilization of affinity ligands via covalent binding.

Two affinity ligands, *p*-aminophenyl-β-D-glucopyranoside and *p*-aminobenzamidine, were covalently coupled to the chemically modified PS beads to make HPLC packings for the chromatographic analysis of concanavalin A (Con A) and trypsin, respectively. Affinity chromatography operated in the HPLC mode is called high-performance affinity chromatography (HPAC) and has been widely used as an analytical tool in biochemical research.

2. Experimental

2.1. Preparation and modification of non-porous PS beads

Non-porous PS beads with an average diameter of 3.7 μm were made by dispersion polymerization in ethanol with 2,2-azobisisobutyronitrile (AIBN) and polyvinylpyrrolidone (PVP) as the initiator and stabilizer, respectively. The apparatus for the polymerization consisted of a water-bath and a 250-ml three-necked round-bottomed flask, equipped with a mechanical (overhead) stirrer, a condenser and a connection with a nitrogen reservoir. The monomer solution was prepared by mixing 22 ml of styrene with 49 ml of ethanol in the flask at 60°C. AIBN (0.4 g) and PVP (1.6 g) were dissolved in 8 ml of ethanol and mixed with the monomer solution. Polymerization was carried out under nitrogen pressure and stirring (150 rpm) at 60°C for 1 day. The resultant PS beads were washed with methanol and dried in a vacuum oven at room temperature for 24 h.

Nitration of PS was carried out in a mixture of acids. Dried PS (10 g) was suspended in a flask containing 20 ml of 99.8% anhydrous acetic acid and stirred for 20 min at 60°C. A mixture of 65% nitric acid (20 ml) and 70% sulfuric acid (23 ml) was cooled to about 5°C and then added to the flask. After reaction for 3 h, the product was diluted with ice–water and washed successively with 0.1 *M* NaOH and distilled water. For the preparation of resins containing amino groups, 8 g of the nitrated PS, denoted PS-NO_2, were suspended in 15 ml of 99.8% anhydrous acetic acid and mixed with 59 ml of 6 *M* HCl containing 20 g of $SnCl_2 \cdot 2H_2O$. The hydrogenation reaction mixture was stirred for 2.5 days and the product was washed with 0.1 *M* NaOH and distilled water. The zeta potential of the non-porous beads was measured with a Zetasizer 3 (Malvern Instruments), in which beads were dispersed in 0.02 *M* phosphate buffer (pH 7).

2.2. Preparation of affinity adsorbents

Beads of the aromatic amino-containing PS, denoted PS-NH_2, were covalently coupled with *p*-aminophenyl-β-D-glucopyranoside and *p*-aminobenzamidine. PS-NH_2 (5 g) and 1,4-diazo[2,2,2]bicyclooctane (DABCO) (0.5 g) were mixed with 99.8% anhydrous acetic acid and stirred at 50°C for 15 min under nitrogen pressure. The cross-linker hexamethylene diisocyanate was then added to the above solution. After stirring for 8 h at 50°C, the unreacted hexamethylene diisocyanate was washed out with anhydrous acetic acid. The diisocyanate-activated PS was resuspended in fresh anhydrous acetic acid with the ligand, *p*-aminophenyl-β-D-glucopyranoside (0.01 g) or *p*-aminobenzamidine (0.5 g), and stirred for 16 h. The final product was washed with methanol and dried in a vacuum oven. The amount of *p*-aminobenzamidine bound to PS was determined spectrophotometrically, measuring the *p*-aminobenzamidine content of the acid hydrolysate.

2.3. Affinity chromatography of concanavalin A

The non-porous PS beads coupled with *p*-aminophenyl-β-D-glucopyranoside were slurry

packed into a 5.0 cm × 4.6 mm I.D. stainless-steel column using a Model CPP-085 column packer (Chemco). Pure Con A (Fluka) dissolved in 0.05 *M* Tris–HCl buffer containing 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 0.1 *M* NaCl (pH 7.4) was applied to the HPAC column at a flow-rate of 1.5 ml/min. The retained component was eluted with 0.01 *M* acetic acid buffer containing 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 0.01 *M* NaCl (pH 4.8). A mixture of Con A and bovine serum albumin (BSA) (Sigma) was also used as the sample injected on to the column. Peaks eluted from the HPAC column were detected at 280 nm using a Gilson Model 115 UV spectrophotometric detector. The peak area was integrated with an SIC Chromatocorder 12 integrator (System Instruments).

2.4. Affinity chromatography of trypsin

The affinity adsorbents coupled with *p*-aminobenzamidine were slurry packed into a 1.0 cm × 4.6 mm I.D. stainless-steel column using the same column packer. Trypsin (Type III, obtained from Sigma) dissolved in 0.05 *M* Tris–HCl buffer containing 0.25 *M* NaCl and 1 m*M* EDTA (pH 7.5) was applied into the HPAC column at a flow-rate of 1 ml/min. The elution buffer was 0.1 *M* glycine–HCl buffer containing 0.1 *M* NaCl (pH 2.6). Eluted peaks were detected at 280 nm and integrated with the SIC Chromatocorder 12 integrator. The peak data were also collected via a PCL-812 A/D converter (Advantech) connected to a personal computer.

For batch adsorption of trypsin, *p*-aminobenzamidine-immobilized PS (0.1 g) was placed in each tube with 10 ml of buffered trypsin (Type II, obtained from Sigma) solution with different initial concentrations. The buffer used was Tris–HCl (50 m*M* Tris–HCl, 0.5 *M* NaCl, 0.2 *M* $CaCl_2$, pH 7.8). The tube was gently rotated end-over-end at 4°C until an equilibrium between the proteins in the solution and on the adsorbents was reached. The concentration of protein was determined by measuring the UV absorbance at 280 nm.

3. Results and discussion

3.1. Properties of non-porous PS beads

PS beads with a monodisperse distribution of size ranging from 0.5 to 13.2 μm were prepared. The size distribution as shown in Fig. 1 was narrow. The average diameter of the non-porous beads was 3.7 μm and the surface area was 1.56 m^2/g, determined with an ASAP 2000 instrument (Micromeritics Instruments), and calculated with the BET equation using nitrogen as adsorbate. It was observed that the particle size of PS was controlled by the amounts of monomer, initiator and stabilizer and the type of dispersion medium, i.e., solvent, used for polymerization. When the solvent was methanol instead of ethanol, smaller beads (average diameter 2.0 μm) could be obtained. Both 3.7- and 2.0-μm PS beads are suitable for packings of HPLC columns.

3.2. Modification of PS beads

For utility as affinity adsorbents, PS should be chemically modified to become hydrophilic and contain functional groups for ligand immobilization. In the nitration procedure, sulfuric acid was present as a catalyst for the production of tran-

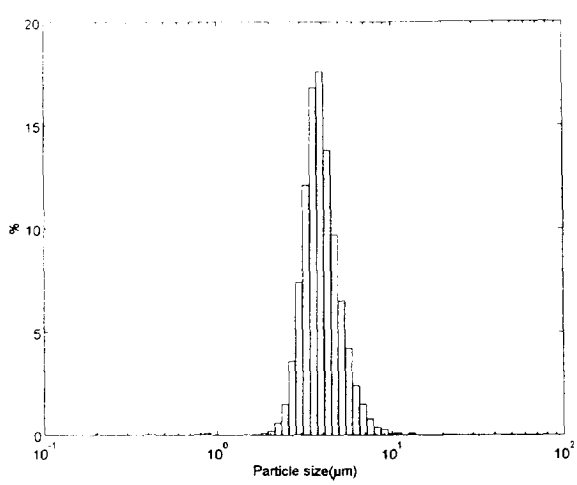

Fig. 1. Particle-size distribution of the polystyrene beads prepared by the method of dispersion polymerization.

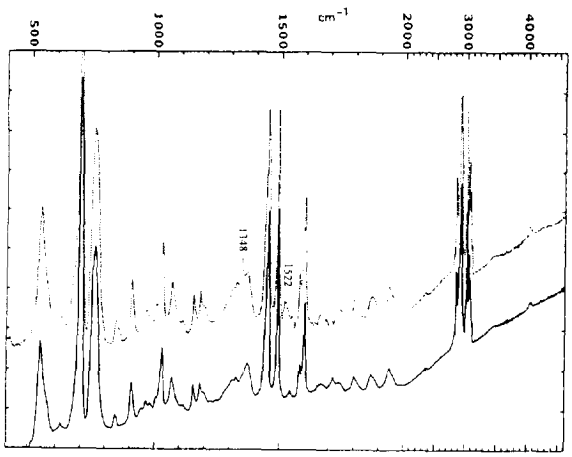

Fig. 2. FT-IR spectra of the prepared non-porous PS (solid curve) and nitrated PS (dashed curve).

sient nitronium ion (NO_2^+) during reaction. However, the long-term exposure of PS in sulfuric acid could cause undesirable sulfonation. No sulfonation was observed on the surface of PS that was exposed to 70% sulfuric acid for 3 h. However, sulfonation became evident when the time exceeded 3 h, which could be detected from the decline in the zeta potential of the PS beads. Therefore, the nitration of PS in the presence of sulfuric acid must be concluded within 3 h to avoid any possible sulfonation that could occur. In comparison with the unmodified PS, the FT-IR spectra of the resultant nitrated PS contain two additional peaks at 1522 and 1348 cm^{-1}, as shown in Fig. 2. These peaks were contributed by NO_2 groups on the benzene rings. Results from elemental analysis of the PS-NO_2 are given in Table 1. The experimental N:O ratio, which is close to the theoretical value (0.5), suggests that the nitration of PS was successful. No significant

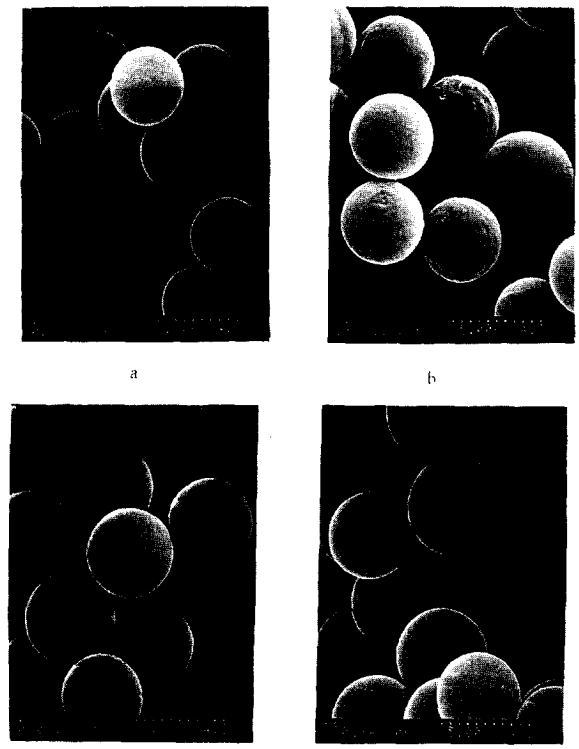

Fig. 3. Scanning electron micrographs of (a) non-porous PS beads, (b) PS-NO_2, (c) PS-NH_2 and (d) PS coupled with *p*-aminobenzamidine.

deformation of the PS beads after nitration and successive hydrogenation was observed on the scanning electron micrographs (Fig. 3).

The PS-NO_2 was converted into aromatic amino-containing PS by hydrogenation in acetic acid with HCl and $SnCl_2$ at 60°C for 2 days. The zeta potential of the beads changed significantly from −35 to −19 mV. An increase in zeta potential suggests that NO_2 groups were reduced

Table 1
Characterization of nitrated PS by elemental analysis

Run No.	C (%)	H (%)	N (%)	O (%)	N:O
1	91.14	8.01	0.28	0.57	0.56
2	91.02	8.04	0.29	0.65	0.51

to NH_2 groups. The zeta potential of the unmodified PS was −32 mV, which approached that of nitrated PS.

3.3. Immobilization of affinity ligands

The immobilization of low-molecular mass ligands on the surface of PS via the method of covalent binding is described here. The reactant hexamethylene diisocyanate played the role of a cross-linker and also a spacer between PS and the ligand. Diisocyanate reacted with the amino group on the benzene ring at one end and on the amino group of the ligand at the other end. The reaction of diisocyanate with amino-containing PS under a dry nitrogen pressure could be concluded in 8 h using DABCO as the catalyst. DABCO is an effective base catalyst for the reaction of amines and isocyanate. The unreacted diisocyanate should be removed by washing with acetic acid. For further reaction of immobilizing ligands, fresh acetic acid and DABCO were added to the isocyanate-activated PS and stirred for 16 h. The amount of *p*-aminobenzamidine bound to PS was determined as 1.1 μmol/g. This value corresponds to 0.7 μmol/m^2, which is higher than that reported [8] for coupling to porous silica (Fractosil 500) beads, 0.32 μmol/m^2. The specific surface area of the porous silica is 43.46 m^2/g and the density of *p*-aminobenzamidine ligands immobilized on the silica is 13.8 μmol/g.

3.4. High-performance affinity chromatography of Con A

When a 5-cm long column was operated at a flow-rate of 1.5 ml/min, the retained component of the Con A sample (20 μl) could be eluted by a stepwise change of pH from 7.4 to 4.8, as shown in Fig. 4. The non-retained peak in the Con A chromatogram constitutes ca. 20% of the total proteins. The retention time of the non-retained component did not change with sample size, as shown in Fig. 5, indicating that this retention time (0.32 min) is the time for the mobile phase to pass through all void spaces.

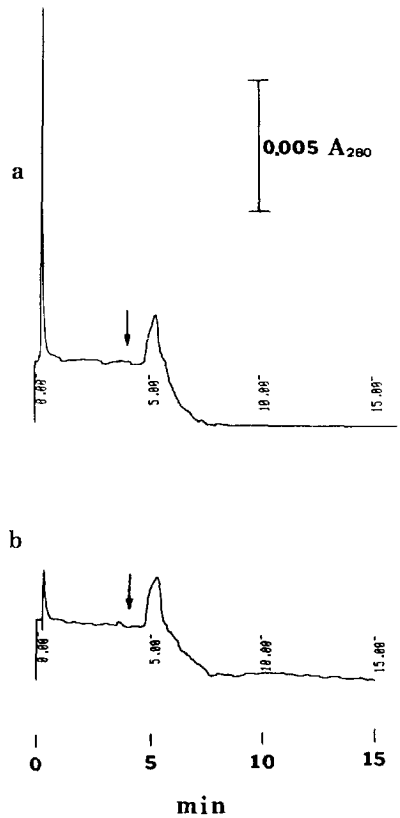

Fig. 4. Analysis of 20-μl samples containing (a) 40 μg each of Con A and BSA and (b) 40 μg of Con A only, using the column packed with *p*-aminophenyl-β-D-glucopyranoside–PS. The arrows indicate stepwise changes in the mobile phase pH from 7.4 to 4.8. Flow-rate, 1.5 ml/min; pressure, 170 bars.

The void volume, which is 0.48 ml, includes the extra-column volume, which was determined as 0.16 ml. The retained portion of the samples containing up to 55 μg of Con A could be completely eluted in 3 min after a stepwise change of pH. A significant shift of the baseline was observed owing to the change of buffers during pH elution. The composition of the starting buffer (Tris–HCl) was different from that of the elution buffer (acetic acid). However, the quantitative properties of the elution peaks were not affected by the shift of the baseline. The problem of the shift in baseline became even worse when elution was effected with a buffer containing competing free ligand, glucose. This

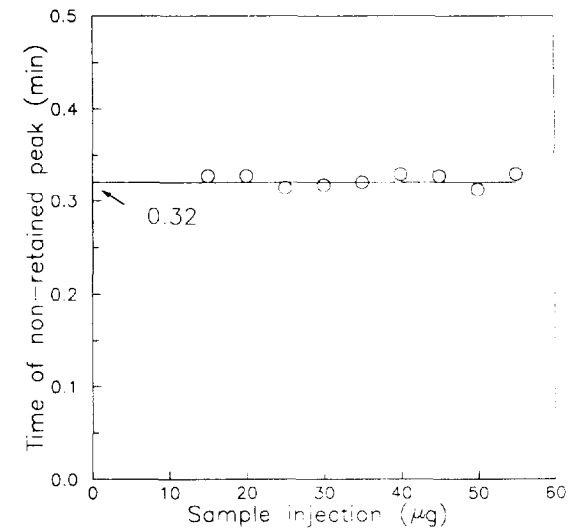

Fig. 5. Variation of retention time of the non-retained peak with sample loading. Flow-rate of effluent, 1.5 ml/min.

suggests that the use of the elution buffer should be subjected to further investigation.

Fig. 6 shows that when 20-μl samples with Con A content ranging from 15 to 55 μg were prepared as standards, a straight line was obtained in the plot of peak area versus Con A content. A straight line could also be obtained in the plot of peak height versus Con A content up to 40 μg. Each data point in Fig. 6 represents an average value resulting from two or three experimental runs. When a sample containing equal amounts (40 μg) of Con A and BSA was applied to the column, the chromatogram as shown in Fig. 4a was obtained. Results from sodium dodecyl sulfate polyacrylamide gel electrophoresis of the peak fractions indicated that no BSA appeared in the retained fraction. Comparison of Fig. 4b and a suggests that the retained peaks in each instance are almost identical in their shape and area. The data indicate that this affinity column is suitable for the chromatographic determination of Con A. The error in the HPLC analysis using the affinity column was within 4%.

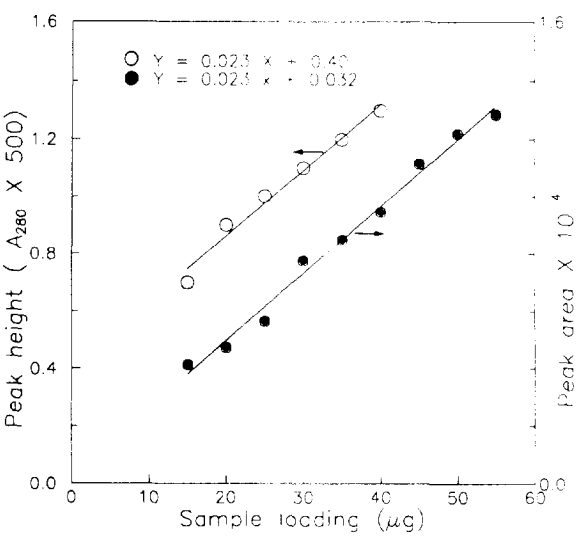

Fig. 6. Calibration graphs of peak height (peak area) versus Con A concentration. The HPAC column (5 cm × 0.46 cm I.D.) was operated at a flow-rate of 1.5 ml/min and 20-μl samples were injected.

3.5. *Affinity chromatography of trypsin*

The utility of the 1-cm column packed with the *p*-aminobenzamidine-immobilized PS for trypsin (Type III) chromatography is demonstrated in Fig. 7. Elution was achieved by a stepwise change of buffers from Tris–HCl (pH 7.5) to glycine–HCl (pH 2.6). A linear relationship was observed between the elution peak area and the trypsin content in the sample (Fig. 8). The retention time of the retained peak was approximately 1.7 min. In contrast to the elution of Con A, no significant shift of the baseline was observed in the elution of bound trypsin. The small fluctuations that appeared on the peaks in Fig. 7a and b were caused by the truncation of the digits of the data collected by the A/D converter. For a sample containing up to 200 μg of trypsin, bound proteins could be eluted completely in ca. 4.5 min following the stepwise change of pH. This suggests that the affinity column was suitable for rapid micropreparative chromatography even though it is only 1 cm long. The total void volume of this column was determined as 0.22 ml, which includes the extra-column volume, 0.14 ml.

The density of bound trypsin (Type II crude)

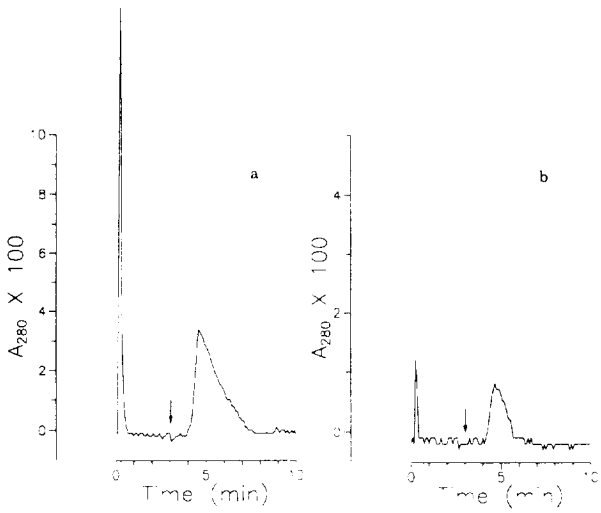

Fig. 7. Chromatograms of trypsin samples (20 μl) with concentrations of (a) 10 and (b) 0.5 mg/ml. The HPAC column (1 cm × 0.46 cm I.D.) packed with *p*-aminobenzamidine-immobilized PS was operated at a flow-rate of 1 ml/min. The arrows indicate stepwise changes in mobile phase pH from 7.5 to 2.6.

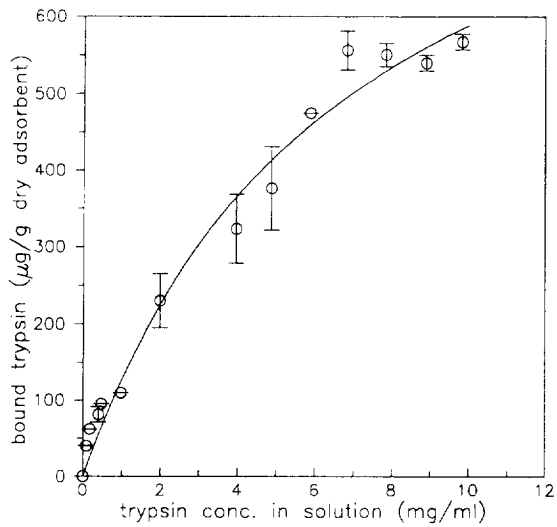

Fig. 9. Adsorption isotherm for trypsin (Type II, Sigma) and *p*-aminobenzamidine-immobilized PS. A 0.1-g amount of adsorbent was placed in each tube with 10 ml of buffered trypsin solution with different initial concentrations. The buffer used was Tris–HCl (50 m*M* Tris–HCl, 0.5 *M* NaCl, 0.2 *M* $CaCl_2$, pH 7.8). The tubes were gently rotated end-over-end at 4°C for 6 h. Protein adsorption was determined by measuring the loss of solution protein.

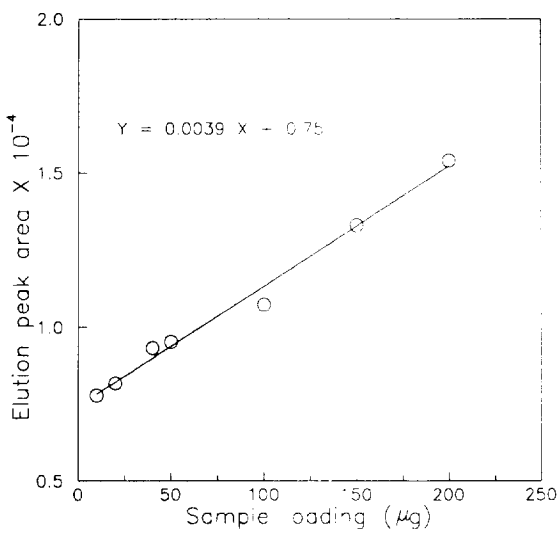

Fig. 8. Calibration graph of peak area vs. trypsin concentration. The HPAC column (1 cm × 0.46 cm I.D.) was operated at a flow-rate of 1 ml/min and 20-μl samples were injected.

was plotted against the equilibrium trypsin concentration in the solution (Fig. 9). The maximum binding capacity was observed to be ca. 550 μg/g. The adsorption capacity at this level due to the use of non-porous adsorbents with a low specific surface area suggests that the prepared affinity adsorbent is suitable for the analysis of protein samples of microgram size. The solid line in Fig. 9 was generated from the best fit of the data with the Langmuir model. It is evident that the equilibrium relationship is not exactly of Langmuir type.

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References

[1] Y.-F. Maa and C. Horvath, J. Chromatogr., 445 (1988) 71–86.
[2] M.A. Rounds and F.E. Regnier, J. Chromatogr., 443 (1988) 73–83.
[3] Y. Miura and J.S. Fritz, J. Chromatogr., 482 (1989) 155–163.
[4] L.M. Warth, J.S. Fritz and J.O. Naples, J. Chromatogr., 462 (1991) 165–176.
[5] R.F. Strasburg, J.S. Fritz and J.O. Naples, J. Chromatogr., 547 (1991) 11–19.
[6] S. Wongyai, J.M. Varga and G.K. Bonn, J. Chromatogr., 536 (1991) 155–164.
[7] A. Tuncel, A. Denizli, D. Purvis, C.R. Lowe and E. Piskin, J. Chromatogr., 634 (1993) 161–168.
[8] R.-M. Chang and W.-C. Lee, J. Chem. Technol. Biotechnol., 59 (1994) 133–139.