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Research Article

Synthesis and characterization of (R)-phenylephrine bonded non-porous polystyrene-divinylbenzene for fast separation of proteins by mixed-mode liquid chromatography

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ABSTRACT

A mixed-mode liquid chromatography PSDVB-PE solid support based on (R)-phenylephrine bonded non-porous polystyrene-divinylbenzene (PSDVB) was prepared and characterized for the fast separation of proteins. The support consisting of both anion-exchange and hydrophobic moieties was synthesized by bonding of the (R)-phenylephrine to the non-porous PSDVB particles *via* hydroboration, bromination and nucleophilic substitution reactions. The support with a surface coverage of 420 µmol/g was characterized with regard to its physical and chromatographic properties. The reverse phase and anion-exchange behaviors of this new stationary phase were investigated by injection of acidic, neutral and basic molecules at a wide pH range (pH 2 to 11.0) of eluents. The retention behaviors of the tested compounds (e.g. ethylparaben, benzoic acid, benzene and aniline) were studied by modifications of pH values and amounts of organic modifiers in the mobile phase in an isocratic elution. Fast separation of six proteins (e.g. cytochrome c, immunoglobulin G, ribonuclease A, human serum albumin, ovalbumin, and conalbumin) within 3 min showed many advantages over non-modified PSDVB support. Applications for separation of proteins in papaya latex, egg white, and human serum were demonstrated. Compare to the reverse phase column, the mixed-mechanism column provided a flexible and versatile method for fast separation of proteins.

1. INTRODUCTION

Biomolecules, especially protein therapeutics, are gaining popularity for medical treatments including cancers, cardiovascular diseases, infectious, genetic disorders and other diseases[1-2]. Quality assurance and control of biomolecules are of great importance to ensure their quality, safety and efficacy. During the past decades, analytical method developments of biomolecules have been an active area in pharmaceutical and biopharmaceutical researches due to the increased usage of protein therapeutics[3]. Many methods have been developed for purification and analysis of these biomolecules including electrophoresis[4], high-performance

liquid chromatography (HPLC)[4,5], and immunological[6] method. Among these techniques, HPLC, based on the interaction between analytes and stationary phases is the most widely used technique[7]. Traditionally, only one dominant interaction between the analytes and stationary phase is involved in chromatographic separation such as reversed-phase liquid chromatography (RPLC), hydrophilic interaction chromatography (HILIC), ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC). However, the single interaction may not sufficient to provide satisfactory resolution for complex biomacromolecules, mainly proteins[8]. Novel stationary phases with additional interaction mechanism are required to provide better protein separation[9]. Mixed-mode or multimode chromatography has attracted much attention to improve separation selectivity and resolution of protein[10-12]. Various multifunctional stationary phases have been of interest in the separation of biological molecules such as amino acids[13], nucleic acids[14,15], peptides[16,17] and proteins[18,19]. Mixed-mode stationary phases could be obtained by combining two types of stationary phases on a column[20] or employing the chemical derivatization of the sorbents with multifunctional ligands[19,21]. Historically, silica-based stationary phases are mostly employed in mixed-mode chromatography due to their excellent mechanical strength and efficiency. However, silica is known to dissolve at pH above 7.0, thus limits its uses in alkali eluents.

Highly cross-linked polystyrene-divinyl-benzene (PSDVB) supports show excellent mechanical properties and good chemical stability over a wide range of pH, which enable them to operate under various pH ranges of the mobile phase[22]. PSDVB particles are very hydrophobic and can be used directly as a reversed-phase matrix[23], however, modified PSDVB supports are of interest to many researchers in order to improve efficiency as well as selectivity against certain targeted compounds[24-27]. Octadecyl (C18) bonded to non-porous PSDVB beads have been successfully used for the separation of single- and double-stranded nucleic acids with high resolution[28-29]. Proteins are biomolecules consisting of both hydrophobic and charged moieties, ion-exchangers based on PSDVB can be alternatives for protein separation.

Presently, new PSDVB-based mixed mode beads carrying reversed-phase and weak anion-exchange sites were synthesized and characterized for fast separation of proteins. The supports were prepared by coupling of (R)-phenylephrine[30] on the surface of non-porous PSDVB particles. The physical and chromatographic properties of the new supports were evaluated on neutral, acidic and basic small molecules. Finally, the supports were applied for fast separation of different proteins including papaya latex, egg white, immunoglobulin G (IgG) and human serum albumin (HSA). The developed PSDVB-based mixed mode support and the optimized LC condition would serve as a novel method for rapid separation of biomolecules

2. MATERIALS AND METHODS

2.1 Reagents

HSA, conalbumin, lysozyme and ovalbumin from chicken egg white, IgG from human serum, papain and chymopapain from papaya latex, ribonuclease A (RNase A) from bovine pancreas, myoglobin from horse heart, and cytochrome c (Cytc) from bovine heart, styrene, divinylbenzene (DVB) (55%), 1-chlorododecane (1-CD), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), potassium peroxosulfate and phosphorus penta-bromide (PBr_5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzoyl peroxide (BPO), 1M borane (B_2H_6) in THF, sodium chloride (NaCl), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), sodium tetraborate decahydrate, aniline, hydrogen peroxide (30%) and methanol (MeOH) were of analytical grade from Merck (Darmstadt, Germany). (R)-Phenylephrine hydrochloride (pharmaceutical grade) was from Iwaki Seiyaku (Tokyo, Japan). HPLC grade acetonitrile (ACN) was from JT baker (Phillipsburg, NJ, USA). Water was deionized and double-distilled.

2.2 Instrumentation

The chromatographic system from Thermo Separation Products (San Jose, CA, USA) was equipped with a Spectra system pumps P4000, a Spectra system autosampler AS3000 and a Spectra system detector UV6000. Chromatographic data were recorded and processed with ChromQuest

4.2.34 version 3.1.6 data acquisition system (Thermo Finnigan, San Jose, CA, USA) chromatographic software. Determination of C/H/N was performed using 2400 CHN Elemental Analyzer (Perkin Elmer, Norwalk, USA). The scanning electron microscope was Jeol (JSM-6400, Tokyo, Japan) and the Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectrophotometer was Nicolet 6700, Thermo Scientific (MA, USA). The solid state nuclear magnetic resonance (NMR) spectrometer 400 MHz was Bruker Avance III HD 400 WB (Karlsruhe, Germany) at 75 MHz.

2.3 Synthesis of (R)-phenylephrine bonded non-porous PSDVB

Non-porous PSDVB beads with a mean diameter of 2.2 μm were prepared by a two-step microsuspension method[23]. The PSDVB beads were chemically modified by hydroboration, bromination, and nucleophilic substitution with (R)-phenylephrine (Supported document 1) as described below.

Hydroboration was done by suspending dried PSDVB beads (3 g) in anhydrous THF (60 mL). While stirring, a 1 M solution of B_2H_6, THF (4 mL) was dropwise added at room temperature (RT) under nitrogen. After 3 h of stirring at RT, water (1 mL) was dropwise added followed by 3 M NaOH (1.5 mL). Hydrogen peroxide (30%, 1.5 mL) was added at such a rate that the temperature of the reaction mixture was between 30-50°C. The solution was stirred for 1 h at RT and the hydroxylated PSDVB (PSDVB-OH) beads were washed with water, acetone, and then dried at 80°C for 2 h.

Bromination was performed using a modified method described by Eliel et al[31]. PBr_5 (~100 mg) was dissolved in DCM (2-3 mL) and was slowly added to the cooled and well-stirred mixture of PSDVB-OH (3 g) in DCM (40 mL). The mixture was continuously stirred for 3 h, added with ice water and stirred for another hour. The obtained brominated PSDVB (PSDVB-Br) beads were washed with water, $NaHCO_3$ solution (4.2%), acetone, and then dried at 80°C for 2 h.

Nucleophilic substitution with (R)-phenylephrine was prepared by dissolving (R)-phenylephrine (0.2 g) with DMF (20 mL). The mixture was added to the suspension of PSDVB-Br (2 g) in DMF (40 mL). The mixture was refluxed for 2 hours under nitrogen and then was allowed to cool to RT. Then, the suspension was washed with water, $NaHCO_3$ solution (4.2%), acetone, and then dried at 80°C for 2 hours.

2.4 Characterization of non-porous PSDVB-PE support

Ligand density of the modified PSDVB-PE was determined by elemental analysis, and the calculation based on the nitrogen contents. The presence of the functional ligands on the PSDVB support were investigated by FTIR and the solid state NMR spectroscopy. All FTIR spectra were recorded from 4000 - 400 cm^{-1} with a resolution of 4 cm^{-1} corresponding to 32 scans per spectra. Mechanical stability of the support was evaluated by measuring the pressure drop across the column using various solvents (e.g. water, MeOH and ACN) at different flow rates. (e.g. 0- 4 mL/min)

2.5 Chromatographic conditions

The PSDVB-PE support was packed into a 50 mm x 4.6 mm i.d. stainless steel column by conventional high-pressure slurry-packing procedures[32]. For small molecules separation, the mobile phase consisting of 30% (v/v) ACN in 30 mM aqueous phosphate (pH 2.4-4.2), Tris (pH 7.0), borate (pH 9.0) and carbonate (pH 11.0) buffers were studied. For protein separation, 0.1% TFA (pH 2.2), 30 mM borate (pH 9.0) and 30 mM carbonate (pH 11.0) containing various ratios of ACN was investigated.

3. RESULTS AND DISCUSSION

3.1 Physical characterization

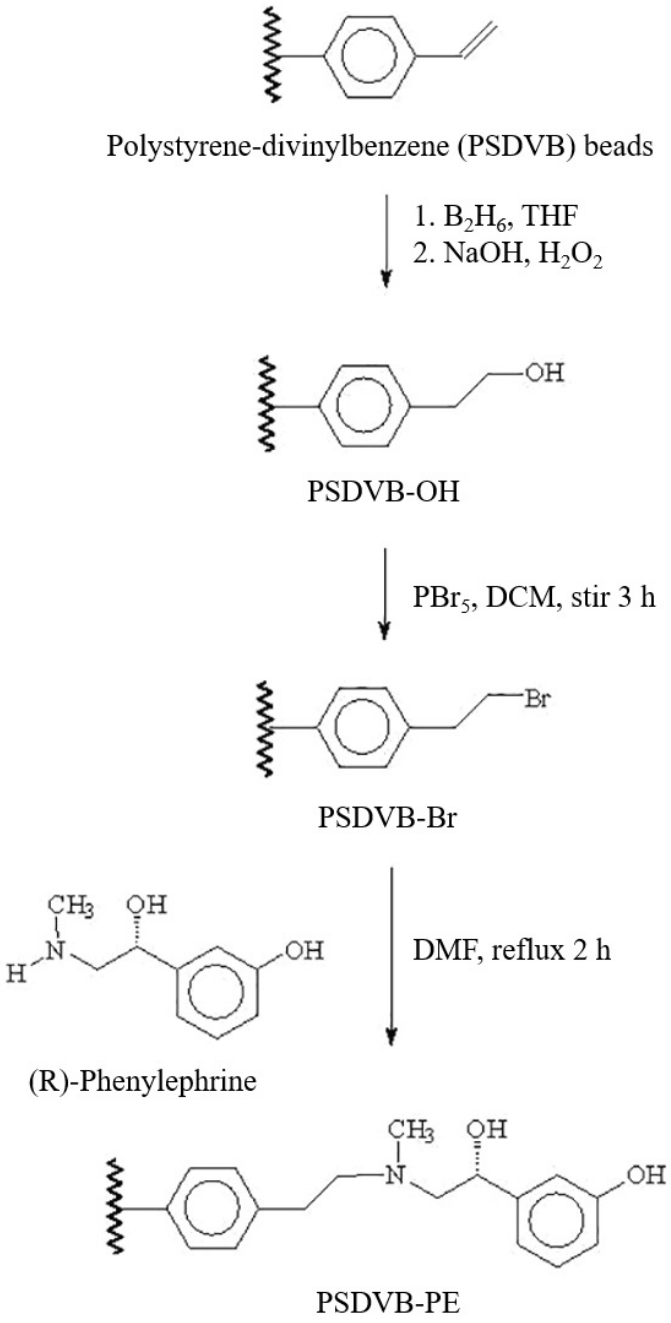
The average diameter of the synthesized non-porous PSDVB beads was 2.2 μ obtained from the scanning electron microscope (Supported document 2).

3.2 Chemical modification of the PSDVB beads

The new stationary phase, PSDVB-PE, was prepared by grafting of (R)-phenylephrine on the bare PSDVB beads via a three-step reaction as depicted in Scheme 1. Initially, hydroxyl groups were added to the free vinyl groups on the PSDVB

beads to obtain PSDVB-OH. Subsequently, hydroxyl moieties were replaced by bromide to give PSDVB-Br using PBr_5 as a reagent. Finally, (R)-phenylephrine was grafted to PSDVB-Br beads by N-alkylation. The IR spectra of PSDVB, PSDVB-OH, PSDVB-Br and PSDVB-PE beads were compared (Supported document 3). The broad peak at the 3700-3200 cm^{-1} showed the hydroxyl character of PSDVB-OH. The bromination of PSDVB-OH was confirmed by the absence of a broad O-H peak of PSDVB-OH. After engrafting of (R)-phenylephrine on PSDVB-Br beads, the IR spectrum exhibits (R)-phenylephrine's characteristic peaks (meta-disubstitution of the aromatic ring at 1900-1682 cm^{-1}, O-H broad peak at 3700-3200 cm^{-1}).

The elemental analysis data of non-modified and modified PSDVB is shown in Table 1. The density of OH, Br, and (R)-phenylephrine attached to the PSDVB beads were 2275, 870, and 420 μmol/g, respectively.



Scheme 1. Synthesis of PSDVB-PE

Table 1. Elemental analysis data of non-modified and modified PSDVB and density of the attached ligands

Sample	%C	%H	%N	Ligand density (μmole/g)
PSDVB	92.26	8.72	0	-
PSDVB-OH	88.02	8.34	0	2275
PSDVB-Br	84.83	8.22	0	870
PSDVB-PE	86.26	8.52	0.59	420

The ^{13}C NMR spectra of PSDVB and PSDVB-PE is shown in Figure 1. The identification of the detected carbon of PSDVB is shown in Table 2. The absence of peak 2 and 4 in the PSDVB-PE spectra indicated that the addition of hydroxyl ligand to the residual vinyl groups was successful. The presence of the (R)-phenylephrine ligand was observed by the enlargement of peak 5, 6, and 7 resulting from the addition of (R)-phenylephrine.

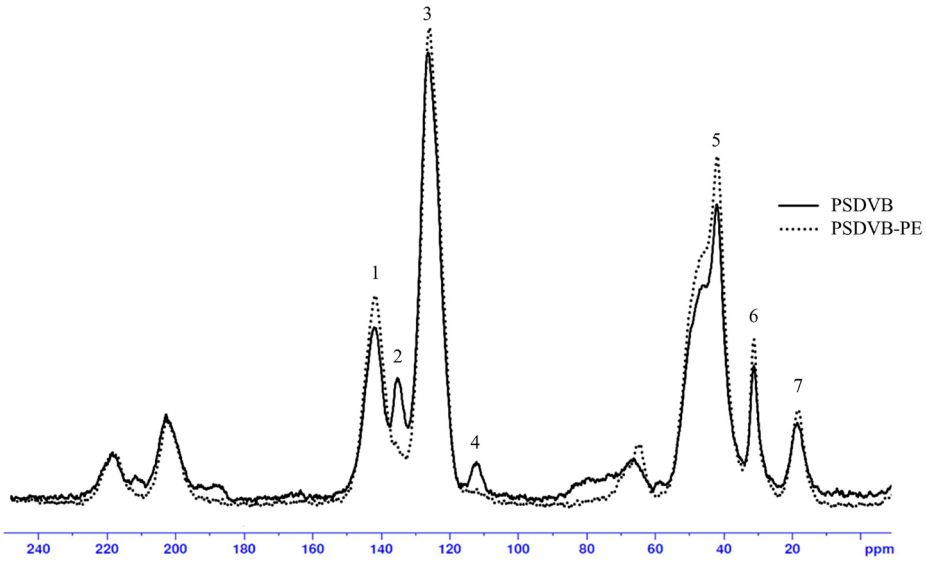
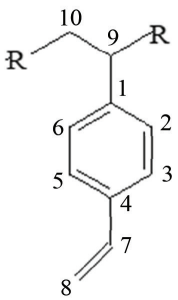


Figure 1. ^{13}C NMR of the PSDVB and PSDVB-PE

Table 2. ^{13}C NMR chemical shift and assignment of PSDVB

Compound	Peak no.	Chemical shift (ppm)	Assignment
PSDVB			
	1	144.2	1,4
	2	137.2	7
	3	127.9	2,3,5,6
	4	113.8, 113.3	8
	5,6	44.0, 40.2	9,10
	7	28.9	7
	8	15.6	8

3.3 Evaluation of the mechanical stability

Evaluation of the mechanical stability of column packing can be done by measuring the pressure drop across the column upon perfusing it with various solvents and flow rates. Methanol, water, and acetonitrile was pumped through the column packed with PSDVB-PE beads at a flow rate up to 4.0 mL/min. A good linear relationship was obtained up to 3,000 psi indicating that the packing materials were rigid and stable up to this pressure limit (Figure 2). This high pressure is well above the operating range of most HPLC applications.

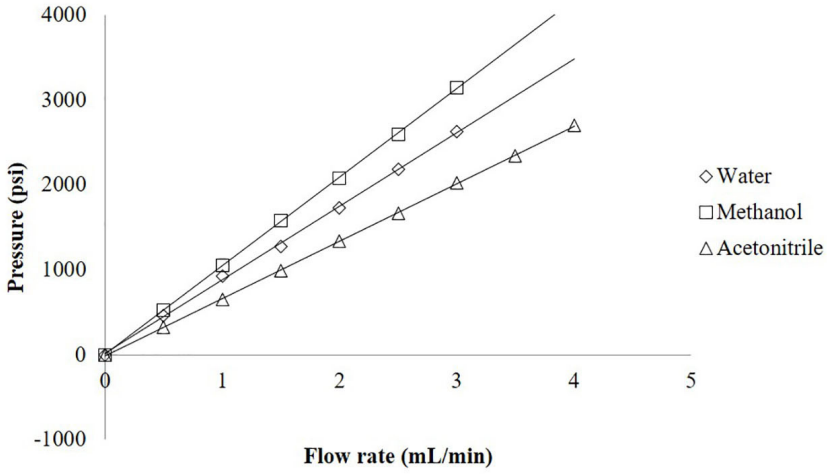


Figure 2. Mechanical stability of PS-DVB-PE column

3.4 Chromatographic behaviors of the PSD-VB-PE column on small molecule separation

3.4.1 Effects of organic modifier concentrations

Performances of PSDVB-PE column on the separation of small molecules were done using ethylparaben, benzoic acid, and aniline as model analytes for neutral, acidic and basic compounds, respectively. Selectivity of the PSDVB-PE supports was modified by varying amounts of organic modifiers in the mobile phase. Ethylparaben, benzoic acid and aniline were isocratically chromatographed using 0.1% TFA in water (solvent A) and 0.1%TFA in ACN (solvent B) as the eluent. Capacity factors (k′) were calculated and plotted as a function of the acetonitrile content of the eluent. Increasing amount of ACN from 20 to 40% (v/v) resulted in the decreased retention of ethylparaben, benzoic acid and aniline. The dependence of log k′ on the concentrations of ACN was very close to linear for all three compounds under conditions of the experiments, as expected for a regular reversed-phase system (Figure 3).

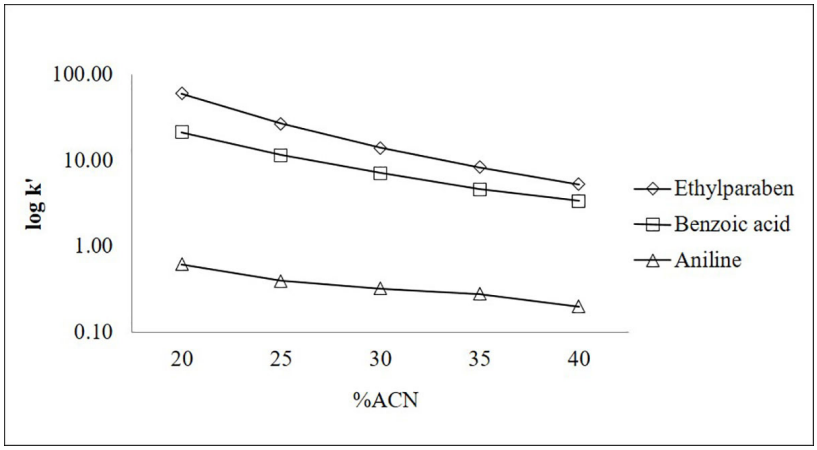


Figure 3. Effect of organic solvent on capacity factors

3.4.2 Effects of eluent pH

Effects of eluent pH on the capacity factors of neutral, basic and acidic compounds on non-modified PSDVB and PSDVB-PE is shown in Figure 4. No change in retention was observed for a neutral compound (i.e. benzene) between pH 2.4 and 11.0. To evaluate the hydrophobicity of the column, benzene was separately injected to the non-modified PSDVB and PSDVB-PE columns under the same conditions. The average capacity factors obtained from these two columns were 50 and 34, respectively, indicating that the PSDVB-PE phase was less hydrophobic. On the other hand, capacity factors of basic compound such as aniline (pK_b 9.8) decreased remarkably at lower pH. The decrease in retention of aniline was 30 fold when the pH changed from 4.2 to 2.4 because the amino groups of both the analytes and stationary phase became protonated and ion-repulsion becomes the predominant interaction at lower pH. At pH above 5.1, hydrophobic interaction was enhanced due to the decreased protonation. Thus, variation of solvent pH had pronounced effects on selectivity as shown in the pH profile.

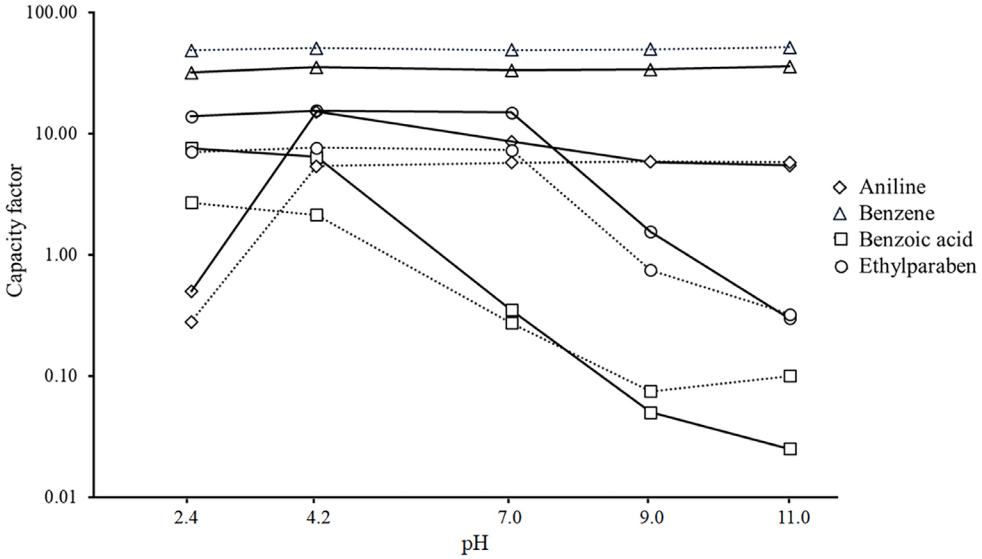


Figure 4. Effects of pH on the capacity factors of the investigated small aromatic compounds on PSDVB (....) and PSDVB-PE (-) column

Capacity factors of benzoic acid (pK_a 4.2) and ethylparaben (pK_a 8.4) decreased as the eluent pH was higher than their pK_a. This effect can be explained by the weakening of the ionic interaction due to the decreased dissociation of the analytes and an increase in the ionic interaction between the buffer anions and the amino groups of the stationary phase. At pH lower than the pK_a of the respective analyte, benzoic acid and ethylparaben were highly retained since the molecules were uncharged and hydrophobic interaction became predominated. The retention behaviors of acidic compounds on PSDVB-PE was in agreement with that of acidic xenobiotic metabolites, (e.g. t,t-muconic acid) on a methylstyrene-divinylbenzene-based, strong anion-exchange column[33]. Also, similar retention was observed on the separation of substituted aromatic acids on a mixed bed stationary phase packed with C18 and strong anion-exchange bonded silica particles[34]. In summary, chromatographic behaviors of the investigated small molecules on PSDVB-PE sorbents were mainly based on reversed-phase and weak anion-exchange chromatography.

3.5 Chromatographic behaviors of the PSDVB-PE column on protein separation

Effects of eluent pH (pH 2.2, 9.0 and 11.0) on the separation of ten proteins were investigated using a linear gradient elution from 20 to 80% ACN in 4 min, at 80 °C. The retention of each protein on the PSDVB-PE column is summarized in Table 3. The average peak width at half height ($W_{0.5}$) of the tested proteins at pH 2.2, 9.0 and 11.0 were

0.29, 0.15 and 0.14 min, respectively. Reduction of peak widths in alkali eluents was desirable for protein separation to improve the separation efficiency. The retention of each protein, hence, selectivity, was differently affected by pH of the eluent.

The selectivity of the protein separation using the non-modified PSDVB and PSDVB-PE columns under the same chromatographic conditions were compared (Figure 5). Under the same acidic condition, the PSDVB-PE column provided a better separation with enhanced selectivity for the six proteins. Evidently, the use of borate buffer at a higher pH (pH 9.0) gave a baseline separation of six proteins in 3 min (Figure 5c).

Table 3. Effects of pH on the capacity factors of the investigated proteins on PSDVB-PE column

	Capacity factor (k´)		
Eluent pH*	2.2	9.0	11.0
Conalbumin	13.8	12.4	9.7
Papain	13.0	11.2	8.6
Ovalbumin	11.9	10.8	8.5
HSA	11.3	9.8	8.1
Chymopapain	10.8	8.8	7.7
Lysozyme	10.0	8.6	6.7
RNase A	9.2	7.4	5.8
IgG	8.4	6.4	5.2
Cytc	7.6	4.8	3.9
Myoglobin	7.3	3.9	3.2

* Aqueous starting eluent: 0.1%TFA (pH 2.2), 30 mM Na2B4O7 (pH 9.0), 30 mM Na2CO3 (pH 11.0).

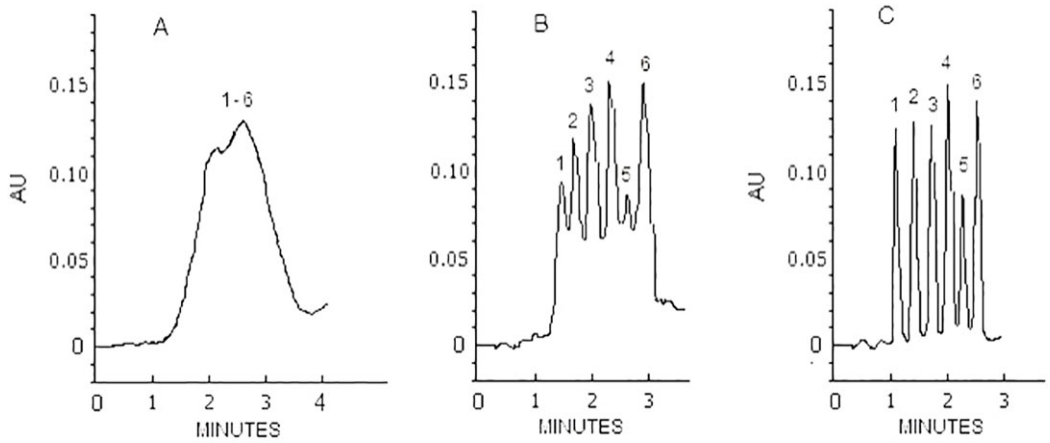


Figure 5. Effects of stationary phases and eluents on the separation of proteins. Column: (A) PSDVB (2.2µ, 50 x 4.6 mm i.d.), (B) and (C) PSDVB-PE (2.2µ, 50 x 4.6 mm i.d.). Flow rate: 2 mL/min. Aqueous starting eluent: (A) and (B) 0.1%TFA (pH 2.2), (C) 30 mM Na2B4O7 (pH 9.0). Linear gradient in 4 min from 20 to 80% acetonitrile in the starting eluent. Temperature: 80°C. UV Detector: 280 nm. Samples: (1) 100 ng of Cytc, (2) 100 ng of IgG, (3) 100 ng of RNase A, (4) 100 ng of HSA, (5) 50 ng of ovalbumin, (6) 100 ng of conalbumin

3.6 Applications

Chromatographic separations of some natural protein mixtures (1 mg/mL in water) were conducted on the PSDVB-PE column using 30 mM borate buffer (pH 9.0) in ACN as the eluent. Results suggested the capability of the PSDVB-PE column for fast separation of biomolecules (within 3 min). The PSDVB-PE column was applied for separation of enzymes from fresh papaya latex and egg white proteins. Papaya latex proteases are composed of four major cysteine proteases: papain, chymopapain, glycyl endopeptidase, and caricain [35]. Chymopapain and papain have found a wide application, especially in pharmaceutical and food industries. Chromatogram of fresh Carica papaya latex is shown in Figure 6. Four outstanding peaks, including papain and chymopapain, were readily resolved within 3 min. Separation of egg white proteins was also investigated as shown in Figure 7. Ovalbumin (54%), conalbumin (12%), ovomucin (3.5%), and lysozyme (3.4%) are among the major proteins in egg white [36]. Five peaks appeared in the chromatogram and they were baseline resolved within 3 min.

In order to examine the ability of the PSDVB-PE column for protein separation in biomedical research, serum samples were subjected to the chromatographic analysis. Two frozen serum samples (1 ml each) were kindly provided from the Faculty of Medical Technology, Rangsit University, Thailand. The sera came from the leftover blood samples and were de-identified. The patient consented to donate the leftover blood to be used for medical research. Human serum contains two classes of proteins including albumin and globulins. IgG is one of the gamma globulins which are known as antibodies. IgG and HSA were found in the chromatogram of pooled normal serum as shown in Figure 8A. Serum from a patient with high fever was examined and the increase of IgG peak was obviously observed (Figure 8B).

The fast separation of some proteins in this study provided the potential applicability of PSDVB-PE column for practical protein samples. The development of such a mixed-mode stationary phase can play an important role in meeting the challenge of protein and biopolymer separation.

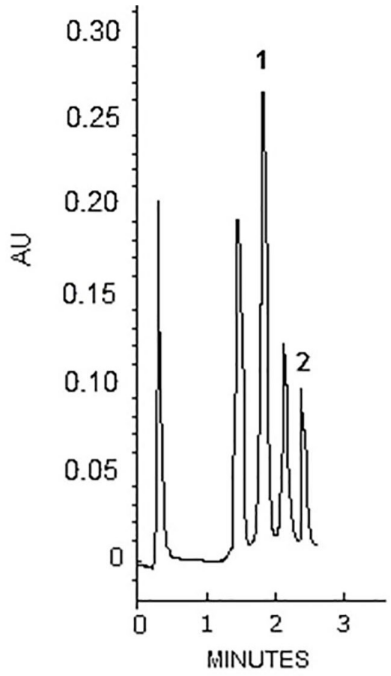


Figure 6. Chromatogram of papaya latex. Column: PSDVB-PE (2.2µ, 50 x 4.6 mm i.d.). Flow rate: 2 mL/min. Aqueous starting eluent: 30 mM Na2B4O7 (pH 9.0). Linear gradient in 4 min. from 20 to 80% acetonitrile in the starting eluent. Temperature: 80°C. UV Detector: 280 nm. Peaks: (1) chymopapain, (2) papain. Sample size: 40 µg

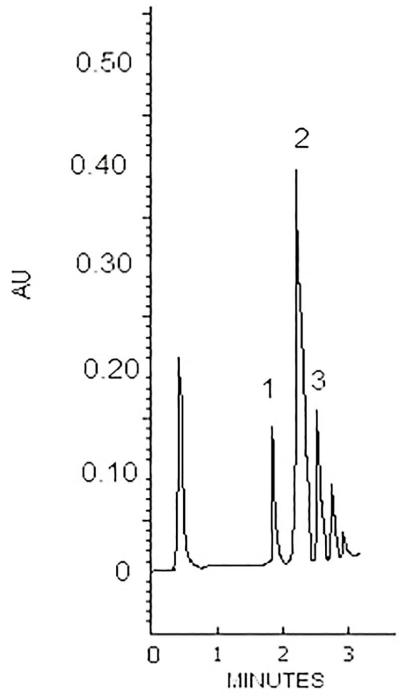


Figure 7. Chromatogram of egg white proteins. Chromatographic conditions as in Figure 5. Peaks: (1) lysozyme, (2) ovalbumin, (3) conalbumin. Sample size: 3 µg

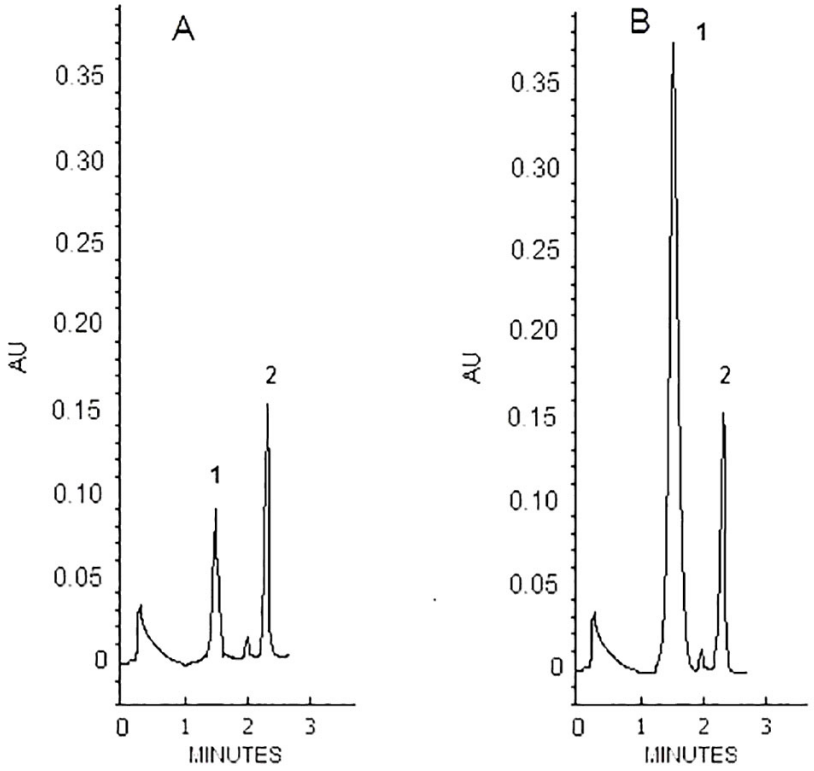


Figure 8. Chromatograms of human serum obtained from (A) pooled normal serum and (B) serum from a patient with high fever. Chromatographic conditions as in Figure 5. Peaks: (1) IgG, (2) HSA. Sample size: 100 µg

4. CONCLUSIONS

This work revealed that the combination of reversed-phase and weak anion-exchange chromatographic mechanism using (R)-phenylephrine-bonded non-porous polystyrene-divinylbenzene column allowed the rapid and simultaneous separation of neutral, acidic and basic compounds. We hypothesized that the mechanism was based on the mixed-mode LC separation. The chromatographic conditions were simple by using a gradient elution of borate buffer (pH 9.0) and acetonitrile and the separation was achieved in 3 min. Applications for fast separation of proteins by the developed PSDVB-PE column provided better separation efficiency over non-modified PSDVB support. The PSDVB-PE column shows potentials for proteins and biopolymer analysis.

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Conflict of interest (If any)

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