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Synthesis and characterization of protein bonded stationary phases for HPAC

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SYNTHESIS AND CHARACTERIZATION OF PROTEIN BONDED STATIONARY
PHASES FOR HPAC

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Keerthini Manda

May 2007

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ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF PROTEIN BONDED STATIONARY PHASES FOR HPAC

by Keerthini Manda

Affinity chromatography is a valuable separation method that allows the target analyte to be determined in a very complex sample matrix. This principle can be applied to HPLC where the selector is the stationary phase. In the method described in this study, the linker (activated 7-octene-1, 2-diol and diol from AGE) would be attached to the silica surface using the silanization/hydrosilation method followed by carbonyldiimidazole activation. Next affinity phases were prepared by coupling proteins (trypsin and papain) to the activated diol compounds. Bonded stationary phases were characterized using spectrometric methods and elemental analysis.

Preliminary chromatographic evaluation of protein-bonded columns is done by the Neue test. Further chromatographic evaluation of these columns is tested by aqueous normal phase retention and chiral separations. Finally, the affinity of the protein bonded phase was tested by separating trypsin inhibitor and antipain to determine the desired specificity.

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I INTRODUCTION

A. Chromatography

Chromatography is a powerful analytical method in separation science. In any chromatographic technique, the components of a sample mixture are separated based on their distribution between the mobile phase and the stationary phase. A column contains a stationary phase while the mobile phase carries the sample mixture through it. Sample components that are strongly held by the stationary phase spend a longer period of time in the column and travel slowly with the mobile phase. In contrast, components that are weakly retained by the stationary phase move faster.

According to the nature of the mobile phase and the stationary phase, chromatographic methods can be classified into two types: (1) Gas chromatography, in which the mobile phase is a gas and the stationary phase is either solid or liquid on a solid support, and (2) liquid chromatography, in which the mobile phase is liquid and the stationary phase is a liquid on a solid adsorbent, a solid, or an ion exchange resin. When compared to gas chromatography, liquid chromatography provides separation of non-volatile solutes and ionic samples [1]. Before the 1970's, liquid chromatography was commonly used in the paper and thin layer formats. However, these chromatographic techniques had limited success because of poor efficiencies and long analysis times. This led to the development of high-performance liquid chromatography (HPLC) in which separation is carried out at pressures up to 6000 psi. High-performance liquid chromatography is the most widely used analytical separation technique because of its

sensitivity, resolution, speed, accuracy and above all, its applicability to wide range of compounds such as pharmaceuticals, food products, and biochemicals.

A typical HPLC instrument consists of an injector, a solvent reservoir, a pump, a column, a detector, a recorder, and a waste reservoir. Sample solution flows with the mobile phase through the stationary phase, where the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase at different rates and elute from the column after different times. The interaction of the stationary phase and the sample with the mobile phase determines the degree of migration and separation of the components in the sample solution. The detector detects the separated components and the signals are digitized and sent to a recorder in order to analyze the data.

HPLC is divided into two types based upon the relative polarities of the mobile phase and stationary phase: (1) normal-phase chromatography, where the mobile phase is non-polar and the stationary phase is polar, and (2) reversed-phase chromatography, where the stationary phase non-polar, often a hydrocarbon, and the mobile phase is polar such as water, methanol, or acetonitrile. Among the different available separation modes, reversed phase is the most popular mode of HPLC because of its excellent selectivity towards non-polar and polar solutes [2]. However, mobile phases with an organic modifier are needed to obtain the best separation of polar solutes in the RP-HPLC. These modifiers can damage the columns used for RP-HPLC. Recently a third type of chromatographic strategy has been developed on stationary phases utilizing silicon-hydride based particles with a bonded phase, called aqueous normal phase

chromatography (ANP) [3]. This mode is similar to normal phase as a highly polar analyte retains longer than the non-polar analyte but the mobile phase has some water as part of the binary solvent. In addition, retention must increase as the amount of the non-polar solvent in the mobile phase increases. The ANP process is useful in distinguishing the hydride stationary phases from typical hydrophilic stationary phases. The hydride stationary phases can also retain non-polar compounds by a traditional reversed-phase mechanism. Therefore ANP is a useful mode of chromatography for separation of polar solutes on silica-hydride stationary phase that also possesses reversed phase capabilities as opposed to a hydrophilic interaction chromatography (HILIC) process, which can only separate polar solutes.

High-performance affinity chromatography (HPAC) is another important mode of HPLC and it combines the specificity of an affinity mechanism with the efficiency, sensitivity, and speed of operation of HPLC techniques [4]. HPAC has emerged as the most popular separation technique for the separation of biomolecules from complex biological mixtures. In affinity chromatography, separation of proteins occurs on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. The technique provides high selectivity, high resolution, and high separation capacity for the proteins of interest. Affinity chromatography is the only technique that uses biological recognition for the purification of a biomolecule, which has become a powerful tool for the separation of biologically active species. In affinity chromatography, biological interaction between ligand and the desired target molecule can be the result of electrostatic or hydrophobic interactions, van der Waal's forces or

hydrogen bonding. The desired target molecule can then be eluted from the affinity medium by a reversible interaction, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength, solvent or temperature.

Affinity chromatography requires a ligand that can be covalently attached to a chromatography matrix and the coupled ligand must retain its specific binding affinity for the target molecules. The binding between the ligand and target molecule must be reversible to allow the target molecule to be eluted in an active form.

B. Silica Surface

The most commonly used chromatography matrix for HPAC is porous silica material, which has excellent mechanical properties. However, it is unstable toward alkaline conditions. AstrosilTM silica was used in this research work, which has a high surface area compared to other silica types. Native silica contains a three dimensional framework of siloxane moieties (-Si-O-Si) with acidic silanol groups (-Si-OH) on the surface [5]. These acidic silanol groups can cause irreversible adsorption of proteins through strong hydrogen bonding and dipole-dipole interactions that result in peak tailing and loss of chromatographic resolution. To overcome these problems, chemically modified versions of silica have received considerable attention. The purpose of silica surface modification is to mask these polar silanol groups and to transform the surface into a hydrophobic material.

C. Surface Modifications

The most commonly established synthetic technique for the modification of silica surface is organosilanization, in which porous silica is reacted with organosilanes to yield

a Si-R functionality attached to the support through a Si-O-Si (Siloxane) linkage as shown in reactions 1 and 2 below.

A monomeric (reaction 1) or polymeric phase (reaction 2) can be achieved using different organosilane reagents [6]. This process has some limitations: (1) it produces a hydrolytically unstable Si-O-Si-C linkage under moderately acidic or slightly alkaline conditions, and (2) it yields an incomplete surface coverage, which results in the exposure of a substantial number of surface silanols.

Another approach for the surface modification is chlorination of silica, which involves two steps. This reaction yields a direct Si-C linkage as shown in reactions 3 and 4.

OR

The first step is the chlorination of the silica surface by reacting with a thionyl chloride reagent. The second step involves an organometallic reaction, which bonds the desired ligands to the surface. Even though the Si-C bond is more hydrolytically stable than the Si-O-Si-C linkage, there are several disadvantages that limit the commercial use of this method. The first one is that the chlorinated intermediate is hydrolytically unstable. The second limitation is that the metal salt by-products formed during the alkylation process may easily contaminate the surface [6].

To overcome these limitations, a new approach has been developed by Pesek, *et al* for the preparation of bonded stationary phases that is called the silanization/hydrosilation technique as shown in reactions 5 and 6.

Silanization

Hydrosilation

This method involves two-steps, which leads high coverage of the silica surface, and high hydrolytic stability of the bonded group [7]. The first step is silanization that

involves the preparation of a silica hydride intermediate by reacting surface silanols with triethoxysilane (TES) in the presence of water, an acid catalyst, and an appropriate solvent. The hydride intermediate formed in this step is more stable compared to the chlorinated intermediate formed in the chlorination process. The second step is hydrosilation; it involves a reaction between the silica hydride intermediate and a terminal olefin in the presence of transition metal complex. The most commonly used catalyst is hexachloroplatinic acid in 2-propanol, also referred as Speier's catalyst. This step results in a direct Si-C linkage (8, 9). The silanization/hydrosilation process has a number of advantages over the other surface modification methods: (1) the silanization results in higher column efficiency due to a reduction in surface silanols, and (2) a large number of organic moieties can be bonded using a variety of catalysts. However, there are two problems that could affect the overall usefulness of the method. First, in the hydrosilation reaction Speier's catalyst forms a complex with silica hydride during the transition step of the olefin addition. The intermediate species thus formed can be of considerable size, which may result in lower yield. The other problem of using Speier's catalyst is that the reaction between hydride and metal may result in the deposition of Pt (0) on the surface. An alternative catalyst for the hydrosilation process is a free radical initiator such as tert-butyl peroxide [10]. In this study the hydrosilation reaction is carried out using both Pt and a free radical catalyst. The organic moieties used for bonded phases are 7-octene-1, 2-diol and allyl glycidyl ether.

D. Diol Bonded Phases

Diol bonded silica was developed in the mid 1970s to overcome the nonspecific interactions of native silica with biological molecules [4, 11]. This modified silica surface provides a support with a hydrophilic, neutral coating on silica that has low nonspecific binding for many biomolecules while also providing a functional group that could be easily modified for ligand immobilization. This modified diol bonded support also increases the stability of the silica support, which would otherwise be unstable due to the hydrolysis at a pH of 7 or higher buffer solution. In this study 7-octene-1, 2-diol and allyl glycidyl ether were used for the preparation of the diol bonded stationary phases (see reactions 7, 8a and 8b) [12]. These bonded groups (7-octene-1, 2-diol and diol from allyl glycidyl ether) serve as linkers for the attachment of proteins in HPAC.

Hydrosilation of 7-octene-1, 2-diol

Catalyst- Hexachloroplatinic acid

Hydrosilation of Allyl glycidyl ether

Catalyst- Hexachloroplatinic acid and t-butyl peroxide

Hydrolysis

D. Activated Diol Bonded Phase

Covalent immobilization is the most common approach for attaching ligands to the silica support. This involves activation of the silica, followed by coupling of the ligand to the activated material. Functional groups such as hydroxyl can be used in this process. Several activation methods have been developed to activate hydroxyl groups in order to couple proteins. Among those, carbonyldiimidazole (CDI) and tosyl chloride methods are frequently used methods for covalent immobilization [13]. In this study, 7-octene-1, 2-diol and diol from allyl glycidyl ether were activated using the carbonyldiimidazole method (see reactions 9 and 10). These groups are reacted with 1, 1'-CDI to give imidazolylcarbamate-activated silica.

Activation of 7-octene-1, 2-diol with carbonyldiimidazole method

Activation diol via allyl glycidyl ether with carbonyldiimidazole method

F. Protein Bonded Affinity Stationary Phases

Trypsin (a single polypeptide chain of 223 amino acid residues) and papain (a single polypeptide chain of 212 amino acid residues) proteins were selected to synthesize affinity phases. A nucleophilic substitution reaction between the carbonyldiimidazole activated diol groups and primary amines on the protein is then used to form a stable linkage between the protein and support. In this case, a side chain amino group of a protein such as Lys and Arg, and/ or an N-terminal amino group may bind to the activated diol silica stationary phase [14]. Reaction 11 below illustrates the reaction involved in this method.

Coupling of proteins to the activated diol compound

G. Aims of this Work

The major goal of the present study is to synthesize affinity stationary phases and to test the chromatographic ability of these phases by separating trypsin and papain inhibitors. Several steps are involved in the preparation of affinity stationary phases: (1) modification of native silica by silanization/hydrosilation, (2) activation of bonded organic moieties, and (3) coupling of proteins to the activated bonded groups by covalent interactions. 7-octene-1, 2-diol and allyl glycidyl ether were used to modify the silica

stationary phases via hydrosilation on a silica hydride surface. Carbonyldiimidazole and tosyl chloride activation methods were tested to activate hydroxyl groups. Based on the carbon elemental analysis, the carbonyldiimidazole method proved as a better activation method and further activation was done by using carbonyldiimidazole method. Finally, trypsin (from porcine pancreas) and papain (from papaya latex) proteins were covalently coupled to the activated diol carrier.

The modified silica was characterized using different spectrometric methods include DRIFT, C13 CP-MAS NMR, and carbon elemental analysis. These methods were used to evaluate the success of bonding and the surface coverage of these bonded phases.

The other goal is the characterization of the affinity-bonded packings using selected solutes by the Neue test [16]. Further chromatographic evaluation of affinity columns were performed under aqueous normal phase chromatographic conditions using different mobile phase compositions. Later, chiral mixtures were separated using different mobile phase conditions. The final goal is the evaluation of the affinity columns by separating trypsin and papain protein inhibitors.

II EXPERIMENTAL

A. Materials

All the stationary phases were prepared using Astrosil TM silica (Stellar Phases, Langhorne, PA) having a pore size of 105 Å⁰, a particle size of 4.2 microns and a surface area of 340 sq.m/gram. The other chemicals used for this study are listed in Tables 1 and 2.

Table 1. Chemicals used in synthesis.

Chemical Name	CAS Number	Manufacturing Company
Triethoxysilane	78-07-9	Aldrich chemical Co
Dioxane	123-91-1	Fisher chemicals
Toluene	108-88-3	Fisher chemicals
7-Octene-1, 2-diol	85866-02-0	Aldrich chemical Co
Allyl glycidyl ether	106-92-3	Aldrich chemical Co
Hexachloro Platinic Acid	16941-12-1	Aldrich chemical Co
t-butyl peroxide	110-05-04	Aldrich chemical Co
1,1'-Carbonyldiimidazole	530-62-1	Aldrich chemical Co
Tosyl chloride	98-59-9	Aldrich chemical Co
Pyridine	110-86-1	Fisher chemicals
Acetone	67-64-1	Fisher chemicals
Trypsin	9002-07-7	Aldrich chemical Co
Papain	9001-73-4	Aldrich chemical Co

Table 2. Chemicals used in HPLC separation.

A. Chemicals used for Neue test

Chemical Name	CAS Number	Manufacturing Company
Uracil	66-22-8	Matheson Coleman & Bell, Ohio, NJ
Naphthalene	91-20-3	Aldrich chemical Co
Acenaphthene	83-32-9	Aldrich chemical Co
K ₂ HPO ₄	7758-11-4	Fisher chemicals
KH ₂ PO ₄	7778-77-0	J.T Baker

B. Chemicals used for aqueous normal phase separation.

Chemical Name	CAS Number	Manufacturing Company
Amitriptylene	549-18-8	Aldrich chemical Co
Metformin	N/A	Donated to SJSU
Methanol	67-56-1	Fisher chemicals
Acetonitrile	75-05-8	Fisher chemicals

C. Chemicals used in chiral separations.

Chemical Name	CAS Number	Manufacturing Company
R 2,2,2-trifluoro-1-(9-anthryl)ethanol	53531-34-3	Aldrich chemical Co
S 2,2,2-trifluoro-1-(9-anthryl)ethanol	60646-30-2	Aldrich chemical Co
R Bi Naphthol	18531-94-7	Aldrich chemical Co
S Bi Naphthol	18531-99-2	Aldrich chemical Co

D. Chemicals used in protein inhibitor separations.

Chemical Name	CAS Number	Manufacturing Company
Trypsin inhibitor	9035-81-8	Aldrich chemical Co
Antipain	37682-72-7	Fluka
NaH ₂ PO ₄	10049-21-5	Fisher chemicals
Acetic acid	64-19-7	J.T.Baker

B. Instrumentation

1. Diffused Reflectance Infrared Spectroscopy (DRIFT)

DRIFT spectra of samples were obtained from an ATI Mattson Infinity series FTIR equipped with a deuterated triglycine sulfate (DTGS) detector and connected to WinFIRST software. Samples were prepared by mixing the modified silica with 5% (by weight) KBr and finely grinding the mixture and filling the DRIFT accessory cup with a smooth sample surface. The instrument was purged with nitrogen gas to provide a

moisture free sample atmosphere. Spectra were collected in the region of 450-4000 cm^{-1} with a resolution of 2 cm^{-1} and 100 scans were averaged against pure KBr as a reference.

2. Nuclear Magnetic Resonance Spectroscopy (NMR)

Carbon-13 solid state NMR with cross-polarization (CP) and magic angle spinning (MAS) spectra were performed on a Varian 400 MHz spectrometer with VNMR 6.1B software. MAS spectra were measured at a spinning rate of 5 KHz and 300-500 mg of the bonded silica sample was placed in a double bearing ZrO_2 rotor.

3. Elemental Analysis

Carbon analyses of samples were obtained from Desert Analytics (Tucson, AZ) and the results were then used to calculate the surface coverage of the modified stationary phase. The surface coverage was calculated as follows:

$$\alpha_R (\mu\text{mol}/\text{m}^2) = \frac{10^6 P_c}{(100M_c n_c - P_c M_R) S_{\text{BET}}} \quad (12)$$

Where P_c is the carbon percentage difference between in the modified silica and the silica hydride, n_c refers to the number of carbon atoms present in the modified phase, M_c and M_R represents the atomic weight of the carbon atom and formula weight of the organic compound respectively. BET is the nitrogen absorption method to determine the surface area of silica. S_{BET} refers to the specific surface area of the silica material [15].

4. High Performance Liquid Chromatography (HPLC)

Aqueous normal phase chromatographic measurements and chiral separations were made using an Agilent 1100 series HPLC (Santa Clara, CA) equipped with a diode array detector, quaternary pump, and an autosampler connected to the Empower software.

Characterization of affinity packings by the Neue test and the separation of protein inhibitors were performed using an HPLC system from Thermo Separation Products (San Jose, CA), connected to the Chromquest software and equipped with UV-2000 detector, AS-3000 autosampler and P-4000 quaternary pump.

5. Column Packing

Synthesized stationary phases were packed in-house by a procedure described below. Methanol was used as the driving solvent. Approximately 2.5 g of bonded silica was mixed with 30 mL of isopropyl alcohol in a 50 mL beaker followed by 5-10 minutes sonication. This slurry was added to the reservoir and rest of the tube was filled with methanol. The mixture in the reservoir was packed into the column under 5000-psi pressure in the presence of nitrogen gas. In this research, a total of six bonded phases were packed into 10 cm X 0.46 cm and 15 cm X 0.46 cm i.d. stainless steel columns (Alltech, Deerfield, IL). Packed columns are showed below.

1. Trypsin bonded allyl glycidyl ether (Pt) column, 100 mm X 4.6 mm i.d.
2. Trypsin bonded allyl glycidyl ether (FR) column, 150 mm X 4.6 mm i.d.
3. Trypsin bonded 7-octene-1, 2-diol (Pt) column, 100 mm X 4.6 mm i.d.
4. Papain bonded 7-octene-1, 2-diol (Pt) column, 150 mm X 4.6 mm i.d.
5. Papain bonded allyl glycidyl ether (FR) column, 150 mm X 4.6 mm i.d.
6. Papain bonded allyl glycidyl ether (Pt) column, 150 mm X 4.6 mm i.d.

C. Procedures

1. Preparation of Silica Hydride

Silica hydride intermediate was prepared by using the silanization method as previously described by Chu et al., [7]. Prior to the synthesis, 15 g of silica was weighed and dried overnight at 100 °C in a vacuum oven. In a typical procedure, 15 g of dried silica was transferred to a 3-neck round bottom flask equipped with a condenser, a drying tube, a thermometer, an addition funnel with an equalizing tube, a heating mantle, a magnetic stir plate, a magnetic stir bar and a stopper. Then 600 mL of HPLC grade dioxane was added followed by 7.29 mL of 2.3 M HCl solution. The mixture was heated to about 70 °C, and then 90.15 mL of dioxane was transferred into the addition funnel equipped with a stopper. Later, under argon, 20.85 mL of triethoxysilane was added to the addition funnel containing dioxane solution. Then the TES/dioxane mixture was added drop wise into the 3-neck round bottom flask over a period of 20-30 minutes with constant stirring. The temperature was raised to 90 °C and the reaction mixture was allowed to reflux for 90 min. After a cooling period, the product was filtered using a vacuum and then the solid silica hydride layer was collected on the filter apparatus. The silica hydride product was washed three times each with 150 mL of dioxane (50 mL each time), followed 150 mL portions of toluene and 150 mL portions of diethyl ether. The final product was dried at room temperature overnight to evaporate the ether, and then dried in a vacuum oven at 110 °C for at least 6 hours.

2. Hydrosilation Using Speier's Catalyst

2.1 7-Octene-1, 2-diol Bonded Phase

Prior to hydrosilation, all the glassware used was dried in the oven and Astrosil silica hydride was dried overnight in a vacuum oven at 110 °C. A solution of 4.07 mL of 7-octene-1, 2-diol in 62.8 mL of toluene and 2.35 mL of 10 mM hexachloroplatinic acid in 2-propanol were added to a 100 mL round bottom flask. The reaction mixture was heated at a constant temperature of 70 °C for 1 hour while being stirred. After this “induction period”, when a clear solution was obtained, 4.0 g of hydride silica were added slowly to the diol/catalyst solution. The reaction was allowed to proceed for 96 hours at 100 °C with continuous stirring. After cooling, the bonded silica was filtered and washed three times using 30 mL of toluene for each washing; followed by similar washings with dichloromethane and diethyl ether. The final ether solvent was allowed to evaporate at room temperature overnight and the product was then dried under vacuum at 110 °C overnight [12].

2.2 Allyl Glycidyl Ether Bonded Phase

A similar procedure as described above but under nitrogen was used for the allyl glycidyl ether bonded phase. A solution of 4.07 mL of allyl glycidyl ether (kept under nitrogen) in 62.8 mL of toluene and 2.35 mL of 10 mM hexachloroplatinic acid in 2-propanol were added to a 100 mL round bottom flask. The reaction mixture was heated at a constant temperature of 70 °C for 1 hour. After a one-hour “induction period”, 4.0 g of Astrosil hydride silica were added slowly to the ether/catalyst solution. Then the reaction was allowed to proceed for 96 hours at 100 °C. After cooling, washings were

done with the 30 mL portions of toluene, followed by dichloromethane and diethyl ether. Later, the hydrolysis process on ally glycidyl ether obtained a diol, in which the epoxy material was placed in a stirred 0.05 M HCl solution at 60 °C for 4 hours. The final product was filtered and dried under vacuum at 110 °C for 6 hr [12].

3. Hydrosilation Using Free Radical Catalyst

3.1 Allyl Glycidyl Ether Bonded Phase

Hydrosilation of the silica hydride by free radical was carried out according to the method previously described by Pesek et al [12]. Prior to hydrosilation, all the glassware used was dried in the oven and silica hydride was dried overnight in a vacuum oven at 110 °C. A mixture of 62.8 mL toluene, 4.07 mL of allyl glycidyl ether and 80 µL of t-butyl peroxide was added to a 100 mL round bottom flask. This mixture was heated to about 70 °C for 1 hour. Then 4 g of silica hydride were slowly added to the flask. The flask was flushed with nitrogen and all the joints were sealed. The reaction was allowed to proceed for 100 hours at 100 °C with continuous stirring. The final product was washed with 30 mL portions of toluene, followed by diethyl ether and dichloromethane. After that, in order to form the diol from allyl glycidyl ether the same hydrolysis procedure was followed as described above where epoxy material was placed in a stirred 0.05 M HCl solution at 60 °C for 4 hours. The final product was dried at room temperature and later dried overnight in the vacuum oven at 110 °C.

4. Carbonyldiimidazole Activation Method

4.1 7-Octene-1, 2-diol by Free Radical Catalyst

Prior to activation, all the glassware used was dried in the oven and 7-octene-1, 2-diol bonded silica was dried overnight in a vacuum oven at 110 °C. In a typical procedure, a suspension of 150 mg of diol silica in 5 mL of dried dioxane and 243.23 mg of carbonyldiimidazole was added to a round bottom flask. The reaction mixture was stirred at room temperature for 30 minutes. The activated diol silica was washed with dioxane, diethyl ether and acetone. The product was dried under reduced pressure in a desiccator [13].

4.2 Diol from Allyl Glycidyl Ether by Pt and Free Radical Catalysts

A similar procedure as described above was used for allyl glycidyl ether activation. Here, a suspension of either 150 mg of diol silica from allyl glycidyl ether by FR or diol silica from allyl glycidyl ether by Pt in 5 mL of dried dioxane and 243.23 mg of carbonyldiimidazole was added to a round bottom flask. The mixture was shaken for 30 minutes at room temperature. The product was washed with dioxane, diethyl ether, and acetone and dried under reduced pressure in a desiccator [13].

5. Tosyl Chloride Activation Method

5.1 7-Octene-1, 2-diol by Free Radical Catalyst

Prior to activation, all the glassware used was dried in an oven and 7-octene-1, 2-diol bonded silica was dried overnight in a vacuum oven at 110 °C. A suspension of 150 mg of diol silica in 5 mL of dried acetone and 285.98 mg of p-toluenesulphonyl chloride was added to a round bottom flask. After that, 395.5 mg of pyridine was added to the

flask. The reaction mixture was stirred for 60 minutes at room temperature. The activated silica was washed with dioxane, diethyl ether and acetone. The final activated silica was dried under reduced pressure in a desiccator [13].

5.2 Diol from Allyl Glycidyl Ether by Pt and Free Radical Catalysts

A similar procedure as described above was used for allyl glycidyl ether activation. A suspension of either 150 mg of diol silica from allyl glycidyl ether by free radical or diol silica from allyl glycidyl ether by Pt in 5 mL of dried acetone and 285.98 mg of p-toluenesulphonyl chloride was added to a round bottom flask. After that, 395.5 mg of pyridine was added to the flask. The mixture was stirred at room temperature for 60 minutes. The final product was washed and dried under reduced pressure in a desiccator [13].

All activated compounds were analyzed by carbon elemental analysis and it was observed that the carbonyldiimidazole method provided a higher carbon percentage for diol groups than compared to the tosyl chloride method. Further activation of large quantities (2 g) of diol silica was done by the carbonyldiimidazole method using similar procedures as described above.

6. Protein Coupled Bonded Phases

6.1 Coupling of Trypsin and Papain to the Activated 7-Octene-1, 2-diol

In a typical procedure, a suspension of 2 g of activated 7-octene-1, 2-diol silica in a 100 mL of 0.1 M phosphate buffer and either 200 mg of trypsin or papain were added to a round bottom flask. Then the reaction mixture was stirred at 4 °C for 2 days. The final product was washed with 0.1 M phosphate buffer [13].

6.2 Coupling of Trypsin and Papain to the Activated Diol from AGE

A similar procedure as described above was used for coupling of proteins to the diol groups of allyl glycidyl ether. Here, a suspension of activated diol groups from allyl glycidyl ether in 100 mL of 0.1 M phosphate buffer and either 200 mg of trypsin or papain were added to a round bottom flask. The reaction mixture was stirred at 4 °C for 2 days. The final product was washed with 0.1 M phosphate buffer.

III RESULTS AND DISCUSSION

The goal of this study was to explore the feasibility of coupling proteins to a silica support by means of a covalent immobilization method. Elemental analysis, DRIFT spectra, and CP-MAS NMR spectra were used to monitor success of the coupling process.

A. Elemental Analysis

Table 3 shows the surface coverage values for diol bonded phases that were synthesized by the hydrosilation process. The surface coverage was calculated from the carbon content of the bonded material according to equation (12). From elemental analysis, it appears each of the two compounds tested in this work were successfully attached to the silica hydride surface. Results show that the hydrosilation of allyl glycidyl ether had higher surface coverage with both a Pt catalyst and a free radical compared to that of hydrosilation of 7-octene-1, 2-diol with the Pt catalyst.

Table 3. Elemental analysis of diol bonded stationary phases.

Bonded phase	Mw	Catalyst	%C	Surface coverage $\mu\text{mol}/\text{m}^2$
Allyl glycidyl ether	114.14	Hexachloroplatinic acid	8.29	3.89
Allyl glycidyl ether	114.14	t-butyl peroxide	7.24	3.34
7-octene-1, 2-diol	144.21	Hexachloroplatinic acid	7.61	2.63

Table 4 shows the surface coverage values for all diol bonded phases that were activated using the carbonyldiimidazole and tosyl chloride methods. Results show that the carbonyldiimidazole activation of 7-octene-1, 2-diol and allyl glycidyl ether bonded

phases had higher surface coverage compared to the tosyl chloride activation of 7-octene-1, 2-diol and allyl glycidyl ether bonded phases.

Table 4. Elemental analyses of activated bonded phases.

Activated Bonded phase	Mw	Diol Bonded phase	%C	Surface coverage $\mu\text{mol}/\text{m}^2$
Carbonyldiimidazole	162.2	7-octene-1,2-diol (Pt)	8.61	3.61
Carbonyldiimidazole	162.2	Allyl glycidyl ether (Pt)	10.63	4.68
Carbonyldiimidazole	162.2	Allyl glycidyl ether (FR)	8.74	3.68
Tosyl chloride	190.65	7-octene-1,2-diol (Pt)	7.97	3.40
Tosyl chloride	190.65	Allyl glycidyl ether (FR)	9.26	4.10
Tosyl chloride	190.65	Allyl glycidyl ether (Pt)	7.95	3.39

B. DRIFT Spectroscopic Evaluation

DRIFT spectra provide spectroscopic confirmation of the bonding process and also functional group information about the organic group attached to the surface.

1. Silica Hydride

The DRIFT spectrum of the Astrosil silica hydride intermediate is shown in Figure 1. The appearance of the strong band at 2250 cm^{-1} represents Si-H stretching which confirms the success of the silanization reaction. The broad peak centered between $3800\text{-}3000\text{ cm}^{-1}$ is due to H-bonded silanol groups and adsorbed water. A band near 3750 cm^{-1} is assigned to the OH stretch from non-hydrogen bonded silanols. The other peaks in the spectrum are due to various fundamental vibrations of the silica matrix.

Figure 1. DRIFT spectra of Astrosil silica hydride.

2. 7-Octene-1, 2-diol Bonded Phase

The DRIFT spectrum of 7-octene-1, 2-diol bonded to silica hydride using a Pt catalyst is shown in Figure 2. The presence of strong C-H stretching bands in the region of $2800\text{--}3000\text{ cm}^{-1}$ indicates the success of bonding the 7-octene-1, 2-diol moiety to the silica hydride surface. Also, after the hydrosilation reaction, the Si-H band was reduced in intensity with reference to DRIFT spectra of Astrosil silica hydride (Figure 1), which indicates that bonding has taken place.

Figure 2. DRIFT spectra of 7-octene-1, 2-diol bonded phase.

3. Allyl Glycidyl Ether Bonded Phase

The allyl glycidyl ether bonded phase was prepared using both Pt as well as t-butyl peroxide. DRIFT spectra for both the bonded phases are shown in Figure 3. Both spectra show a C-H band in the range of 2800-3000 cm^{-1} , which indicates the success of hydrosilation bonding. Both spectra show that after the hydrosilation reaction, the Si-H stretching is reduced in intensity indicating that bonding has taken place.

Figure 3. DRIFT spectra of AGE by FR (A) and AGE by Pt (B).

4. Carbonyldiimidazole Activated 7-Octene-1, 2-diol (Pt), AGE (Pt) and AGE (FR) Bonded Phases

The 7-octene-1, 2-diol bonded phase by Pt catalyst and the allyl glycidyl ether bonded phase by both Pt catalyst as well as t-butyl peroxide were activated with

carbonyldiimidazole in order to couple proteins. DRIFT spectra of all activated bonded phases are shown in Figure 4. A change observed in the DRIFT spectra of all activated bonded phases, when compared to the all bonded phases before activation (Figures 2 and 3), is a good indication that the surface morphology is changed after the activation. Also, all activated spectra show a band at 1700 cm^{-1} which represents the C=O frequency of carbodiimidazole compound. Results confirm the activation of all diol compounds.

5. Protein Attached Bonded Phases

Trypsin and papain proteins were coupled to the all carbonyldiimidazole activated diol bonded stationary phases. DRIFT spectra of all protein coupled bonded phases are shown in Figure 5 and 6. A change in DRIFT spectra of all protein coupled bonded phases, in comparison to the activated bonded phases (Figure 4) was observed. Again, this is a good indication that the surface morphology is changed after the protein attachment.

C. Solid-state C-13 CP-MAS NMR Spectroscopic Evaluation

1. Trypsin and Papain Coupled Allyl Glycidyl Ether (Pt) Stationary Phases

Both trypsin and papain proteins were coupled to the ally glycidyl ether (Pt) stationary phase using appropriate conditions. Both spectra (see Figures 7A and 7B) show a similar pattern except the C=O peak at 170 ppm in trypsin attached AGE (Pt). A peak at 6.674 ppm (A) and 6.067 ppm (B) represents the carbon directly bonded to the silica surface. This confirms that the organic moiety is actually bonded to the surface. A large peak at 20.460 ppm (A) and 20.035 ppm (B) represents the methylene group next to the surface bonded carbon. The peaks at 69.165 ppm, 61.391 ppm (A) and 69.347 ppm,

61.270 ppm (B) are due to the remainder of the carbon atoms in the allyl glycidyl ether molecule. Each of these carbons is bonded to an oxygen atom and accounts for the downfield chemical shift. The peak at 170 ppm in a trypsin attached allyl glycidyl ether (Pt) represents the C=O group, which confirms the coupling of trypsin to the allyl glycidyl ether. The broad nature of the peak in the CP-MAS spectrum is due to an NMR spectrum effect, most likely a significant decrease in the relaxation times of some carbons. Association of adjacent bonded moieties also results in a sharp decrease in the spin-spin relaxation time (t_2) that leads to the broadening and eventual disappearance of some resonances.

2. Trypsin and Papain Coupled 7-Octene-1, 2-diol (Pt) Stationary Phases

Both spectra (see Figures 8A and 8B) show a similar pattern except the C=O peak at 169.793 ppm in trypsin attached 7-octene-1, 2-diol (Pt). Peaks at 10.01 ppm (A) and 9.82 ppm (B) represent the carbon directly attached to the silica surface. The methylene groups appear between 22-30 ppm in A and B. Two peaks at 63.821 ppm, 70.015 ppm (A) and 70.136 ppm, 63.881 ppm (B) represent the CH₂-O and CH₂-OH, respectively. The peak at 169.793 ppm in trypsin coupled 7-octene-1, 2-diol (Pt) represents the C=O group.

Figure 4. DRIFT spectra of carbonyldiimidazole activated 7-octene-1, 2-diol (Pt) (A), AGE (FR) (B), and AGE (Pt) (C).

Figure 5. DRIFT spectra of trypsin coupled AGE by FR (A), papain coupled AGE by FR (B), and papain coupled AGE by Pt (C).

Figure 6. DRIFT spectra of trypsin coupled AGE by Pt (A), papain coupled 7-octene-1, 2-diol by Pt (B), and trypsin coupled 7-octene-1, 2-diol by Pt (C).

Figure 7. Carbon-13 CP-MAS NMR spectra of trypsin coupled AGE by Pt (A) and papain coupled AGE by Pt (B).

Figure 8. Carbon-13 CP-MAS NMR spectra of trypsin coupled 7-octene-1, 2-diol by Pt (A) and papain coupled 7-octene-1, 2-diol by Pt (B).

3. Trypsin Coupled Allyl Glycidyl Ether (FR) Stationary Phase

The broad peak at 30.41 ppm (see Figure 9) represents both the carbon that is directly bonded to the hydride surface and the methylene carbon that is located adjacent to the bonded carbon. The peaks at 60.48 ppm and 68.80 ppm are due to the remainder of the carbon atoms in the allyl glycidyl ether molecule. Each of these carbons is bonded to an oxygen atom and accounts for the downfield chemical shift. A peak at 170 ppm is due to the presence of C=O group.

Figure 9. Carbon-13 CP-MAS NMR spectra of Trypsin coupled AGE by FR.

D. Chromatographic Studies

1. Characterization of Stationary Phases by Neue Test

The Neue test is used to define the hydrophobicity and silanophilic activity (pH 7) of the newly synthesized stationary phases. The purely hydrophobic analytes like naphthalene and acenaphthene are used to measure hydrophobicity. A basic analyte like amitriptylene is used to measure silanophilic activity. Uracil is used as the marker for the column void volume. The structures of the solutes used for this test are shown in Figure 10.

Figure 10. Structures of the solutes used for Neue test.

Solutes were isocratically separated using a 20 mM K₂HPO₄/KH₂PO₄ buffer at pH 7 and methanol in the ratio of 35.0%: 65.0%. Two different flow rates were used, 1.4 ml/min and 1 ml/min based on the length of the column and the injection volume was 10 ul. The detection wavelength was set at 254 nm for naphthalene and acenaphthene and 211 nm was used for amitriptylene. All six synthesized stationary phase columns were used for this chromatographic test. Hydrophobic interactions are measured using the following equation.

$$\ln(k) = V \times V - 3.068 \quad (13)$$

Where V is the molar volume of the analyte (naphthalene or acenaphthene), V is the hydrophobicity parameter of the packing and k is the retention factor of hydrophobic compound.

Silanophilic interactions at pH 7 are calculated using the following equation.

$$S = \ln(k_{\text{amitriptylene}}) - 0.7124 \times \ln(k_{\text{acenaphthene}}) + 1.9748 \quad (14)$$

Where S is the silanophilic activity parameter, $k_{\text{amitriptylene}}$ is the retention factor of amitriptylene and $k_{\text{acenaphthene}}$ is the retention factor of acenaphthene.

1.1 Hydrophobic Interactions

Naphthalene and acenaphthene were eluted close to the void volume on all protein coupled silica stationary phase columns. A chromatogram on papain bonded 7-octene-1,2-diol column is shown in Figure 11 as an example. This is a typical elution behavior for all polar group incorporated stationary phases. The hydrophobicity of all protein coupled silica stationary phases is shown in Table-5. Poor hydrophobic interactions are observed for all protein coupled columns, which confirm the polar nature of the

stationary phases. Similar hydrophobic behavior is observed for all protein coupled columns. Further evaluation of the polarity of these stationary phases is done by aqueous normal phase testing.

Table 5. Hydrophobicity of protein bonded stationary phases.

Column	Retention time (tR)		Hydrophobicity	
	Naphthalene	Acenaphthene	Naphthalene	Acenaphthene
Papain coupled allyl glycidyl ether (Pt)	1.849	2.501	-0.0106	-0.0062
Trypsin coupled allyl glycidyl ether (Pt)	1.584	1.751	-0.0037	-0.0016
Papain coupled allyl glycidyl ether (FR)	1.288	1.315	-0.0085	-0.0067
Trypsin coupled allyl glycidyl ether (FR)	1.508	1.549	-0.0046	-0.0035
Papain coupled 7-octene-1, 2-diol (Pt)	1.584	1.740	-0.0037	-0.00177
Trypsin coupled 7-octene-1, 2-diol (Pt)	1.576	1.755	-0.0038	-0.0016

Figure 11. Chromatogram of uracil (A), acenaphthene (B) and naphthalene (C) on papain coupled 7-octene-1, 2-diol (Pt) column.

1.2 Silanophilic Activity at pH-7

Amitriptylene was retained on all protein coupled stationary phases. Figure 12 shows the chromatogram of amitriptylene on papain bonded 7-octene-1, 2-diol column as an example. Silanophilic interactions of amitriptylene on all the protein coupled stationary phases are shown in Table 6. Papain coupled AGE (Pt) and trypsin coupled AGE (FR) bonded phases had the lowest amount of silanophilic activity among all the protein coupled bonded phases.

Figure 12. Chromatogram of amitriptylene on papain bonded 7-octne-1, 2-diol (Pt) column.

Table 6. Silanophilic activity of protein coupled stationary phases.

Column	Retention time (tR)	Silanophilic activity (S)
Papain coupled allyl glycidyl ether (Pt)	6.951	0.5
Trypsin coupled allyl glycidyl ether (Pt)	5.137	1.19
Papain coupled allyl glycidyl ether (FR)	7.294	1.54
Trypsin coupled allyl glycidyl ether (FR)	5.985	0.73
Papain coupled 7-octene-1, 2-diol (Pt)	9.093	2.30
Trypsin coupled 7-octene-1, 2-diol (Pt)	10.635	2.63

2. Aqueous Normal Phase Retention

Four protein coupled silica hydride bonded phases were used for this chromatographic testing in order to evaluate the behavior of the bonded stationary phases: a trypsin bonded 7-octene-1, 2-diol (Pt) column, a papain bonded 7-octene-1, 2-diol (Pt) column, a papain bonded allyl glycidyl ether (FR) column and a papain bonded allyl glycidyl ether (Pt) column. Two polar compounds, metformin (highly polar) and amitriptylene (moderately polar) were used as solutes. Structures are shown in Figure 13. Compounds were separated using acetonitrile/water and methanol/water in the ratios of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90. 0.1% formic acid was added to the mobile phase for ionizing the test samples. A 1.4 mL/min flow rate was used for the 150 mm columns and a 1 mL/min flow rate was used for the 100 mm

columns. The injection volume was 10 μ L and the UV detection was set at 211 nm for amitriptylene and 277 nm for metformin.

Figure 13. Structures of solutes used for aqueous normal phase evaluation.

Amitriptylene showed reversed phase behavior (retention decreases as the amount of either methanol or acetonitrile increases in the mobile phase) on trypsin 7-octene-1, 2-diol (Pt) column using both methanol and acetonitrile solvents. This could be due to the hydrophobic interactions of amitriptylene with the bonded phase. Metformin showed good aqueous normal phase behavior (retention increases as the amount of acetonitrile added to the mobile phase) in acetonitrile and only a slight aqueous normal phase behavior observed in methanol as shown in Figure 14.

For metformin, on the papain 7-octene-1, 2-diol (Pt) and papain allyl glycidyl ether (Pt) columns, increased retention was observed as the amount of either methanol or acetonitrile increases in the mobile phases. This indicates a typical aqueous normal phase behavior. Increased retention was observed for amitriptylene when the organic solvent percentage (methanol, acetonitrile) in the mobile phase increased from 50% to 90% or decreased from 50% to 10%. It can be concluded that amitriptylene has both reversed

phase and aqueous normal phase behavior on the papain 7-octene-1, 2-diol (Pt) and papain allyl glycidyl ether (Pt) bonded phases as shown in Figures 15 and 16.

Amitriptylene and metformin retention times increased when the organic solvent (methanol and acetonitrile) percentage increased from 10% to 90% on papain AGE (FR) columns, which confirms that the papain AGE (FR) bonded phase has aqueous normal phase behavior for both metformin and amitriptylene as shown in Figure 17.

Figure 14. Retention map of trypsin bonded 7-octene-1, 2-diol (Pt) column with (A) methanol/water +0.1% formic acid and (B) acetonitrile/water +0.1% formic acid.

Figure 15. Retention map of papain bonded 7-octene-1, 2-diol (Pt) column with (A) methanol/water +0.1% formic acid and (B) acetonitrile/water +0.1% formic acid.

Figure 16. Papain bonded allyl glycidyl ether (Pt) column tested with (A) methanol/water +0.1% formic acid and (B) acetonitrile/water +0.1% formic acid.

Figure 17. Papain bonded allyl glycidyl ether (FR) column tested with (A) methanol/water +0.1% formic acid and (B) acetonitrile/water +0.1% formic acid.

3. Chiral Separation

For chiral separations, a mixture of R, S 2, 2, 2-trifluoro-1- (9-anthryl) ethanol and a mixture of R, S binaphthol were used as the test solutes. The structures of the solutes used for this test are shown in Figure 18.

(i) S 2, 2,2-trifluoro-1- (9-anthryl) ethanol (ii) R 2,2,2-trifluoro-1- (9-anthryl) ethanol

(iii) (R) - (+)-1, 1-bi (2-naphthol)

(ii) (S)-(-)-1, 1-bi (2-naphthol)

Figure 18. Structure of solutes used for chiral separation.

Four columns were used for this chromatographic testing: trypsin bonded 7-octene-1, 2-diol (Pt); papain bonded 7-octene-1, 2-diol (Pt); papain bonded allyl glycidyl ether (FR) and papain bonded allyl glycidyl ether (Pt). In this study, all HPLC separations were performed isocratically using four different mobile phase compositions with acetonitrile/water ratios: 90:10, 80:20, 20:80 and 10:90. The flow rate was set at 1.4 mL/min for the 150 mm columns and 1 mL/min flow rates were used for the 100 mm columns. The injection volume was 10 μ L and the detection wavelength was set at 254 nm. First the retention times for all the chiral compounds were determined individually and finally mixtures of the chiral compounds were run isocratically. Initially, the papain bonded allyl glycidyl ether (FR), papain bonded allyl glycidyl ether (Pt) and papain bonded 7-octene-1, 2-diol (Pt) columns were tested using two mobile phase compositions in the ratios of 90:10, 80:20 acetonitrile / water. All chiral compounds were eluted at the void volume. So, further evaluation of all four columns was tested using only 20:80, 10:90 acetonitrile / water mobile phase compositions. Under these conditions, all individual chiral compounds showed good retention on all four columns and as the water content increased, the retention times increased as in reversed phase behavior (Tables 7, 8, 9 and 10). Individual isomers showed some separation, but mixtures of isomers showed only one peak on all protein-bonded columns. For example, Figure 19 shows the chromatogram for the individual R, S 2,2,2-trifluoro-1- (9-anthryl) ethanol isomers and also the enantiomer mixture of RS 2,2,2-trifluoro-1- (9-anthryl) ethanol on the papain bonded column. It can be concluded that these protein-coupled columns didn't show any

significant chiral selectivity and individual isomers were retained based on their hydrophobic interactions.

Figure 19. Chromatograms of individual and a mixture of R, S 2, 2, 2-trifluoro-1-(9-anthryl) ethanol isomers on the papain bonded column.

Table 7. Papain bonded allyl glycidyl ether (FR) column.

Solutes	Retention time in four mobile phase (ACN+water)	
	20:80(ACN+water)	10:90(ACN+water)
S 2,2,2 trifluoro1- (9-anthryl) ethanol	2.745	7.181
R 2,2,2 trifluoro1- (9-anthryl) ethanol	2.759	7.187
S binaphthol	2.745	4.748
R binaphthol	2.267	4.709

Table 8. Trypsin bonded allyl glycidyl ether (Pt) column.

Solutes	Retention time in four mobile phase (ACN+water)	
	20:80(ACN+water)	10:90(ACN+water)
S 2,2,2 trifluoro1- (9-anthryl) ethanol	11.601	52.352
R 2,2,2 trifluoro1- (9-anthryl) ethanol	11.608	51.259
S binaphthol	8.339	46.138
R binaphthol	8.365	46.008

Table 9. Papain bonded allyl glycidyl ether (Pt) column.

Solutes	Retention time in four mobile phase (ACN+water)	
	20:80(ACN+water)	10:90(ACN+water)
S 2,2,2 trifluoro1- (9-anthryl) ethanol	13.665	56.485
R 2,2,2 trifluoro1- (9-anthryl) ethanol	13.525	56.493
S Bi Naphthol	10.132	36.786
R Bi Naphthol	9.922	34.716

Table 10. Papain bonded 7-octene-1, 2-diol (Pt) column.

Solutes	Retention time in four mobile phase (ACN+water)	
	20:80(ACN+water)	10:90(ACN+water)
S 2,2,2 trifluoro1- (9-anthryl) ethanol	7.639	36.244
R 2,2,2 trifluoro1- (9-anthryl) ethanol	7.665	36.420
S Bi Naphthol	4.850	18.483
R Bi Naphthol	4.870	18.439

4. Protein Inhibitors Separation

The main goal of this work is to study retention of protein inhibitors on protein coupled bonded phase columns. As mentioned previously, HPAC is the most popular separation technique for the separation of biomolecules from biological mixtures [4].

Trypsin inhibitor (consists of 129 amino acid residues, which inhibits trypsin) and antipain (from a microbial source, which inhibits papain) were evaluated on four affinity bonded phase columns, a trypsin bonded 7-octene-1, 2-diol (Pt) column, a papain bonded 7-octene-1, 2-diol (Pt) column, a papain bonded allyl glycidyl ether (FR) column and a papain bonded allyl glycidyl ether (Pt) column. The mobile phases, 0.01 M phosphate buffer at pH 7 and acetic acid at pH 2.8 were used for the isolation of the protein inhibitors. Phosphate buffer was used as the application buffer, which helps to form the affinity complex between the inhibitor and the protein and acetic buffer was used as the eluting buffer, which releases the retained protein inhibitors. In this study, inhibitors on protein coupled bonded phases were first injected and then run for 30 minutes under phosphate buffer conditions in order to form the affinity complex. The mobile phase was then changed to acetic acid buffer immediately after 30 minutes to elute the retarded protein inhibitors. A 1.4 mL/min flow rate was used for the 150 mm columns and 1 mL/min flow rates were used for the 100 mm columns. The injection volume was 20 μ L and the detection wavelength was set at 280 nm. Results showed that the inhibitors were not eluted under phosphate buffer conditions on all protein-coupled columns, which is a good indication of the formation of the affinity complex between the protein and the inhibitor. After changing the mobile phase to acetic acid buffer, inhibitor peaks were eluted on all protein-coupled columns, which confirms the coupling of proteins to the silica stationary phase. Results are shown in Figures 20, 21, 22, and 23.

Figure 20. Chromatogram of antipain on papain coupled allyl glycidyl ether (FR). Mobile phase: phosphate buffer (A) and acetic acid buffer (B). Flow rate 1 mL/min detection at 280 nm.

Figure 21. Chromatogram of antipain on the papain coupled 7-octene-1, 2-diol (Pt) column. Mobile phase: phosphate buffer (A) and acetic acid buffer (B). Other conditions same as Figure 20.

A

B

Figure 22. Chromatogram of trypsin inhibitor on the trypsin coupled allyl glycidyl ether (Pt) column. Mobile phase: phosphate buffer (A) and acetic acid buffer (B). Other conditions same as Figure 20.

Figure 23. Chromatogram of antipain on the papain coupled allyl glycidyl ether (Pt) column. Mobile phase: phosphate buffer (A) and acetic acid buffer (B). Other conditions same as Figure 20.

IV CONCLUSION

The main goal of this study was to synthesize affinity stationary phases and to characterize these phases by separating protein inhibitors. Trypsin and papain proteins were used to prepare affinity bonded phases and trypsin inhibitor and antipain used as solutes to test the affinity of the protein coupled bonded phases. These affinity stationary phases were prepared using several steps: (1) diol bonded silica stationary phases were synthesized from 7-octene-1, 2-diol and allyl glycidyl ether by hydrosilation process using Pt and free radical catalysts; (2) diol bonded stationary phases were activated using carbonyldiimidazole and tosyl chloride methods; and (3) trypsin and papain proteins were coupled to the activated diol bonded silica stationary phases. These protein bonded silica stationary phases were characterized by carbon elemental analysis, DRIFT spectroscopy, Carbon-13 CP-MAS NMR spectroscopy and HPLC.

The DRIFT spectroscopy results confirmed that the formation of 7-octene-1, 2-diol and allyl glycidyl ether bonded phases via hydrosilation using both Pt and free radical catalyst. Carbon analysis showed that 7-octene-1, 2-diol and diol from allyl glycidyl ether bonded phases activated with carbonyldiimidazole method had higher carbon percentages compared to the bonded phases before activation, which confirms the formation of activated diol bonded phases. Solid state Carbon-13 NMR spectra of the protein bonded phases gave molecular information regarding the coupling of proteins. The results of carbon analysis, DRIFT spectra and NMR spectra, confirmed that silanization/hydrosilation followed by carbonyldiimidazole activation provides a suitable method for the preparation of protein bonded silica based stationary phases.

HPLC characterization of these protein bonded phases by the Neue test showed poor hydrophobicity, which is a typical behavior for all polar bonded phases and also this test showed that the silanophilic activity was < 2.7 . All protein bonded columns showed good aqueous normal phase behavior for the retention of metformin using both mobile phases (methanol/water and ACN/water). Amitriptylene showed both aqueous normal phase and reversed phase behavior. Individual runs of R and S Chiral compounds showed good retention on these protein columns under reversed phase conditions. Mixtures of RS chiral compounds were not separated.

This study shows that the trypsin and papain inhibitors can be successfully retained on their respective protein bonded columns. It confirms the attachment of the proteins to the diol activated stationary phase. Future work should focus on the separation of chiral mixtures by using protein-based columns because of their unique enantioselective properties. Also, further research on aqueous normal phase retention helps to evaluate the behavior of these protein-bonded phases.

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