Development of monolithic column for digestion of proteins and separation of dansyl amino acid enantiomers in capillary liquid chromatography

(キャピラリー液体クロマトグラフィーにおけるタンパク質の消化およびダンシルアミノ酸対掌体の分離のためのモノリスカラムの開発)

MATERIAL ENGINEERING DIVISION
GRADUATE SCHOOL OF ENGINEERING
GIFU UNIVERSITY
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A dissertation submitted to the Gifu University in partial fulfillment of the requirements for the degree of Doctoral of Philosophy in Material Engineering

By

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Contents

Chapter 3

Chapter 4

Preface

Chromatography was first developed by the Russian botanist Mikhail Tswett in 1903 as he produced a colorful separation of plant pigments through a column of calcium carbonate. Chromatography has since developed into an invaluable laboratory tool for the separation and identification of compounds. Although color usually no longer plays a role in the process, the same principles of chromatography still apply. Chromatography is a separation method by using the interaction between mobile phase and stationary phase.

Liquid chromatography or more commonly known as High-performance liquid chromatography (HPLC) are useful analytical chromatographic techniques for separating ions or molecules dissolved in a solvent. Nowadays, they are grown as the leading technique for chemical analysis and related applications, with an ability to separate, analyze, and/or purify any sample. LC is one of the method in the analysis of polymers.

The establishment of capillary technique gives an opportunity for scientists to train more easily on the development and improvement of chromatography methods. The use of miniaturized column LC techniques in analytical chemistry improves separation efficiency, speeds up the separation, reduces solvent consumption, and enhances detection performance with the use of concentration-sensitive detection devices.

This thesis consists of 4 chapters. The first chapter describes about the introduction of chromatography. The second chapter describes about forms of monolithic material that have been used as a matrix support for immobilization of enzymes. In this study, a monolithic enzymatic microreactor was prepared in fused-

silica capillary by *in situ* polymerization of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) in the presence of ternary porogenic mixture of methanol, decanol, and 1,4-butanediol, trypsin was then introduced to form an immobilized reactor (IMER). Enzyme immobilization was smoothly achieved by passing a trypsin solution through the column and kept at 4 ^{O}C for 9 hours. Methods for enzyme immobilization on the microchannel surface have been developed because they can take advantages of the larger surface area of micro reaction system and to reduce the time taken during sample preparation without pressure increase. The purpose of this research was to develop a method of the immobilized enzyme trypsin. The performance of the monolithic microreactor column was demonstrated with the digestion of bovine serum albumin (BSA) and the characterization of the column by using scanning electron microscopy which demonstrates that the porous polymer has homogeneous property across the entire monolith.

The third chapter describes enantioseparation of several dansyl amino acids, which was obtained by using β-cyclodextrin (glucuronyl glucosyl β-cyclodextrin) as a chiral selector. The monolithic phases were prepared using one-pot *in situ* copolymerizations of EDMA, GMA as monomers, and the presence of a ternary porogenic mixture of 1,4-butanediol, decanol, and ethanol. The chiral stationary phase was achieved by keeping the column in the water bath at 60 ^{O}C for 18 hours. The purpose of this research was to develop a chiral stationary phase for chiral separation of dansyl amino acids. The influence of some parameters has been investigated, such as mobile phase, pH, and flow rate of the mobile phase on retention time and enantioselectivity to achieve the maximum possible resolution. Dansyl amino acids chiral separation can be affected by pH. The obtained chiral

monolith column was characterized by scanning electron microscopy (SEM) and demonstrated that the monolith column with a higher concentration of β-cyclodextrin has the larger surface area, smaller pore size compared to the monolith column with a lower concentration of β-cyclodextrin and the porous polymer has homogeneous property across the entire monolith.

Chapter 1

Introduction

1.1 Chromatography

Chromatography is a physicochemical method for separation of complex mixtures and it was discovered at the very beginning of the twentieth century by Russian–Italian botanist M.S. Tswett [1]. His paper "On the new form of adsorption phenomena and its application in biochemical analysis", was presented at the regular meeting of the biology section of the Warsaw Society of Natural Sciences in 1903. Tswett gave a very detailed description of the newly discovered phenomena of adsorption-based separation of complex mixtures, which he later called "chromatography" as a transliteration from Greek "color writing" [2]. Chromatography is usually introduced as a technique for separating and/or identifying the components in a mixture. The basic principle is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent. It is a powerful method in industry, where it is used on a large scale to separate and purify the intermediates and products in various syntheses [3].

There are several different types of chromatography currently in use based on the several combination states of a mobile phase and a stationary phase, involving paper chromatography; thin layer chromatography (TLC); gas chromatography (GC); liquid chromatography (LC); high-performance liquid chromatography (HPLC); ion exchange chromatography; and gel permeation or gel filtration chromatography [4]. Principle and classification of chromatography are shown in Table 1-1.

Table 1-1 Principles of separation techniques

Molecular characteristic	**Physical property**	**Separation techniques**
Polarity	Volatility	Gas-liquid chromatography
	Solubility	Liquid-liquid chromatography
	Adsorptivity	Liquid-solid chromatography
Ionic	Charge	Ion-exchange chromatography
		Electrophoresis
Size (mass)	Diffusion	Gel permeation chromatography
		Dialysis
Shape	Sedimentation	Ultracentrifugation
	Liquid binding	Affinity chromatography

HPLC is the most widely used and versatile chromatography and can be used to determination and separation of organic, inorganic, biological, ionic and polymer materials. The system of HPLC consists of following instruments: solvent reservoir, mobile phase, pump, injector, guard column, analytical column, temperature control, detector and data processor. A chromatography detector is a device used in gas chromatography (GC) or liquid chromatography (LC) to visualize components of the mixture being eluted off the chromatography column. Detector is set down at the end of the instrument.

Detector of HPLC should have specific features such as great linear response as a function of concentration of solute, wide linear dynamic range and high signal to noise ratio [5]. There are many ways to classify detectors: concentration *vs*. mass flow rate; selective *vs*. universal; destructive *vs*. non-destructive; bulk property *vs*. specific property; and analog *vs*. digital, the first three are the most important. Selective *vs*. universal, this classification differentiates

between detectors based on the percentage of analyte capable of being detected by the system. Selective systems will only respond to particular types or classes of compounds, while a universal system will generally be able to detect all solutes.

1.2 Capillary liquid chromatography

Liquid chromatography is the separation of a solution following differential migration in a liquid flowing through a column packed with solid particle and separation technique used in many different areas is to aid the identification and quantification of substances in various matrices.

Table 1-2 Development history of capillary-based separation methods and their related technique

Year	Development capillary separation methods and their related technique	References
1974	μLC	6
1978	Open-tubular capillary LC	7
1978	Packed microcapillary LC	8
1979	Fused-silica capillary	9
1981	Capillary zone electrophoresis	10
1985	Electrokinetic chromatography	11
1987	Capillary electrochromatography	12, 13
1998	Monolithic silica capillary column	14, 15
1992	Capillary array electrophoresis	16
1998	Monolithic silica capillary column	17
2004	Capillary LC	18

The most important part of the liquid chromatography is the column which represents the heart of the system [19]. The development history of capillary-based separation methods and their related technique is shown in Table 1-2 [20].

The column consists of many different kinds of stationary phases of which octadecyl bonded silica (ODS) is the most frequently used as stationary phase in reversed-phase chromatography. Miniaturized liquid separation techniques began in the late 1960's. In 1967, Horváth *et al*. developed stainless steel columns ranging from 0.5 to 1.0 mm internal diameter (i.d.) packed with pellicular particles for separation of nucleotides [21]. In conventional HPLC, columns are usually 10-25 cm length and 2-4 internal diameter. In the literature LC system can be categorized by their column diameter as shown in Table 1-3.

Table 1-3 Classification of liquid Chromatography

Purpose	**Classification**	**I.D./mm**
Analytical	Nano-LC	~0.075
	μLC	0.2-0.8
	Semi-μLC	1.0-2.1
	Conventional LC	4.0-6.0
Preparative	Preparative LC	10~

Columns with the diameter smaller than 0.075 mm are used in nano-LC and its flow rate is less than 1μL/min, considering the column diameter in μLC is 0.2-0.8 mm and its flow rate is 1-20 μL/min. Capillary LC is defined as the one involving nano-LC and μLC [20].

1.3 Monolithic stationary phase in liquid chromatography

Since in the late 1980's monolith columns in capillary liquid chromatography were introduced by Hjertén with the development of the continuous bed. He carried out studies aiming at an improvement in performance of both electrophoresis and chromatography. Eventually, a monolithic column in capillary LC affected and gained attention in various applications and many improvements [22]. The potential advantages of monolithic columns were recognized more than 30 years ago. However, a preparation of common size and capillary columns containing silica-based or organic monoliths became possible only few years ago [19].

Monolithic stationary phase is prepared by polymerization of a monomer in the presence of a porogen and characterized by a bimodal pore structure consisting of large through pores for flow and small diffusion pores for adsorption. Monolithic stationary phase is usually divided into four categories: the inorganic monoliths, the organic monoliths or polymer, the molecularly imprinted polymer, and the particle-fixed monolith [23]. Organic polymer-based monolithic columns were initiated by Svec and Fréchet with the rigid polymer rod in the early 1990s [24]. The preparation of polymer monolithic columns usually uses radical polymerization. Polymer monolith column is prepared by mixing of initiator, monomers, cross-linker, and porogens. This mixture is polymerized *"in situ"* in a capillary column.

The polymer-based monolithic column has some advantages such as good permeability, high stability, facileness of preparation process, and easiness for modification [25, 26]. Nakanishi and Tanaka developed silica-based monolithic

column based on hydrolysis of tetramethoxysilane in acidic solution, suitable porogen, and followed by gel maceration [27, 28]. Silica-based monolithic columns are mostly prepared by using sol-gel technique. Small-sized skeletons and large through-pore is allowed by this process.

1.4 Immobilized enzymatic reactors (IMERs)

Immobilized enzymatic reactors (IMERs) recently have drawn much attention because of the striking advantages such as high substrate turnover rate and ease in coupling with the separation and detection systems [29]. The immobilization of enzymes onto solid materials could be traced back to the 1950s [30]. In the last decades, numerous of enzyme immobilization methods have been developed. It has been found that the monoliths, which are composed of porous solid with facileness of functionality and relatively large through-pores, could offer high rates of mass transfer and high enzyme binding capacity [31]. In brief, proteolytic enzymes could be covalently bonded, trapped, or physically adsorbed onto different carriers, which in turn greatly affect the properties of the immobilized enzyme. In the development of enzyme processes, the use of immobilized enzymes is preferable. Enzyme immobilization is a promising strategy for high throughput digestion. It allows the digestion time to be reduced to a few minutes or even seconds [32, 33]. Several methods are available to immobilize enzymes on supports in conventional reaction apparatus. The use of microreactor in conjugation with enzymatic processes is attracting increasing attention due to the ability, to reduce the time during sample preparation many researchers have introduced enzyme-immobilization reactors in which protein is digested [34]. In recent years, various IMERs with different kinds

of support, such as magnetic particles, fused-silica capillaries, membranes and monolithic material, have been developed. Among them, monoliths with advantages of high permeability and fast mass transfer, are considered as excellent support for enzyme immobilization [35].

BSA is a single polypeptide chain consisting of about 583 amino acid residues and no carbohydrates. The molecular weight of BSA has frequently been cited as 66,430 Da. In aqueous solution, small organic molecules like urea can have severe effects (chemical denaturation) on protein structure, function, and stability. The use of these solutions to stabilize or destabilize proteins depends on the co-solvent. Protein studies are conducted almost exclusively in complex solutions. Urea is widely used as protein denaturant. Two kinds of interaction models are differentiated in the literature. The first, direct interactions between urea and the protein are the main denaturation driving force. The second, urea-induced changes in the water structure are proposed as indirect interactions that drive unfolding [36].

1.5 Chiral stationary phases (CSPs)

High performance liquid chromatography (HPLC) utilizing chiral stationary phases (CSPs) is the most popular and effective method for the separation of enantiomers, because of its robustness, reproducibility, and capability for both analytical and preparative scale chiral separations. The increasing interest in separation of chiral compounds and production of enantiomerically pure compounds have led to enantioselective separation becoming one of the most important analysis tasks. Many chromatographic methods, *i. e*., HPLC, gas chromatography (GC), capillary electrophoresis (CE), thin layer chromatography

(TLC), supercritical fluid chromatography (SFC) have been frequently used for resolution of enantiomers of interest [37].

The use of CSPs in LC containing various kinds of chiral selectors; ligand-exchange, protein and glycoprotein, cyclodextrin, polysaccharide, macrocyclic glycopeptide and crown ether, is available for separation of enantiomers and has proved to be one of the most efficient approaches for the direct separation applied to the enantioseparation of different kinds of derivatized amino acids [38, 39, 40]. HPLC using CSPs is a powerful tool for the direct analysis of enantiomers. HPLC on a semi-preparative scale is considered to be one of the most efficient approaches to obtain a small amount of enantiomerically pure compounds in a reasonable time [41].

The most important CSPs can be classified as three types based on their structures. They are macrocyclic, π-π association, and polymeric CSPs. Macrocyclic CSPs includes three groups of chiral selectors. They are chiral crown ethers, cyclodextrin derivatives, and macrocyclic glycopeptides. Macrocyclic CSPs, particularly cyclodextrin-based CSPs, dominate enantiomeric separations in CE and GC, they also are important HPLC CSPs, particularly in the reverse phase and polar organic modes [42]. Cyclodextrins are cyclic oligomers of α-1,4-linked D-glucose units [43]. The cavity is hydrophobic and the exterior rims are hydrophilic. Therefore, non-polar molecules or parts of the molecules will form inclusion complexes with the hydrophobic cavity in the aqueous or hydro-organic solutions. The first successful cyclodextrin-based CSP was introduced by Armstrong be found in 1984. β-Cyclodextrin was bonded to silica gel *via* an ether linkage. This CSP can separate many compounds and is the first CSP used in the reverse phase mode [44].

1.6 Objectives of the research

A capillary liquid monolithic column in the last decade has proved to be an effective alternative and attracted. A wide range of stationary phases have been developed for liquid chromatography and one of the main factors is that there are large differences in the chemical and physical properties of stationary phase materials. The objectives of the present research develop the capillary liquid column for microreactor (Immobilized Enzyme Reactor) and for the separation of chiral compounds as CSPs. Chapter 2 describes the preparation of immobilized microreactor for high throughput online digestion in capillaries of the monolith, binding with enzyme and achieves direct enzyme immobilization. Trypsin is used as one of the most popular digestion enzymes and provides typically active counter to peptides bond. Chapter 3 describes a preparation of capillary liquid chromatography column containing β-cyclodextrin in order to obtain satisfactory column for CSP. The optimized monolithic column was applied for enantioseparation of derivatized amino acids.

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Chapter 2

Development of immobilized enzymatic trypsin microreactor on polymer monolith column for biocatalytic reactions by using capillary chromatography

2.1 Introduction

Proteins regulate many cellular functions and analyzing the presence and abundance of proteins in biological samples is central focuses in proteomics [1]. Within the few years, proteomics as a new subject to understand the various problem is directed toward the identification of all proteins in cells, tissues or body fluids [2]. The proteolytical digestion of protein generally takes place with one or multiple proteases, which usually have their own unique specificities. For example, trypsin is capable of selective cleaving proteins at arginine and lysine residues, which could provide typically peptides in a mass range compatible with MS for amino acid sequence determination [3].

In recent years, several methods have demonstrated the feasibility and have been developed for protein digestion using enzyme immobilized on various supports [4], such as porous monolithic material [5, 6], porous silicon matrix [7], polymer [8], glass [9], and membrane [10, 11]. Among the methods, monolith has several advantages such as high permeability, and fast mass transfer is supposed to be excellent for enzyme immobilization support [12]. Therefore, the application of a monolithic column as a separation medium for bioseparation has received great

attention in recent years [13]. Three types of monolithic stationary phases have been developed: silica-based monolithic columns, organic polymer-based monolithic columns, and organic–silica hybrid monolithic columns [14].

The ordinary in-solution based protein digestion has several obstacles such as still very time consuming for digestion, unavoidable enzyme autodigestion and usually, the sample should be prepared by off-line digestion [15]. Immobilization of the enzyme is a promising strategy for high throughout digestion. Reduced the digestion time and for on-line digestion system directly connected between the immobilized enzyme reactors (IMERs) and separation column. A large number of IMERs have been prepared in capillaries and applied because the monolith has nearly high binding capacity for enzyme, low back pressure, biological inertia, and mechanical stability [16]. Because of easy modification, the porous poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith is included as one of the popular materials used for enzymatic reactor [5, 17]. *In situ* prepared monoliths are chemically attached to the inner wall of capillaries, and retaining frits are not required to support the monolithic matrices.

This study describes the preparation and characterization of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith column for development of bioreactor based on the immobilized trypsin. Trypsin was covalently immobilized on the epoxy-modified monolith support with a single reaction. Several types of effect on enzyme activity were studied, such as flow rate, buffer type, and pH. After the digestion, the separation and detection of the peptides were conducted by capillary liquid chromatography.

2.2 Experimental

2.2.1 Apparatus

The chromatographic measurement was conducted by using a capillary LC system constructed by an L. TEX-8301 microfeeder (L. TEX Corporation, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL, Ito, Fuji, Japan) as a pump, a model M435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA) with injection volume of 0.3 μL, a 100 mm x 0.32 mm i.d. of microcolumn and a UV-1575 intelligent UV/Vis detector (JASCO, Tokyo, Japan) and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The morphology of the column was characterized by an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

2.2.2 Reagents and materials

Glycidyl methacrylate (GMA) (97%) and ethylene dimethacrylate (EDMA) (97%) were obtained from Wako (Osaka, Japan). 3-(Trimethoxysilyl)propyl methacrylate (γ-Maps, 98%) and 2,2'-azobisisobutyronitrile (AIBN) were obtained from TCI (Tokyo, Japan). Trypsin, 0.1 mol/L phosphate buffer, 1 mol/LTris-HCl buffer, 1,4-butanediol, decanol, ethanol, sodium borohydride, bovine serum albumin (BSA) were obtained from Nacalai Tesque (Kyoto, Japan). Benzamidine hydrochloride hydrite was obtained from Sigma-Aldrich (Saint Louis, USA). All solutions used in this study were prepared using ultrapure water prepared in the laboratory using a simplicity UV water purification system (Millipore, Molsheim, France).

2.2.3 Preparation of pre-treatment of the capillary

Initially, the fused silica capillary was flushed with 1M NaOH solution, deionized water, and 1M HCl, respectively for approximately 30 min. 30% (v/v) of γ-MAPS in acetone was poured into the capillary, where after the capillary was sealed in the filled state allowed to react thermally in water bath at 60°C for 24 hours, used for equipping methacrylate groups on the inner of the capillary tube, and followed by rinsing the capillary with acetone (30 min) and then drying using nitrogen gas at least 30 min. With this procedure, the monolith support column could be covalently anchored to the activated inner surface wall, and silanization reaction introduced methacrylic anchoring groups [18].

2.2.4 Preparation of the monolith column

The mixture solutions of monomer, cross-linker, and porogen were prepared. The appropriate solvent was directly added to the porogen mixture. As shown in Table 2-1 monomer, cross-linker and porogen were added in the various ratio. As the solution for the fabrication of the monolith column a mixture of GMA (v/v), EDMA (v/v), 1,4-butanediol(v/v), decanol (v/v), ethanol (v/v), and AIBN (1% with respect to the monomers) was added, followed by sonication for 5 min. Subsequently, the solution was manually introduced into the pretreated capillary using a syringe, both ends of the capillary were sealed and heated at 60°C for 24 h in a water bath for polycondensation and polymerization. After polymerization was completed, the capillary column was flushed with methanol to wash porogenic solvents and other compounds.

2.2.5 Immobilization of the enzyme

The capillary was first rinsed with water for 30 min and followed by trypsin for one-step immobilization through the epoxy groups, because these groups readily reacted with amino groups of the enzyme, as shown in Fig 2-1. The trypsin solution was prepared by mixing 2.5 mg/mL trypsin in phosphate buffer pH 7.6 in the presence of benzamidine (trypsin inhibitor) to prevent autodigestion [19]. Trypsin solution was pumped through the columns for 12 h at 4ºC. Later, the enzyme-immobilized monolith column was rinsed with 5 mg/ml sodium borohydride solution overnight. It was filled with 50 mM Tris-HCl buffer pH 7.0 containing 10 mM $CaCl_2$ and 0.02 % NaN_3 and stored in fridge at 4ºC before use [20].

2.2.6 Denaturation of the protein

The protein sample (bovine serum albumin) was dissolved in 2 mL Tris-HCl buffer solution (10 mmol/L) containing 8 mol/L urea. The protein denaturation was performed at 4ºC for 24 h. Then 14 mL Tris-HCl buffer (10 mmol/L) was added and the denaturation protein solution was introduced into the immobilized-enzyme column [21].

2.2.7 Characterization

The morphology of the monolith surface was also observed by using scanning electron microscopy for characterization of the internal of the monolith.

2.3 Results and Discussion

2.3.1 The preparation of the monolith microreactor

The preparation of enzymatic microreactor was carried out in two steps. The preparation procedures are outlined in Fig 2-1. Firstly thermally initiated *in situ* polymerization was conducted. In this study a mixture of solutions of monomer, cross-linker, and porogens were prepared, then the solution mixed with AIBN. Thermal polymerization was carried through the water bath at 60^{o} C for 24 h. The capillary was rinsed with methanol after polymerization to remove unreacted reagents and porogenic solvents. Secondly, trypsin was attached *via* covalent immobilization onto functionalities of the support. The epoxy-activated monoliths are the most often used enzyme support because of easy fabrication and modification as well as relatively low cost [22]. Due to the direct coupling, the enzyme *via* the epoxy groups can be modified with various reagents.

To increase the amount of the enzyme immobilized on the support, low-temperature (4^{o}C) and long reaction time (24 h) have to be used to raise the enzyme activity. The reactions with an amino group from protein could be handly performed with fast speed even under ambient condition. However in this study, the reaction was conducted for a long time, with slow speed flow rate and under low temperature. Henceforth, sodium borohydride was passed through to improve the stability of the enzyme immobilization.

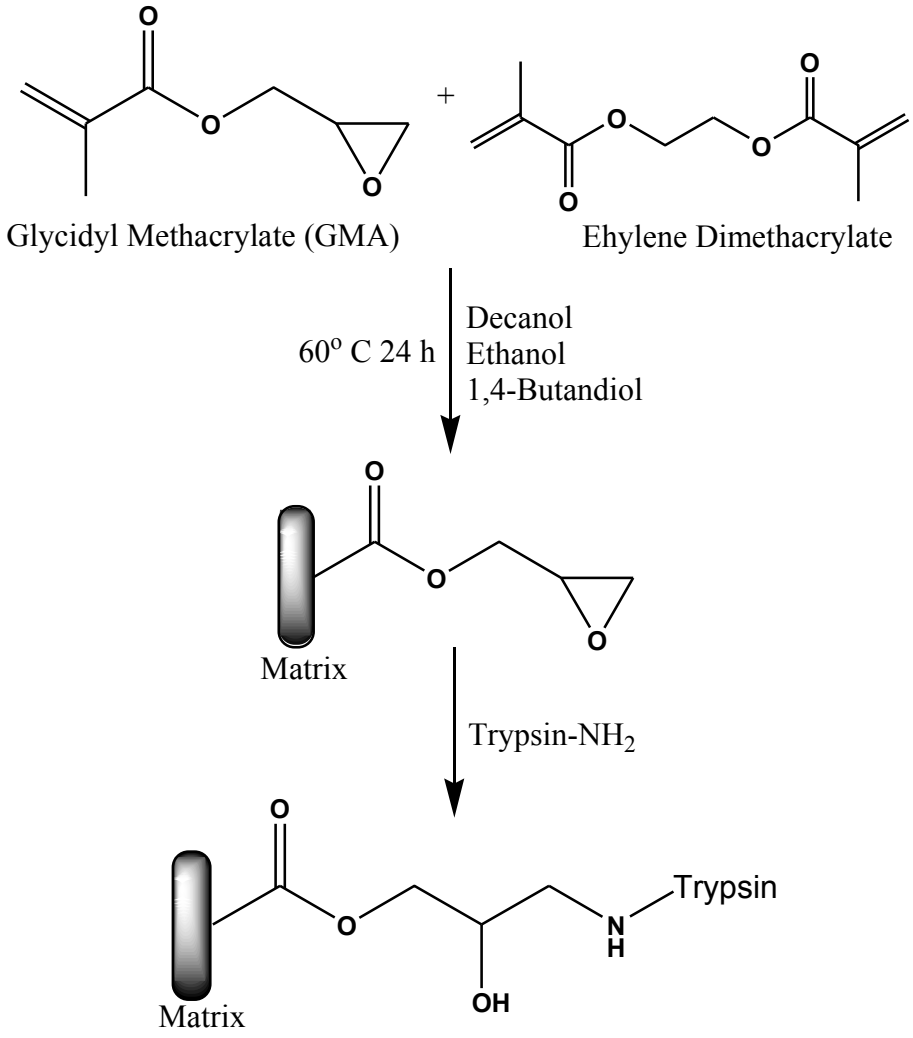

Fig.2-1 Schematic diagram for IMERs preparation

The D3 monolith column in Table 2-1 was chosen as microreactor after several mixtures had been tested. First, the choice of the cross-linker was made but the important factor was to avoid failure during the polymerization. The length of the polymerization chain was too long, resulting in the very small pores and the polymer structure was too weak to resist high flow. Addition of a cross-linker had an impact on the strength of the monolith. The ratio of monomer to cross-linker was fixed and another factor is polymerization time, which was fixed for 24 h.

Table 2-1 Various polymerization conditions

Column	Monomer[a] % (v/v)	Crosslinker[b] % (v/v)	Porogen[c] % (v/v)	Mixture ratio
D1	7.5	2.5	90	10/90
D2	15	5	80	20/80
D3	22.5	7.5	70	30/70
D4	30	10	60	40/60

[a]Glycidyl methacrylate as a monomer
[b]Ethylenedimethacrylate (EDMA) as a cross-linker
[c]Ternary porogens i. e. 1,4-butanediol, decanol, and ethanol (1:0.5:0.1)

2.3.2 Characterization of the monolith

The SEM images of the monolithic supports in capillaries are shown in Fig. 2-2. Theoretically, for the morphology of the monolith if the ratio of porogen increased the through-pore will also increase, when the ratio of the porogen decreased the monolith surface will fully pack and increasing the back pressure. The monomer/porogen ratio is 30/70, which performed the homogeneous porous polymer across the entire monolith. The introduction of trypsin had a little effect on the porous properties of the monolithic column. The macroporous and microporous structure resulted in high throughput and low backpressure.

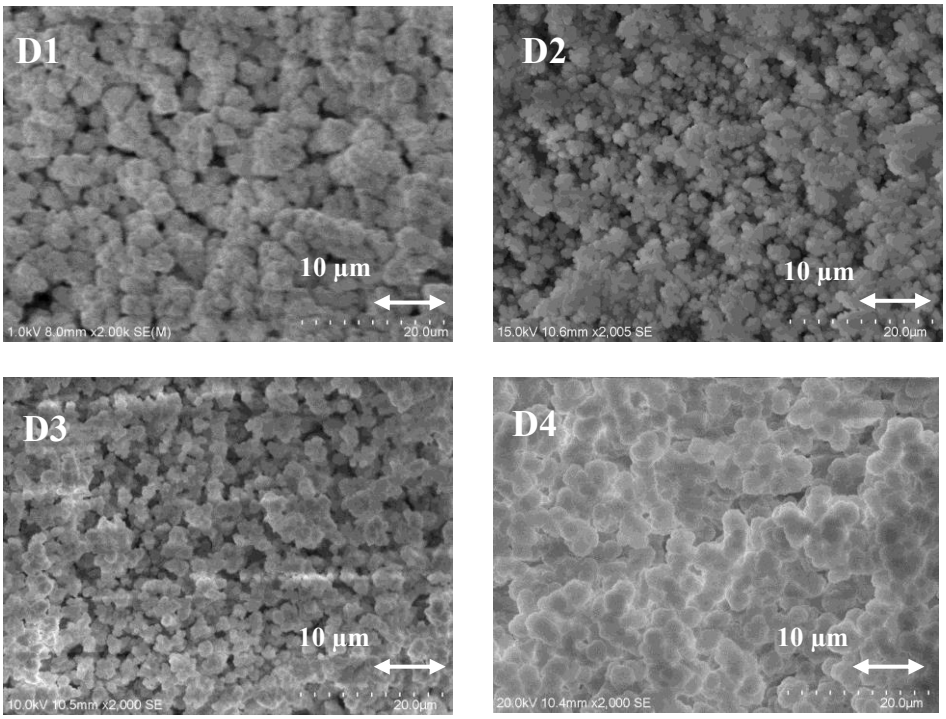

Fig. 2-2 Scanning electron microscopy of the internal structure of monolith in the capillary(magnification 2000x).

The small size and uniform particles of the monolith column achieved a high surface area for enzyme immobilization, and monolith was attached to the inner of the wall of the capillary. Less amount of porogen can also cause the dense structure of the monolith or fully packed. The macroporous structure led to low backpressure. Also, the small particle and the uniformity could provide a high surface area for trypsin immobilization. The porous properties of the support of the monolith have to be adjusted to flow through the pores of the monolith column at a low backpressure. As shown in Fig. 2-3, the relationship between the flow rate and the back pressure demonstrated the monolith column (D3) was mechanically stable until the flow rate 6 µL/min.

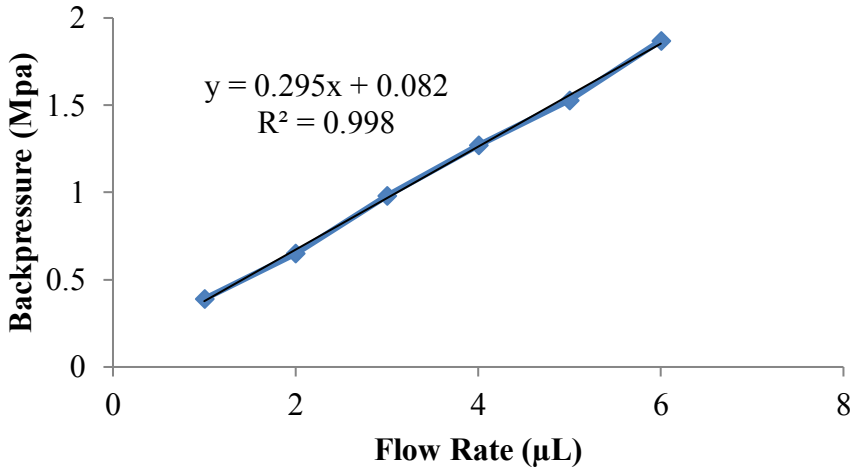

Fig. 2-3 Effect of the flow rate on backpressure. Condition: mobile phase, 0.1 mol/L Tris-HCl buffer. Immobilized column : 100 x 0.32 mm I.D.

2.3.3 Denaturation of the protein sample (BSA)

In aqueous solution, small organic molecules can have severe effects on protein structure, function, and stability. The use of these solutions to stabilize or destabilize proteins depends on the co-solvent. Exactly, protein studies are conducted almost exclusively in complex solutions. Chemical denaturation, with an agent such as urea, is one of the primary ways to assess protein stability, the effects of mutations on stability, and protein unfolding [23]. Proteins can be denaturated by direct interaction whereby urea breaks the hydrogen bonds and other chemical bonding to the polar moieties of the protein and urea denature proteins by decreasing the hydrophobic effect and by directly binding to the amide units *via* hydrogen bonds, particularly peptide groups [24]. The BSA sample solution was examined by adding urea and without urea before passing through the immobilized enzyme microcolumn.

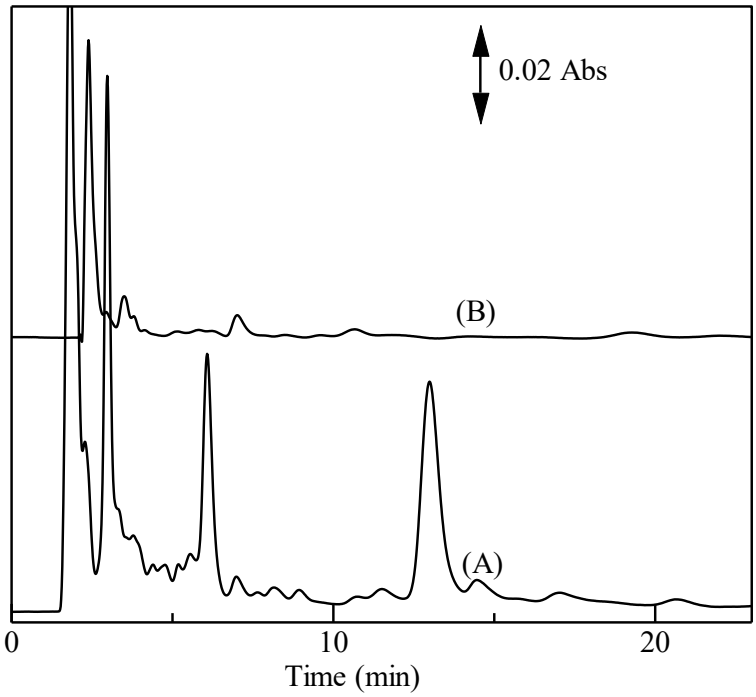

Fig. 2-4 Effect of urea on on-line BSA digestion in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D) (A) with urea (B) without Urea. Condition: mobile phase, 0.1 mol/L Tris-HCl buffer (pH 8.3) with flow rates 3 μL/min for separation and flow rate 1 μL/min for digestion. Separation column: L-column2 ODS (100 x 0.32 mm I.D). Digestion system monitored at 214 nm.

Fig. 2-4 shows the BSA sample solution treated and untreated with urea. It can be seen that the BSA sample solution treated by urea was required to encourage the tryptic effect of the microcolumn.

2.3.4 Application of the monolith enzymatic microreactor for digestion

The important part of the digestion ability of IMERs is the amount of trypsin immobilization onto the monolith column. Theoretically, more immobilization of the enzyme, the higher digestion rate would be expected. Nevertheless, the immobilization methods and the supporting properties materials also could affect the digestion ability of IMERs [25].

A standard protein bovine serum albumin (BSA) was used to test the performance of the trypsin-immobilized microreactor. BSA is a single polypeptide chain consisting of about 583 amino acid residues and no carbohydrates. The molecular weight of BSA frequently has been cited as 66,430 Da. The reference monolith D3 was chosen to verify chromatographic possibilities of the microreactor. The immobilized enzyme monolith column was used for on-line protein digestion and then peptide separation by reversed phase column.

The BSA solution was passed through the enzyme micro-reactor column with several variations of flow rate 1, 2, 3 and 4 µL/min and then the tryptic fragments were injected directly onto the reversed phase separation column, as shown in Fig. 2-5.

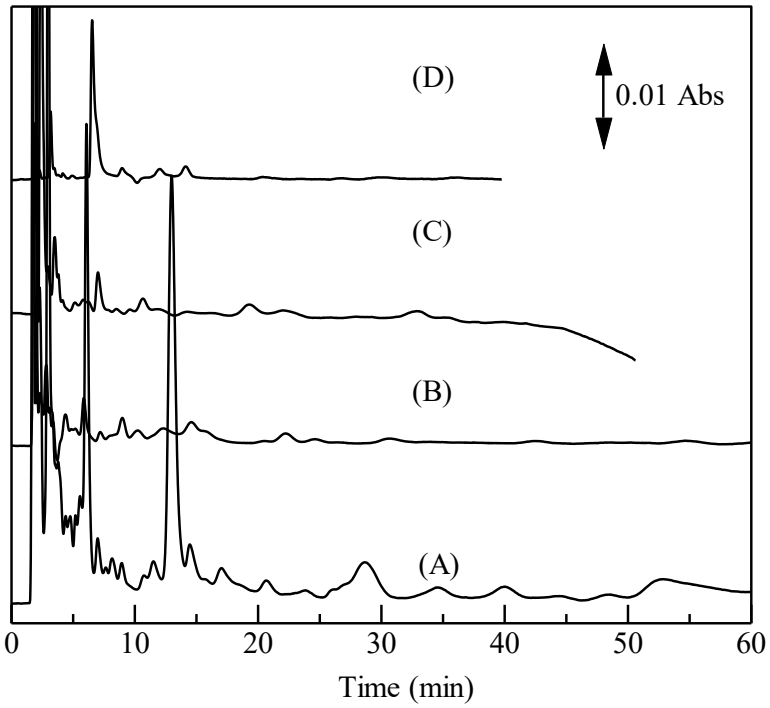

Fig. 2-5 Effect of the sample flow rate on on-line BSA digestion in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D). Condition: mobile phase, 0.1 mol/L Tris-HCl buffer (pH 8.3) with flow rates of digestions (A) 1 (B) 2 (C) 3 and (D) 4 μL/min and separation flow rate 3 μL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D). Digestion system monitored at 214 nm.

The factor that could affect the digestion of the protein is the flow rate of the sample solution or the contact time of the protein inside the immobilized enzyme microcolumn. The effect of the flow rate of the protein digestion was examined by varying several variations of flow rate when the micropump draining the solution to the immobilized enzyme micro-column from 1 to 4 μL/min. In Fig. 2-5 it can be shown that the digestion of the protein increases with decreasing the flow rate and larger peaks are obtained, which shows the longer contact time of the

protein inside the monolith column with the enzyme site digests the more protein. Since the digestion could be expected with 1 µL/min as the optimum flow rate at the lower speed and complete digestion of the protein is observed at 1 µL/min, this flow rate is used for all further experiments.

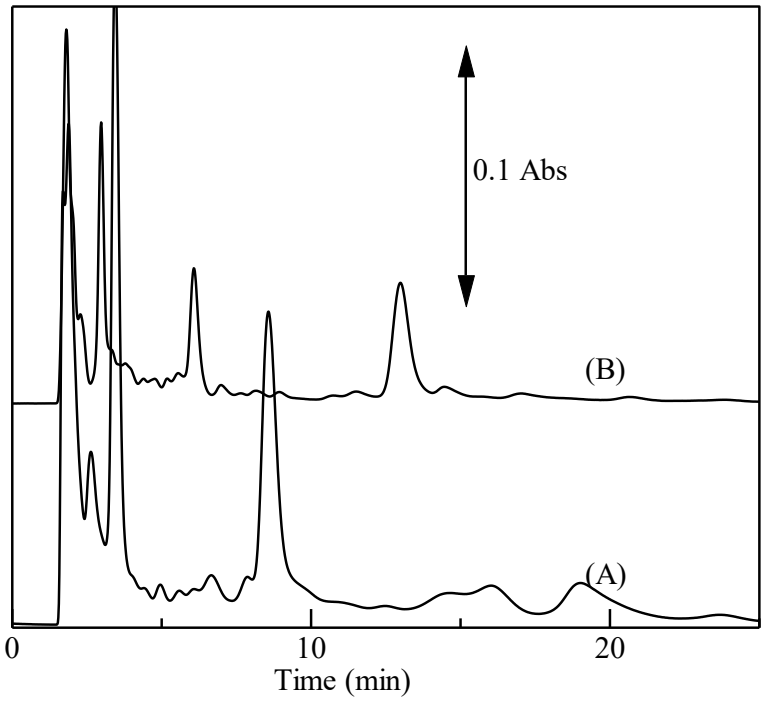

Fig. 2-6 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with (A) 0.1 mol/L Tris-HCl buffer solution (pH 8.3) (B) 0.1 mol/L phosphate buffer solution (pH 8.1) with flow rate for separation 3 µL/min and flow rate for digestion 1 µL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D.).

In this study, the activity of trypsin for BSA digestion was assessed by using buffer solution such as phosphate buffer and Tris-HCl buffer solution as the

mobile phase. Fig. 2-6 shows the chromatograms of the injected sample to the immobilized enzyme microcolumn, for (A) Tris-HCl buffer and (B) phosphate buffer. We found that the digestion in Tris-HCl buffer was more effective than phosphate buffer and showed much higher rate of digestion. Tris-HCl buffer solution is kind of buffer which is breaking open cells for use in molecular biology experiments and completely denaturated because contains salts to regulate the acidity and osmolarity and could establish an ionic strength in the buffer solution.

Enzymes activity are affected by the change of pH. The different enzyme is effective at different pH levels. Extremely high or low pH values generally result in complete loss of activity for the most enzyme. pH is also a factor in the stability of enzymes and there is a region of pH optimal stability. Theoretically, the optimum pH value of trypsin enzyme between 7.8 – 8.7. As can be shown in Fig. 2-7 there are 3 variations of pH that we used as mobile phases. The experiment was conducted with Tris-HCl buffer solution pH 8.3, 7.3 and 5.4. At higher or optimum pH (8.3 and 7.3) the digestion of the injected BSA leads to the presence of multiple peptide fragments. Meanwhile, at lower pH (5.4) the digestion of the injected BSA did not lead to multiple peptide fragments appeared.

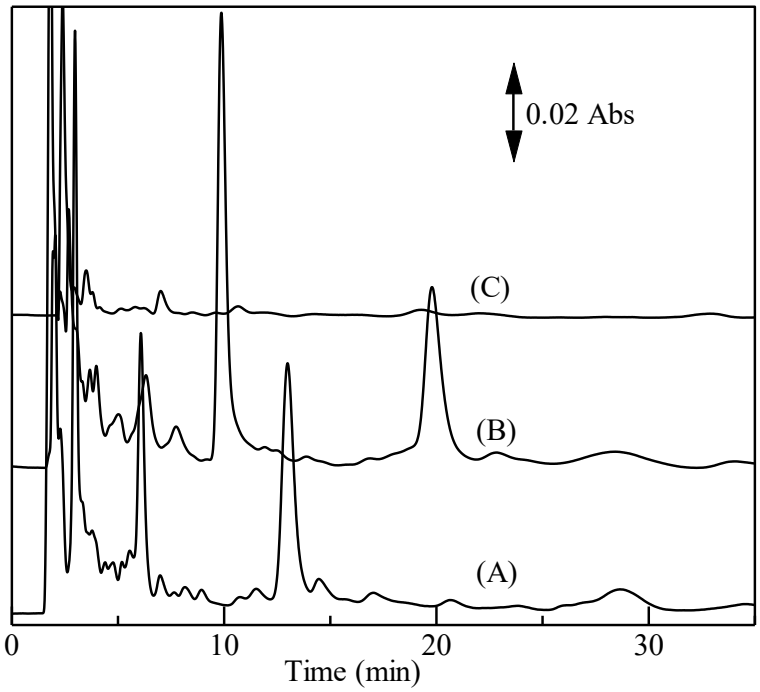

Fig. 2-7 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with 0.1 mol/L Tris-HCl buffer solution pH (A) 8.3 (B) 7.3 (C) 5.4with flow rate for separation 3 μL/min and flow rate for digestion 1 μL/min. Separation column: L-column2 ODS (100 x 0.32 mm I.D.)

Chromatogram for on-line digestion obtained with the capillaries with 0.32 mm I.D. Fig. 2-8 shows a blank run in a capillary with no enzyme and on-line digestion for a microreactor containing the enzyme. The experiment was conducted with a monolith column containing no trypsin as a blank column and immobilized enzyme microcolumn. The digestion of the injected BSA leads to the presence of multiple peptide fragments for the latter case. As can be observed the microcolumn is capable of digesting the injected protein and resulting in the production of many proteolytic fragments of the peptide.

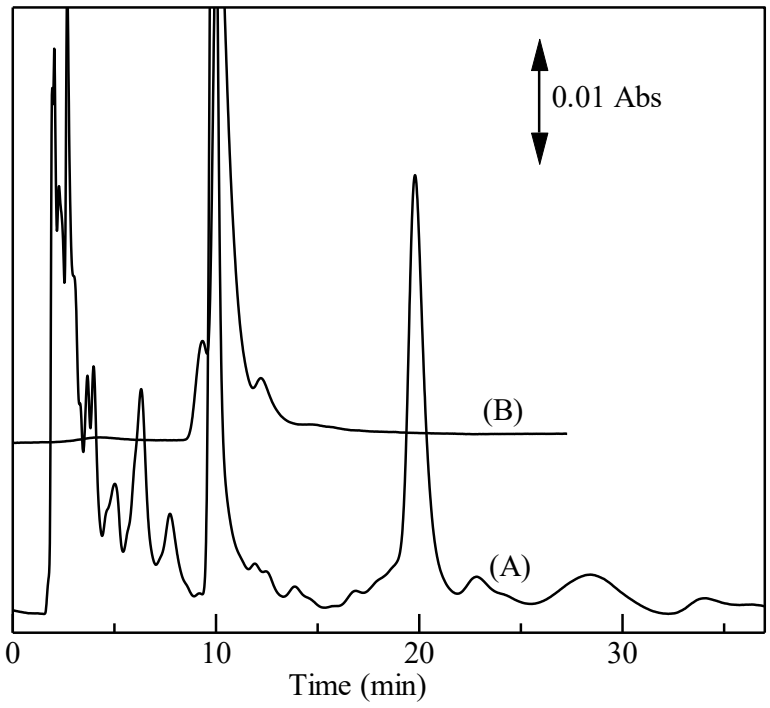

Fig. 2-8 Chromatograms of the injection of BSA in the immobilized-enzyme microcolumn (100 x 0.32 mm I.D.). Digestion system monitored at 214 nm. The experiment was conducted with (A) an immobilized enzyme microcolumn (B) a monolith column containing no trypsin (blank) with flow rate for separation 3 μL/min and flow rate for digestion 1 μL/min.Buffer solution: 0.1 mol/L Tris-HCl buffer solution (pH 8.3). Separation column: L-column2 ODS (100 x 0.32 mm I.D.).

2.4 Conclusions

Monolithic polymer column was successfully used as immobilized enzyme microcolumn and prepared *via* thermally initiated *in situ* polymerization and immobilization of trypsin. Denaturation of the protein (BSA) with urea increased the digestion in the microcolumn. This microreactor was applied to digestion of

protein (BSA). Demonstration of the digestion of the protein in Tris-HCl buffer solution with pH 7.8 was proved more compatible for on-line digestion. This system used microcapillary column for digestion of the protein (immobilized enzyme microcolumn) and for separation of the tryptic fragment.

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Chapter 3

Separation of dansyl amino acids in capillary liquid chromatography using cyclodextrin-bonded chiral monolithic stationary phases

3.1 Introduction

Development of methods for chiral separation has attracted in analytical chemistry. The extending demands for chiral compounds separation and fabrication of enantiomerical compounds in chemistry, biotechnology, pharmacology, etc have conducted the enantioseparation as one of the most necessary analytical assignment. A lot of chromatographic methods have been used and developed for the chiral separation such as gas chromatography (GC) [1], supercritical fluid chromatography (SFC), high-performance liquid chromatography (HPLC) [2, 3], capillary electrophoresis (CE) [4, 5], and micro-liquid chromatography [6].

Several kinds of analytical methods for the enatioseparation of enantiomers have been developed, the most accepted method among them is HPLC[7]. In the recent years, liquid chromatography has been greatly used in the analysis of many biological substances because of its advantages such as high sensitivity, low sample consumption, high enantioselectivity, and quick analysis [6, 8].

Cyclodextrins (CDs) and their derivatives as chiral selectors in liquid chromatography still represent a significant tool for the analysis of structurally different compounds in modern analytical chemistry. The wide use of CDs as the chiral stationary phase is due to their natural chirality and ability to form inclusion complexes with molecules *via* hydrophobic cavity [9]. There are two common ways to use CDs in liquid chromatography: chiral mobile phase additives (CMAs) and chiral stationary phases (CSPs). CDs and their derivatives are useful for chiral selectors as the running buffer additives for separation of racemic drugs were reported by Matsunaga *et al.* [10].

A CD-based chiral stationary phase is one of the most highly used CSP in recent year. Heretofore, direct enantioseparations byHPLC linked with CDs derivatized CSPs have notably expanded into one of the most favored analysis [11]. CDs have many advantages such as stable performance, strong chiral recognition ability, and low cost [12]. Different types of amino acids derivatives have been chirally separated by liquid chromatography. The amino acids stereochemistry performs a significant part in their biological properties. The increasing of observation of amino acids recommends that amino acids have a significant effect in life science [6].

Several studies have described advances in the analysis and chiral separation of amino acids. Tang *et al.* described chiral separation of dansyl amino acids using mono-(3-methyl-imidazolium)-β-CDchloride as the selector[4]. Gabriela *et al.* described the enantioseparation of the chiral amino acids and dipeptides using sulfobutylether-β-CD[13]. Recently, Li *et al.* Used *N*-benzyl-phenethylamine-β-CD for enantioseparation of dansyl amino acids [11].

In this study, the efforts were focused on developing glucuronyl glucosyl β-cyclodextrin (GUG-β-CD) based stationary phases to perform the enantioseparation of chiral derivatives of amino acids by capillary liquid chromatography. GUG β-CD is a single isomer, mono-substituted β-CD with glucuronyl glucosyl group, which has one carboxylic acid group [10]. Among the chiral stationary phases, CD-based stationary phases have many sided purpose for the analytical separation of many different chiral compounds. These CSPs can be prepared by click chemistry or dynamic coating [14,15]. Indirect optical resolution of amino acids is performed in the chiral environment by the interaction of enantiomers with chiral selectors added to the separation buffer [4].

3.2 Experimental

3.2.1 Apparatus

The chromatographic measurement was conducted by using a capillary LC system constructed by an L. TEX-8301 micro feeder (L. TEX Corporation, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 mL, Ito, Fuji, Japan) as a pump, a model M435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA) with injection volume of 0.3 µL, a 100 mm x 0.32 mm I.D. of microcolumn and a UV-1575 intelligent UV/Vis detector (JASCO, Tokyo, Japan) operated at 254 nm and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The morphology of the column was characterized by an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

3.2.2 Reagents and materials

Glycidyl methacrylate (GMA) (97%) and ethylene dimethacrylate (EDMA) (97%), GUG-β–CD were obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-(Trimethoxysilyl)propyl methacrylate (γ-Maps, 98%), 2,2'-azobisisobutyronitrile (AIBN) and acetonitrile (ACN) were obtained from TCI (Tokyo, Japan). 1,4-Butanediol, decanol, ethanol, triethylamine, and acetic acid were obtained from Nacalai Tesque (Kyoto, Japan). Dansyl (5-(Dimethylamino)naphthalene-1-sulfonyl) -DL-amino acids and dansyl-L-amino acids were obtained from Sigma Chemical Company (St. Louis, MO, USA). All solutions used in this study were prepared using ultrapure water prepared in the laboratory using a Simplicity UV water purification system (Millipore, Molsheim, France).

3.2.3 Preparation of the monolith columns

Initially, for the preparation of the monolith, the fused silica capillaries were first pre-treated with γ-MAPS. With this procedure, the monolith support column could be covalently anchored to the activated inner surface wall, and the silanization reaction introduced methacrylic anchoring groups [16]. The mixture solutions of monomer, cross-linker, porogens, and GUG-β–CD were prepared. The appropriate solvent was directly added to the porogen mixture. As the solution for the fabrication of the monolith column, a mixture of GMA, EDMA, GUG-β–CD, 1,4-butanediol, decanol, ethanol, and AIBN (1% with respect to the monomers) was added, followed by sonication for 5 min. Subsequently, the solution was manually introduced into the capillary pretreated column using a

syringe, both ends of the capillary were sealed and heated at 60° C for 18 h in a water bath for polycondensation and polymerization. After polymerization was completed, the capillary column was flushed with methanol to remove the residuals. The column was cut to a total length of 10 cm and 2-5 mm of the capillary was used for SEM analysis.

Table 3-1 Polymer composition mixture used in the preparation of poly(GMA-GUG-β–CD-co-EDMA) monolithic column

Column	GMA: GUG-β–CD (mixture ratio)	Monomer %(v/v) GMA: GUG-β–CD	Crosslinker % (v/v) EDMA	Porogen* % (v/v)	Monomer; Porogen (Mixture ratio)
A1	3:1	40	10	50	50/50
A2	2:1	30	10	60	40/60
A3	1:1	20	10	70	30/70

*Ternary porogensi. e. 1,4-butanediol, decanol, and ethanol (1:0.8:0.2)

3.2.4 Standard solution and sample preparation

The triethylammonium acetate (TEAA) buffer solution was prepared by dissolving an appropriate amount of triethylamine onto ultrapure water, stir under cold condition. Then, the desired pH value was adjusted with acetic acid and stored in the fridge. The ammonium acetate (AMAC) buffer was prepared by dissolving ammonium acetate in deionized water and adding acetic acid [13]. The mobile phases were prepared by mixing the desired amount of ACN and the buffer solution. The concentration of GUG-β-CD was 1 mg/mL in methanol (MeOH) was used as chiral selector solution. MeOH was used as a solvent in the

majority of cases. Stock solutions of dansyl amino acid at concentration 0.5 mg/mL in methanol were prepared. All analytes were detected at 254 nm wavelength.

3.3 Results and Discussion

3.3.1 Preparation of poly(GMA- GUG-β–CD-co-EDMA) monoliths

The preparation of poly(GMA-GUG-β–CD-co-EDMA) monolithic columns was carried out in one step. Three polymer monoliths were prepared *via in-situ* copolymerization. First *in situ* polymerization was thermally initiated. In this study a mixture of solutions of monomer, GUG-β–CD, cross-linker, and porogens were prepared, then the solution was mixed with AIBN. Thermal polymerization was carried through the water bath at 60^{o}C for 18 h. The capillary was rinsed with methanol after polymerization to remove unreacted reagents and porogenic solvents.

The coupling group to epoxy groups can be adopted for cyclodextrin agent. In this study, a one-pot route integration of the GMA-GUG-β–CD monomer and following by copolymerization inside the same vial was to prepare poly(GMA-GUG-β–CD-co-EDMA) monolithic column, as shown in Fig. 3-1. The composition of the reaction mixture has a major effect on the morphology, permeability, and selectivity of the monolith, and the ratio of GMA-GUG-β–CD and the content of the porogens were optimized as shown in Table 3-1.

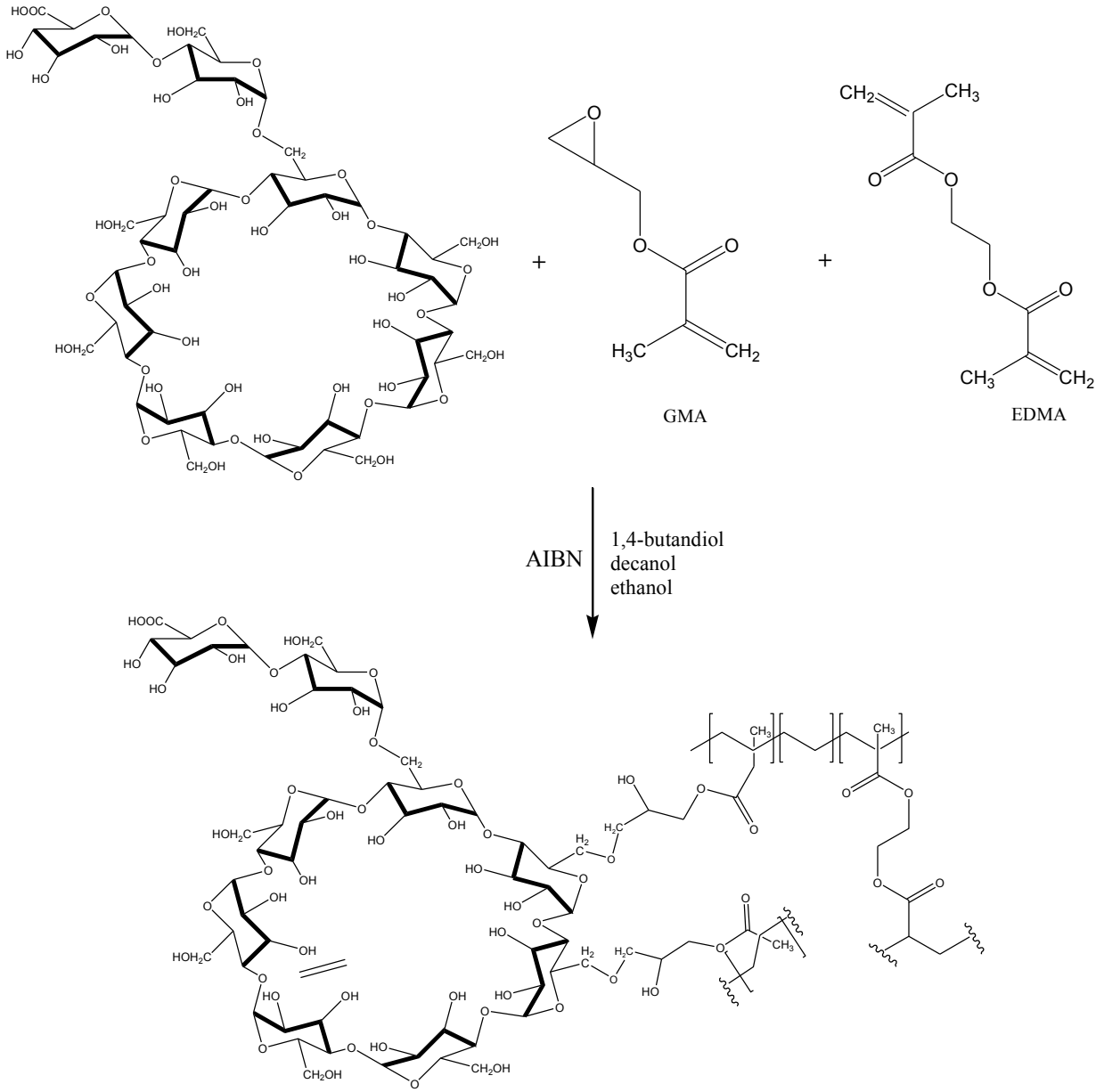


Fig. 3-1 Schematic diagram for chiral stationary phase preparation

The ratio GMA/GUG-β–CD in the reaction mixture could influence the amount of the functional monomer and the skeleton. The mixture ratio of GMA/GUG-β–CD of the monolith was varied from 3:1 (A1) to 1:1 (A3) while keeping the other condition constant. The porous properties of the support of the

monolith have to be adjusted to flow through the pores of the monolith column at a low backpressure. As shown in Fig. 3-2, the relationship between the flow rate and the back pressure demonstrated the monolith column (A2) was mechanically stable until the flow rate 6 µL/min.

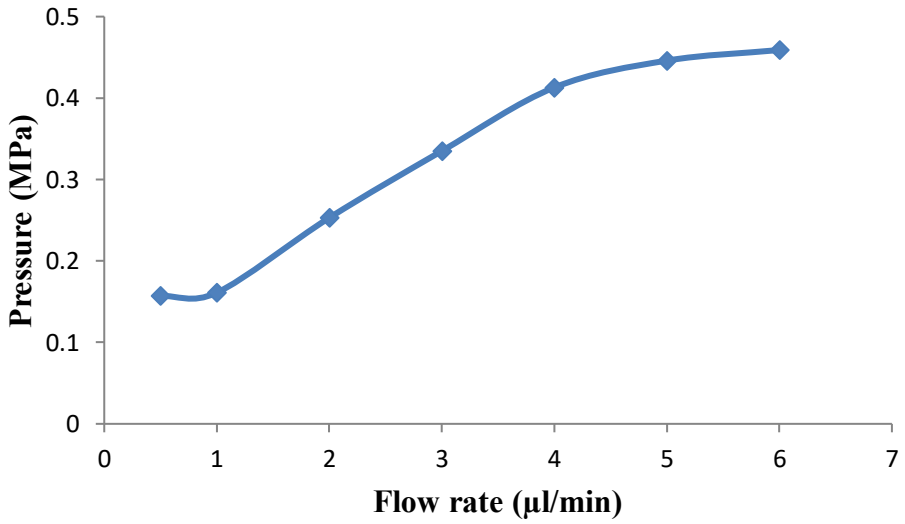


Fig. 3-2 Effect of the flow rate on backpressure. Condition: mobile phase, ACN/100 mM TEAA (pH4.1) 30/70 (v/v). Chiral stationary phase column: 100 x 0.32 mm I.D.

Fig. 3-3 shows the SEM of chiral stationary phase with the optimum conditions. The optimal conditions for polymerization of poly(GMA-GUG-β–CD-co-EDMA) monolithic column were obtained from the column A2. The GMA/GUG-β–CD proportion resulted in a homogeneous matrix. The A1 column was too dense and the A3 column was unavailable because of failure of the polymerization.

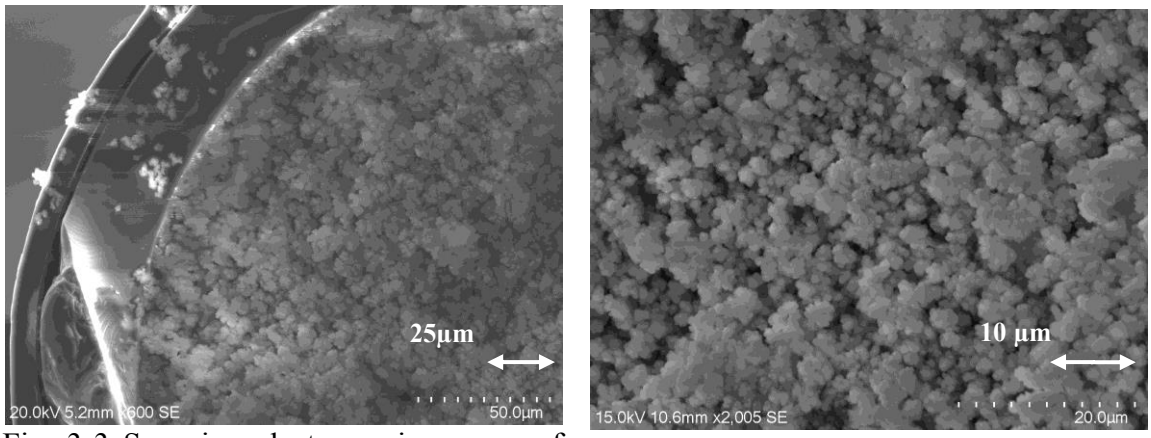


Fig. 3-3 Scanning electron microscopy of poly(GMA-GUG-β–CD-co-EDMA) monolithic column (column: A2), magnification 600x and 2000x, respectively.

3.3.2 Separation of chiral analytes

Enantioseparation of dansyl amino acids using columns containing chiral stationary phase based on β–cyclodextrin derivatives were examined at the analytical level. The retention factor (k) and separation factor (α) for column in the separation of all compounds using a mixture of ACN/TEAA buffer and ACN/AMAC as the eluents were determined. Examination and optimization of the composition of mobile phases for chiral analytes separated on the chiral stationary phases were obtained from derivatized amino acids.

The selection of buffer and operating pH is important for several factors. The main factor is the polarity, and charge of acidic and basic analytes are affected by pH which will in turn affect the solutes interaction with the chiral stationary phase. Obviously, analytes that are ionized will interact with the chiral stationary phase differently than their neutral conjugates.

In reversed-phase chromatography, the most frequently used organic modifiers are methanol and acetonitrile. Changing a solvent that can accept and donate hydrogen bonds (methanol) to one that cannot provide hydrogen bonding (acetonitrile) will often have an effect on the observed separataion. In this study, acetonitrile has been used as organic modifier, and acetonitrile tends to be a stronger eluent than methanol. The use of TEAA buffer (pH 8.3) was not useful for several of the chiral analytes. They were in general eluted at very short retention time. Nearly half of the chiral analytes did not indicate enantioseparation with decreasing content of TEAA buffer in mobile phases. The analytes with a free carboxyl group are negatively charged at pH 8.3. Several enantioseparation of amino acids were achieved at various mobile phase composition. Suitable mobile phase was arranged for ACN and TEAA buffer (pH 4.1) in different ratio of volumes. Under these condition (pH 4.1) the enantiomeric elution order was determined. Partial enantioseparation of chiral analytes was observed in mobile phase ACN/TEAA (pH4.1) 30/70 (v/v), as shown in Fig. 3-4.

Higher resolution values were observed if TEAA (pH 4.1) was used instead of TEAA (pH 8.3). Under this condition (pH 4.1) amino acids were uncharged. These analytes were baseline enantiosepatared with a mixture of ACN/TEAA buffer (pH 4.1) with higher contents of TEAA buffer (30/70 v/v). The analysis time was around 10 min. The eantioselectivity of amino acids was born with two chiral centers. Therefore, to discover the rules of these two chiral aggregations, we performed the enantiomer elution order (EEO) determined by injection of the authentic enantiomer dansyl-L-amino acids and L was found to be

eluted before D, indicating a stronger interaction of D-enantiomer with the chiral selector.

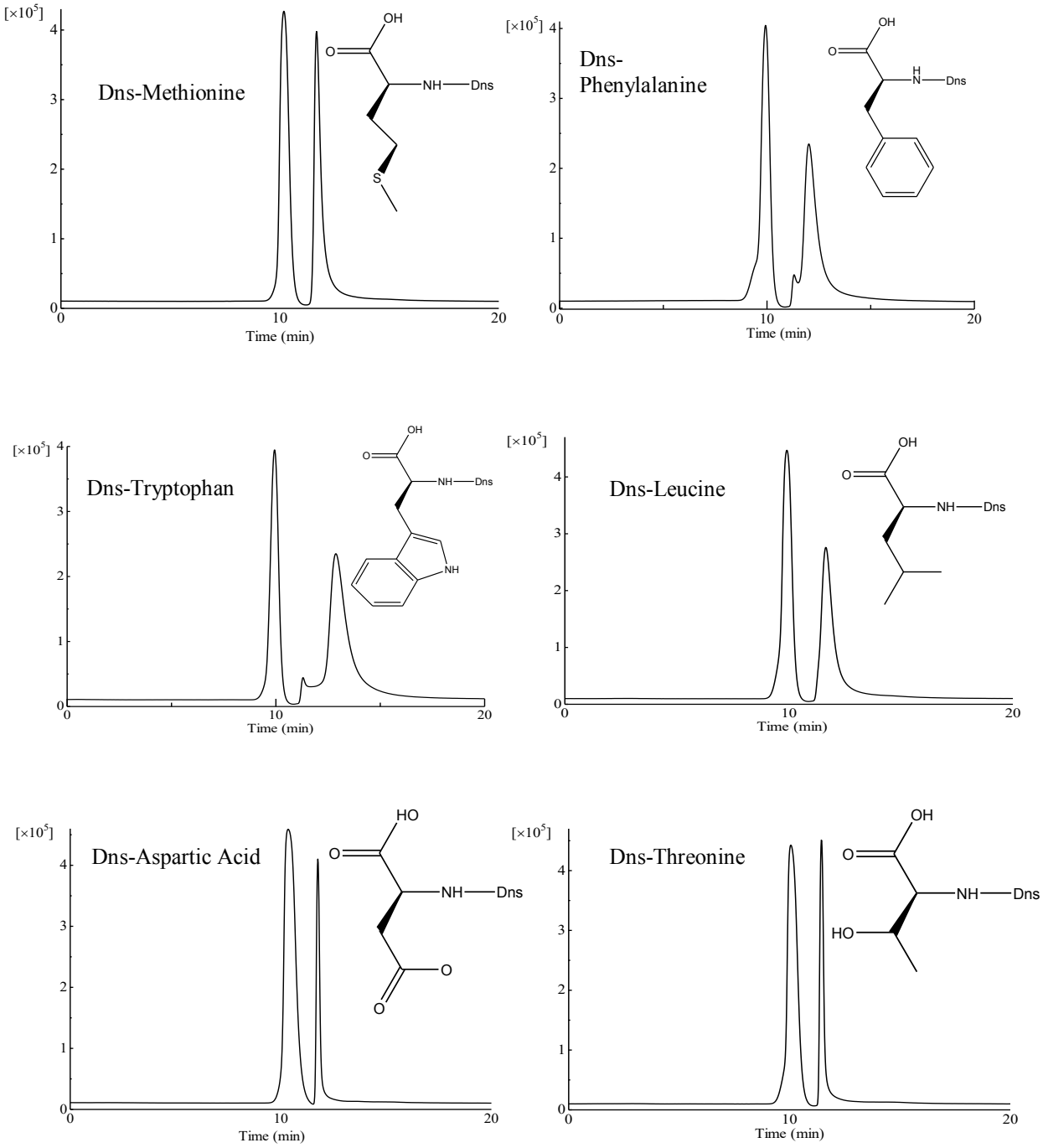
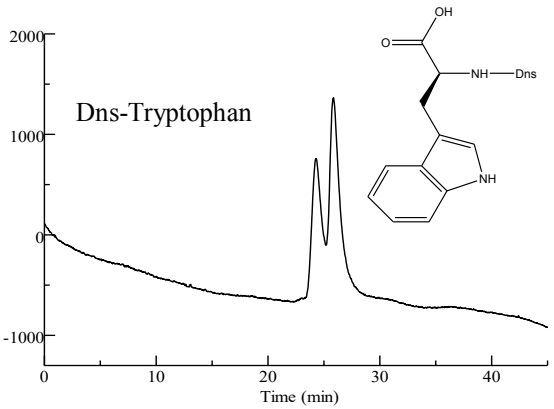
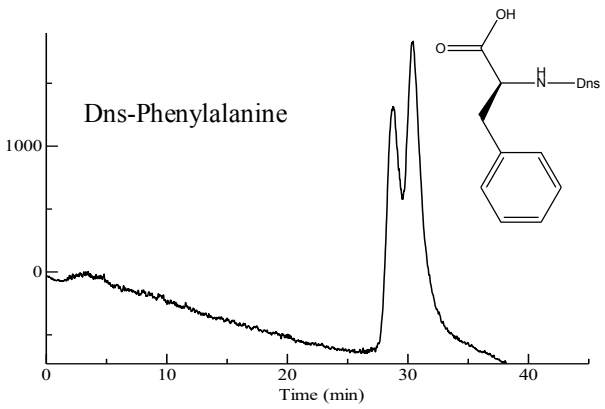


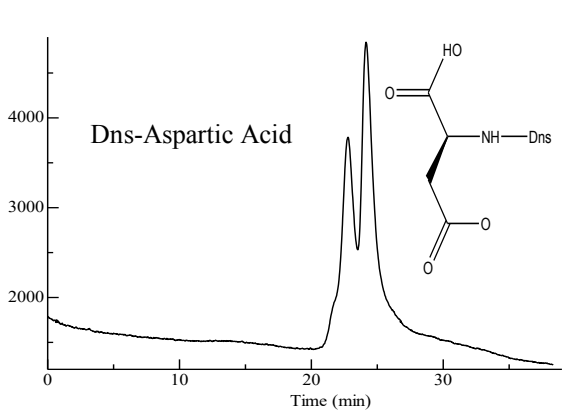
Fig. 3-4 Chromatograms of the separation of chiral analytes with mixture of ACN/100 mM TEAA buffer (pH 4.1) 30/70 (v/v). Conditions: column, 100x0.32 mm I.D.; flow rate, 1µL/min; wavelength of UV detection, 254 nm.

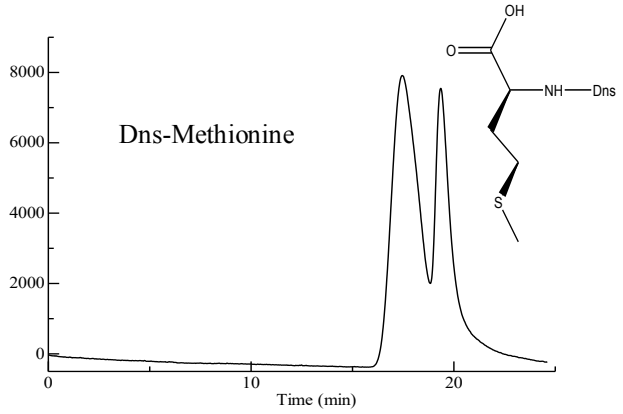
The use of acetonitrile/AMACbuffer (pH 4.7) was useful for several analytes. It could be found that the separation result significantly changed compared to the chiral analytes with mobile phases of ACN/100 mM TEAA buffer (pH 4.1) 30/70 (v/v).

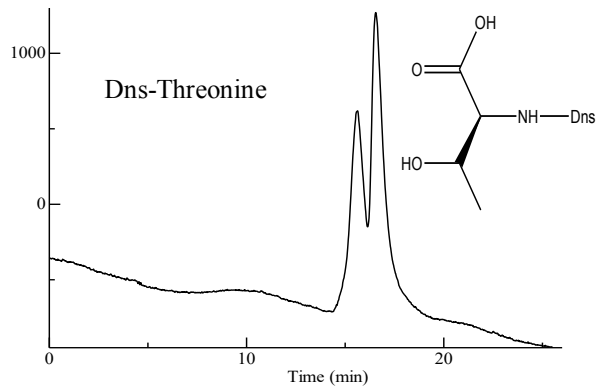
Evidently, they were in general could be eluted at long retention time, resulting from the strong interaction between the chiral selector on CSP and compounds. The analyte that is more tightly bound to the CSP has greater negative free energy and is retained longer. Under these condition the enantiomeric elution order was determined by injection of dansyl-L-amino acids, indicating a weaker interaction of L-enantiomer with the chiral selector, while the peak of the compound obtained still overlaps. Partial enantioseparations of chiral analytes are shown in Fig. 3-5.


Dns-Tryptophan
OH
O
NH—Dns
NH
2000
1000
0
-1000
0
10
20
30
40
Time (min)


Dns-Phenylalanine
OH
O
H
N—Dns
1000
0
0
10
20
30
40
Time (min)


Dns-Aspartic Acid
HO
O
NH—Dns
O
O
4000
3000
2000
0
10
20
30
Time (min)


Dns-Methionine
OH
O
NH—Dns
S
8000
6000
4000
2000
0
0
10
20
Time (min)


Dns-Threonine
OH
O
NH—Dns
HO
1000
0
0
10
20
Time (min)

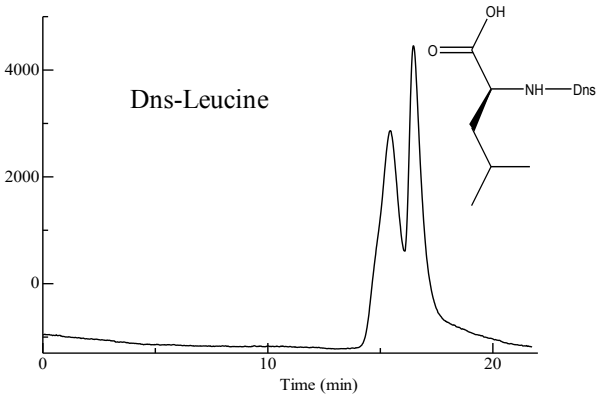

Dns-Leucine
OH
O
NH—Dns
4000
2000
0
0
10
20
Time (min)

Fig. 3-5 Chromatograms of the separation of chiral analytes with mixture of ACN/60 mM AMAC buffer (pH 4.7) 30/70 (v/v).Conditions: column, 100x0.32 mm I.D.; flow rate, 1μL/min; wavelength of UV detection, 254 nm.

3.3.3 Effect of pH

The effect of pH on retention and enantioselectivity are shown in Fig. 6 and Table 3-2. The retention was obtained for Dansyl-DL-threonine on the poly(GMA-GUG-β–CD-co-EDMA) monolithic column. The effect of pH on enantioselectivity (α) for Dansyl-DL-amino acids was favored at lower pH.

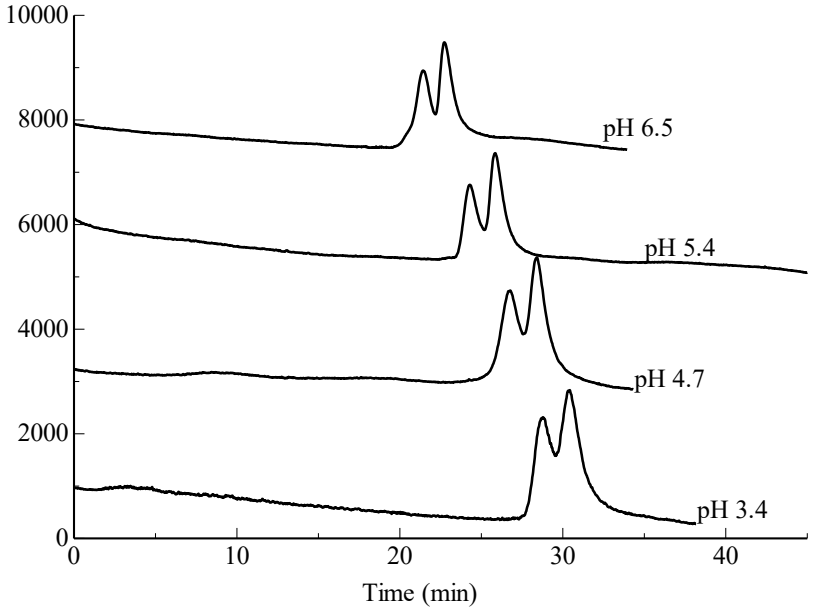


Fig. 3-6 The influence of pH on retention of Dansyl-DL-threonine. Conditions: column, 100x0.32 mm I.D.; mobile phase, ACN/60 mM AMAC buffer (pH 4.7) 30/70 (v/v);flow rate, 1μL/min; wavelength of UV detection,254 nm; column temperature, 20°C.

It is well known that chiral recognition mechanism is based on the specific short-range interaction and hydrogen bond, following the nonspecific long-range interactions (hydrophobic and ionic forces) which allow the approach of solute to the chiral selector [17].

Table 3-2 Enantioseparation data on cyclodextrin of Dansyl-amino acids.

Amino Acids	**Retention Factor (k)[(a)]**	**α [(a)]**	**Retention Factor (k)[(b)]**	**α [(b)]**
Methionine	k_1 = 4.752; k_2 = 6.182	1.30	k_1 = 8.386; k_2 = 10.296	1.23
Phenylalanine	k_1 = 4.612; k_2 = 7.052	1.53	k_1 = 19.686; k_2 = 21.406	1.09
Tryptophan	k_1 = 4.742; k_2 = 7.942	1.67	k_1 = 15.086; k_2 = 16.895	1.12
Leucine	k_1 = 4.712; k_2 = 6.612	1.40	k_1 = 6.085; k_2 = 7.435	1.22
Asp. Acid	k_1 = 5.152; k_2 = 6.322	1.23	k_1 = 13.667; k_2 = 15.314	1.12
Threonine	k_1 = 4.642; k_2 = 6.172	1.33	k_1 = 6.306; k_2 = 7.256	1.15

[a]Separation of chiral analytes with mixtureof ACN/100 mM TEAA buffer (pH 4.1) 30/70 (v/v)

[b]Separation of chiral analytes with mixtureof ACN/60 mM AMAC buffer (pH 4.7) 30/70 (v/v)

With a constant organic modifier and buffer composition the chromatograms at various buffer pH are shown in Fig. 3-6, *i.e.*, the effects of pH on retention times of Dansyl-DL-threonine. The retention time of the solutes decreased with the buffer pH varied in the range of operational pH (3.4 – 6.5). Since at higher pH the carboxylic acid of chiral solute is mainly ionized, the

interaction between the dansyl group and β-CD decreases, leading to the decrease in the separation factor (α).

3.4 Conclusions

Poly(GMA-GUG-β–CD-co-EDMA) monolithic columns were prepared *via* the copolymerization of β–CD in one-pot approach. The prepared column was investigated for enantioselective separation of derivatized amino acids. The polymer monolith is less time consuming and can be easily reproduced, with further option of variation of monomer ratio composition. The enantioselective separation was conducted under reversed phase conditions.

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Chapter 4

Conclusions and future perspective

4.1 Conclusions

Monolithic column stationary phases as one of the methods to achieve the satisfaction in the analysis, simple preparation, rapid and good separations have been developed.The objectives of the present research are to develop the capillary columns for microreactor (Immobilized Enzyme Reactor) and for the separation of chiral compounds as chiral stationary phase (CSP).

Chapter 2 describes the preparation of immobilized microreactor for high throughput online digestion in capillaries of the monolith, binding with enzyme and achieve direct enzyme immobilization. Trypsin is used as one of the most popular digestion enzymes and provides typically active counter to peptides bond. Monolithic polymer column was successfully used as immobilized enzyme microcolumn and prepared *via* thermally initiated *in situ* polymerization and immobilization of trypsin. Denaturation of the protein (BSA) with urea increased the digestion in the microcolumn. This microreactor was applied to digestion of protein (BSA). Demonstration of the digestion of the protein in tris-HCl buffer solution with pH 7.8 was proved more compatible for on-line digestion. This system used microcapillary column for digestion of the protein (immobilized enzyme microcolumn) and for separation of the tryptic fragments.

Chapter 3 describes a preparation of capillary liquid chromatography column containing β-cyclodextrin in order to obtain satisfactory column for chiral stationary phase. The optimized monolithic column was applied for

enantioseparation of derivatized amino acid. Poly(GMA-GUG-β–CD-co-EDMA) monolithic columns were prepared *via* the copolymerization of β–cyclodextrin in one pot approach. The prepared column was investigated for enantioselective separation of derivatized amino acids. The polymer monolith is less time consuming and can be easily reproduced. Further option of variation of monomer ratio composition will be necessary. The enantioselective separation was conducted under reversed phase conditions.

4. 2 Future Perspective

Liquid chromatography (LC) is an important technique in analytical chemistry, because this method offering rapid analysis, excellent selectivity, and higher of column efficiency. The ease and flexibility of capillary columns preparation allows their wider applications.

Development of the monolith column as microreactor still remains as a challenging task due to the fact the high porosity and great uniform skeletal structure (support for the enzymes) can affect the separation of peptide fragments, and still needs to try other conditions for digestion and for separation.

Nowadays, analyze of biological samples and the introducing suitable functional groups as active site into monolith column for samples separation are still well known. Separation of racemic mixtures has been considered a more attractive tool to obtain the individual enantiomers [1]. The long-term stability and optimizing of the chiral stationary phase still remains as a challenging task and still needs to try other conditions for developing chiral separation. LC as one of the microfluidic techniques, along with monolithic capillary columns offer a

cheaper alternative to conventional HPLC. This combination allows short analysis time, reduced sample size and low consumption of solvents which enables analysis under environmentally friendly conditions [2].

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List of figures

Chapter 2

Chapter 3

List of tables

Chapter 1

Chapter 2

Chapter 3

List of publications

1. R. Putri, L. W. Lim, T. Takeuchi

   Development of Immobilized Enzymatic Trypsin Micro-reactor on Polymer Monolith Column for Biocatalytic Reactions in Capillary Liquid Chromatography. Globalize Research Journal of Chemistry, accepted for publication.

2. R. Putri, L. W. Lim, T. Takeuchi

   Separation of Dansyl Amino Acids in Capillary Liquid Chromatography Using Cyclodextrin-Bonded Chiral Monolithic Stationary Phases. Globalize Research Journal of Chemistry, accepted for publication.

List of presentations

[1] "Development of Immobilized Enzymatic Trypsin Microreactor on Polymer Monolith Column for Biocatalytic Reactions by Using Capillary Chromatography ", The 8th Asia-Pacific Symposium on Ion Analysis, Chiba, Japan, September 1-2nd 2015 (Oral Presentation)

[2] "Development of Immobilized Enzymatic Trypsin Microreactor on Polymer Monolith Column for Biocatalytic Reactions by Using Capillary Chromatography "Analytical Technology Towards Life Innovation" Makuhari-Messe, Japan., September 3-4th 2015 (Poster Presentation)

[3] "Chiral separation of dansyl amino acids in capillary liquid chromatography using cyclodextrin as chiral stationary phase", The 9th Asia-Pacific Symposium on Ion Analysis, Shaoxing, China, November 20-23rd 2017 (Oral Presentation)

Curriculum vitae

Radhia Putri was born on March 7th, 1988 in Padang, West Sumatera, Indonesia.

1994 – 2000	Elementary School (SDN 25), Padang, West Sumatera, Indonesia
2000 – 2003	Junior High School (MTsN Model), Padang, West Sumatera, Indonesia
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2013	Research student at Toyohide Takeuchi's Laboratory, Faculty of Engineering, Gifu University, Gifu, Japan
2011 – 2013	Master degree (M.Sc) at Chemistry Department, Mathematics and Natural Sciences Faculty, Andalas University, Padang, West Sumatera in 2013.
2015 – 2018	Doctoral degree (Ph.D.) at Material Engineering Division, Graduate School of Engineering, Gifu University, under supervisor Prof. Toyohide Takeuchi and Associate Prof. Lee Wah Lim.

Doctoral degree (Ph.D.) study was financially supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, (Mombukagakusho scholarship

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Japan, March 2018

Radhia Putri