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Research article

Immobilization of trypsin onto porous methacrylate-based monolith for flow-through protein digestion and its potential application to chiral separation using liquid chromatography

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ABSTRACT

Monolithic columns for analytical applications have attracted the researcher's attention. In this work, the laboratory-made organic-polymer monolithic column is modified with trypsin and further applied as a nano-biocatalyst microreactor and a stationary phase for separating chiral compounds by liquid chromatography. The monolith was synthesized by in-situ copolymerization of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) or trimethylolpropane trimethacrylate (TRIM) as a crosslinking agent, with porogen of 1,4-butanediol/propanol/water (4:7:1 v/v) and AIBN as the radical polymerization initiator inside PEEK and silicosteel tubings (1.0 mm i.d × 100 mm) at 60 °C for 12 h. A total monomer ratio (%T) and crosslinking agent (%C) of 40:25 and 28:12 were applied to prepare poly-(GMA-co-EDMA) and poly-(GMA-co-TRIM), respectively. The produced monoliths were further modified by introducing trypsin (10 mg/L) through the ring-opening reaction of the epoxide group existing in the monolithic column. The trypsin-immobilized poly-(GMA-co-EDMA) monolithic column was applied as the nanobiocatalyst microreactor for online/flow-through and rapid digestion of β-casein sample into its peptide fragments. The trypsin-immobilized poly-(GMA-co-TRIM) column has potential application to be used as the HPLC stationary phase for the separation of R/S-citronellal enantiomers.

1. Introduction

Enzymes are a catalyst with high potential for application in the analytical field because enzymes are useful for producing substances in an environmentally friendly manner [1]. The proteolytic enzyme that is widely used in proteolysis is trypsin. The proteolysis process can be carried out in solution (homogeneous) or by solid-phase digestion (heterogeneous) [2]. The enzyme immobilization method inside the microfluidic reactor containing nanostructured porous materials has several advantages, such as long-term storage stability, fast reaction rate, preventing enzyme autolysis reactions, and easy-to-use [3].

Several mechanisms of immobilization techniques include physical adsorption (via ionic interactions, hydrogen bonding, hydrophobic interactions, or van der Waals forces), crosslinking of enzymes in the polymeric pore support, and covalent bonding [4]. The selection of a suitable immobilization technique can avoid enzyme instability. In several studies, trypsin was immobilized into a different support material, such as a membrane polymer [5], silica capillary column [6], porous glass [7], silica monolith [8], silica hybrid monolith [9], and organic polymer monolith [10, 11].

The presence of mesoporous characters (2–50 nm) in the monolith plays a significant role because these pores can act as active sites for enzyme immobilization [4]. Monolithic columns with narrow inner diameter tubing have currently come into a new trend in HPLC. The low mobile phase consumption of conventional HPLC columns is proportional to the low dead volume characteristics and the separation efficiency of the monolithic column phase [12]. Monoliths have a flow-through pore structure providing a low-pressure drop, improving mass transfer kinetics, and facilitating high flow rates [13, 14]. In a monolith, interconnected pores and skeleton allow mobile phases to pass

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through the column by convention, causing the diffusion effects to be lower than conventional packed particle columns [15].

Enzyme immobilization to the polymeric monolith matrix commonly occurs via the opening-ring reaction of the epoxy group existing in GMA as a functional monomer. Further activation with a dialdehyde is aimed to improve the surface area of the monolith [16]. Trypsin can act as a chiral selector because it has many chiral centers originated from its constituent amino acids, providing selectivity towards chiral compounds. Thus, trypsin immobilization onto a support material was applied as a chiral stationary phase (CSP) [17]. The protein-based CSP has attracted the attention of researchers because of its unique enantioselective properties. The advantages of protein-based CSP include the use of a liquid mobile phase in reverse-phase HPLC. It has the selectivity to separate enantiomers from various compounds, and the analysis is more effective without going through derivatization [18]. A trypsin-based CSP study was able to separate enantiomers from N- and O-derivatives of amino acids lysine, histidine, and arginine [17]. In simple words, enzyme activity and site of chiral recognition influence the separation of the enantiomeric compounds.

The exploration of new chiral stationary material-based monoliths and separation by enantioselectivity techniques using HPLC is currently being developed due to their essential role in the separation efficiency and overall back pressure of the column [19]. The utilization of trypsin-immobilized monolith is expected to possess an excellent ability to separate isomeric compounds when operated as chiral stationary phases (CSP) in HPLC. Besides CSP, a large number of trypsin-immobilized monoliths were also reported for protein digestion and peptide analysis [2, 20, 21, 22]. In addition to trypsin, immobilization of chymotrypsin on monoliths or hybrid magnetic materials is also used for protein hydrolyzation [23, 24], and peptide synthesis [25], but is rarely applied to CSP [26]. This work focuses on examining the potential of trypsin-immobilized onto the methacrylate-based organic polymer monolithic columns as a nanobiocatalyst microreactor for high-speed and online digestion of proteins, and chiral stationary phase (CSP) for the separation of natural products.

2. Experimental

2.1. Chemical and materials

Ethylene glycol dimethacrylate (EDMA), pyridine, 2,2′-azobisisobutyronitrile (AIBN), glycidyl methacrylate (GMA), 3-(trimethoxysilyl) propyl methacrylate (MAPS), and R/S-citronellal were purchased from Sigma-Aldrich (Singapore). Trypsin, β-casein, HPLC grade acetonitrile (ACN), sodium hydroxide (NaOH), ethanol, 1-propanol, sodium carbonate (Na_2CO_3), and 1,4-butanediol were obtained from Merck. Hydrochloric acid 37% and acetone from Smart Lab Indonesia, trimethylolpropane trimethacrylate (TRIM) from Tokyo Chemical Industry (Japan). All chemicals for monolith synthesis and applications are used without purification.

2.2. Instrumentation

Union, column end plugs 1/16", PEEK finger-tight Nut produced by Supelco and syringe pump made by GL Sciences Tokyo Japan were employed to prepare the monolithic columns and their modification. Silicosteel and PEEK tubings (1 mm i.d × 1/16 inch o.d) were purchased from Restek (USA) and GL Sciences (Japan). Chromatographic system UFLC 20 (Shimadzu, Japan) was used for separation of analytes. The reverse phase C12 monolithic column (1.0 mm i.d × 100 mm) [27] was applied for separation of β-casein tryptic digest sample.

2.3. Preparation of poly-(GMA-co-EDMA) monolithic column

Before use, the polyetheretherketone (PEEK) tubing as a column housing was treated according to our previous work [28, 29]. This tubing is filled with sulfuric acid/water (1:1) and left to stand at room temperature for 6 h with both ends of the tubing closed. Sulfonation was carried out at room temperature. The tubing was rinsed with water until neutral (pH 7). Then, the tubing was refilled with 1 M GMA in acetone, left to stand at room temperature for 4 h with both ends of the column closed, then rinsed with ethanol three times. The solution filling and washing process of the tubing were carried out using a syringe. In the last step, the PEEK tubing was washed with acetone, cut into 100 mm length, and is ready to be filled with the mixture of monomer, crosslinker, and porogens for preparation of monolithic columns.

After the PEEK tubing as a column housing was ready, poly-(GMA-co-EDMA) monoliths were prepared according to the procedure reported in the previous works [13, 30, 31] with slight modification. The total volume mixture of functional monomer, crosslinker, and porogen was 2 mL in the following composition: 0.6 mL of GMA (%T 40) as a functional monomer, 0.2 mL of EDMA (%C 25) as crosslinker, and 1,4-butanediol/1-propanol/H_2O as porogen with a volume ratio of 4:7:1 (v/v). The mixture was homogenized by a sonicator for 5 min, then added with the radical initiator, AIBN (1% w/v), and homogenized again for further 5 min. The homogeneous solution was injected using a syringe into the sulfonated PEEK column. After both ends of the column were closed using PEEK fingertight plugs, the mixture was thus polymerized in an oven at temperature of 60 °C for 12 h. After the polymerization reaction was complete, the poly-(GMA-co-EDMA) column was connected to an HPLC pump. Then, the column was washed with ethanol and water for 1 h at a flow rate of 0.05 mL/min to remove unreacted monomer residues and a porogen.

2.4. Preparation of poly-(GMA-co-TRIM) monolithic column

The silicosteel tubing as a column housing was filled with 0.2 M NaOH and left for 30 min, washed with water, then filled with 0.2 M HCl and left for further 30 min. This procedure was repeated twice before washing the tubing three times each with water and acetone. MAPS (30%) in acetone and pyridine with a volume ratio of 30:65:5 was filled into the silicosteel tubing for this tubing's inner wall activation [13, 30]. Both ends of the tubing were closed and left at room temperature for 12 h. This activation step was repeated two times. In the last step, the silicosteel tubing was washed with acetone and cut into 100 mm length.

Poly-(GMA-co-TRIM) monoliths were prepared using the same procedure by replacing the EDMA with trimethylolpropane trimethacrylate (TRIM). To prepare a poly-(GMA-co-TRIM) monolith, the mixture composition (2 mL) consist of functional monomer GMA 0.493 mL (%T 28) and TRIM crosslinker 0.067 mL (%C 12), 5.6 mg AIBN (1% w/v) and porogen of 1,4-butanediol/1-propanol/H_2O (4:7:1, v/v). The reaction was carried out by in-situ copolymerization for 12 h at 60 °C. After the polymerization reaction was complete, the poly-(GMA-co-TRIM) monolithic column was washed with ethanol and water for 1 h using an HPLC pump at a 0.05 mL/min flow rate. The monolith column's permeability was measured according to the HPLC pump pressure drop and further identified by SEM.

2.5. Trypsin immobilization onto poly-(GMA-co-EDMA) and poly-(GMA-co-TRIM) monolithic columns

Trypsin is attached covalently to the surface of the monoliths at room temperature. For trypsin immobilization, as much as 10 mg/mL trypsin in 0.005 M benzamidine hydrochloride and 0.1 M carbonate buffer (pH 10.5) was passed through the monolithic columns using a dedicated syringe pump (GL Sciences, Japan) at room temperature for 1, 3, 5, and 7 h. The trypsin-immobilized monolithic columns were washed by flowing 1 M NaCl in a 0.1 M carbonate buffer (pH 10.5). Finally, the columns were rinsed again by flowing 0.005 M benzamidine hydrochloride in 0.1 M carbonate buffer (pH 10.5). The two washing steps above were carried out at room temperature for 1 h with a flow rate of 0.01 mL/min. A schematic representation of the chemical route used for immobilization of trypsin to poly-(GMA-co-EDMA) monolith is shown in Figure 1.

Especially for the poly-(GMA-co-TRIM) monolith column, additional preparation was carried out by introducing 1% glutaraldehyde in 0.1 M ammonium bicarbonate (pH 8) to the column at room temperature for 2 h with the flow rate of 0.05 mL/min. The purpose of this procedure is to provides a larger surface-active area of the monolith, which may result in better interaction with analytes [32, 33]. The column was then washed by flowing ethanol for 1 h at a flow rate of 0.05 mL/min. Before use, the trypsin-modified monolithic columns were stored at the refrigerator. A schematic representation of the chemical route used for immobilization of trypsin to poly-(GMA-co-TRIM) monolith is shown in Figure 2.

2.6. Application of trypsin-immobilized poly-(GMA-co-EDMA) and trypsin-immobilized poly-(GMA-co-TRIM) monolithic columns

The trypsin-immobilized poly-(GMA-co-EDMA) monolithic column as a nanobiocatalyst microreactor was applied to the online/flow-through digestion of β-casein samples. This protein (1000 mg/L) was dissolved in a mixed solution of 50 mM ammonium bicarbonate and 2 mM $CaCl_2$ pH 8. The β-casein solution was passed through the microreactor by employing the dedicated syringe pump (GL Sciences, Japan) at various flow rate of 1, 5, and 10 μL/min and digestion temperature of 37 °C, 45 °C, and 50 °C by placing the column inside CTO-20A oven (Shimadzu, Japan) to obtain sufficient digestion conditions. The resulted β-casein digest was collected in 1 mL vial. For off-line/batch-wise digestion, β-casein (1000 mg/L) in 50 mM ammonium bicarbonate and 2 mM $CaCl_2$ pH 8 was incubated at 37 °C for 15 h. The peptide profile identification of digested β-casein was then carried out using the reverse-phase C12 monolithic column with dimension of 1 mm i.d × 100 mm [27] by applying the gradient elution with the mobile phases of 0.05% TFA in water and acetonitrile/water (4:1 v/v) in 0.05% TFA. The peptides were detected at a wavelength of 214 nm at flow rate of 0.1 mL/min.

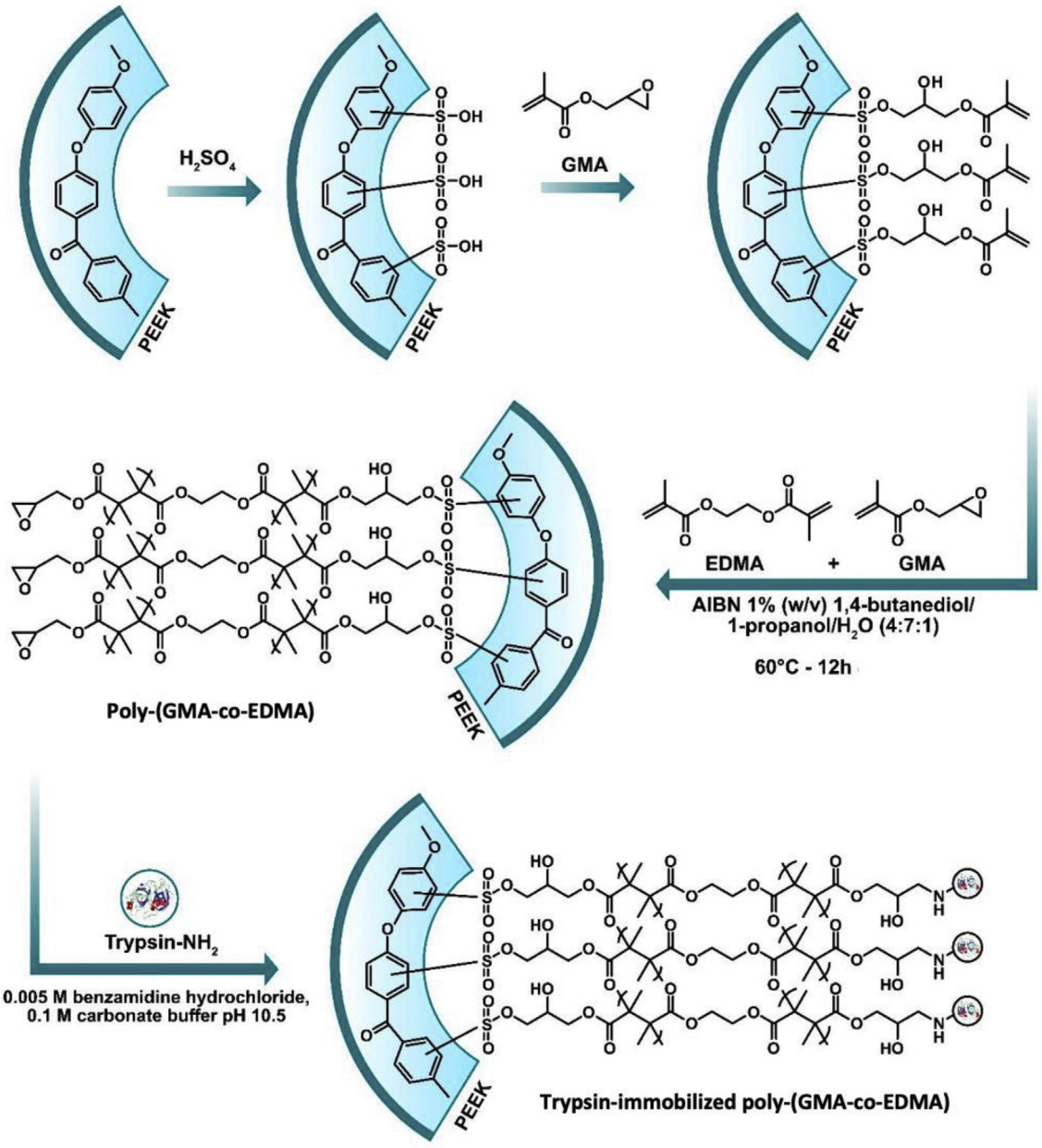

Figure 1. The chemical route for surface modification of PEEK tubing 1.0 mm i.d. × 1/16°.d and immobilization of trypsin onto poly-(GMA-co-EDMA) monolith inside the tubing.

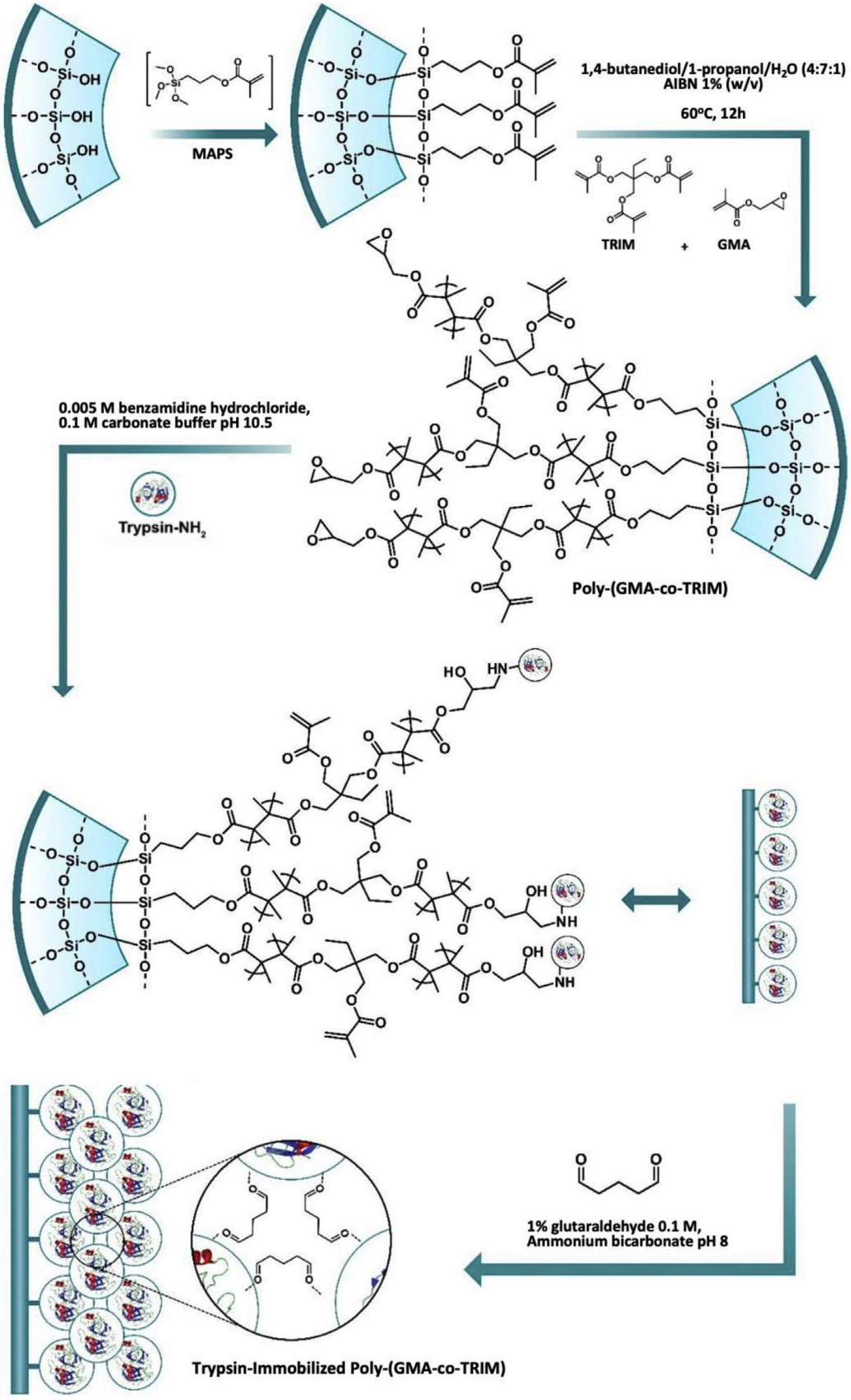

Figure 2. The chemical route for surface modification of silicosteel tubing 1.0 mm i.d. × 1/16°.d and immobilization of trypsin onto poly-(GMA-co-TRIM) monolith inside the tubing.

The trypsin-immobilized poly-(GMA-co-TRIM) monolithic column is connected to the HPLC system and used as a chiral stationary phase (CSP) column to identify R/S-citronellal compounds. The isocratic or gradient elution methods using the appropriate acetonitrile/water solvent composition were optimized. All analytes were detected at a wavelength of 290 nm, and the flow rates were varied from 0.02 mL/min to 0.1 mL/min.

3. Result and discussion

3.1. Preparation and characterization of tyrpsin-immobilized poly-(GMA-co-EDMA) and tyrpsin-immobilized poly-(GMA-co-TRIM) monolithic columns

Large numbers of monoliths were commonly synthesized inside fused silica capillary tubing with an inner diameter of 75–250 μm [34, 35, 36, 37] because of the increasing difficulty of preparing homogeneous monoliths as the diameter increases. Capillary-scale columns and miniaturized-chromatographic systems perhaps is required for analytical and green chemistry purposes. However, in term of easy-to-use and preparative purposes, a slightly larger inner diameter is more suitable. From this point of view, microbore columns with an inner diameter of 0.5–1.5 mm seems to be the best options as they can be easily connected to a standard commercially available HPLC system with minor modifications [13, 27, 28, 30]. This is the reason why monoliths in this work were prepared inside 1 mm i.d column tubing.

The monolith is prepared by in-situ copolymerization in the column tubing, therefore, the monolith formed must be covalently attached (chemical interaction) to the inner wall of the column, avoiding slip off and come out from the column under operating pressure. Accordingly, before being used as a column housing for monolith preparation, the PEEK and silicosteel tubings were treated first using sulfonation and silanization processes, respectively. The chemical anchoring to the inner wall of PEEK tubing is quite difficult because of no existence functional groups on its surface. For this reason, sulfonation was carried out to functionalize the inner wall surface of PEEK tubing (Figure 1). The presence of the sulfonate group provides advantages for further modification through the introduction of the methacryloyl group, allowing the chemical bonding of monolith with the inner wall of PEEK tubing. Additionally, the methacryloyl group is also used to terminate the sulfonation process, preventing degradation of PEEK tubing [28, 29].

The silanization process in the silicosteel tubing was carried out by hydrolyzing the column using a solution of hydrochloric acid and sodium hydroxide. MAPS solution was filled into the silicosteel column to facilitate covalent bonds forming between silanol group existing in the inner wall surface of the column and the monolith polymer via methoxy group of the MAPS (Figure 2).

Shu et al. [28] reported a comparison of the use of PEEK and silicosteel tubings as column housing for the preparation of methacrylate-based monoliths by in-situ copolymerization. It was found that the microglobule sizes of the monolith prepared inside PEEK tubing were larger than that of the monoliths synthesized inside silicosteel tubing. Other facts showed that monoliths made inside silicosteel tubing have higher back-pressure but better column performance for analytes separation than that made inside PEEK tubing. In our experiments, solid white monoliths slipped out of the columns when the back-pressure exceeded 15 MPa and 20 MPa for monoliths fabricated in PEEK and silicosteel tubings, respectively, indicating monoliths bonded more strongly in the inner wall surface of silicosteel tubing. Burke et al. [38] compared the EDMA and TRIM crosslinkers with the functional monomer GMA for monolith fabrication. The results showed that TRIM improves the kinetic reaction and produces more homogeneous monoliths with smaller pore sizes. This is because TRIM has three methacrylate groups, while EDMA only has two methacrylate groups used to react with GMA. These results indicated that TRIM provides better analytical separation of analytes. Based on the above facts and as a compromise between permeability and column performance, in our experiment, trypsin-immobilized poly-(GMA-co-EDMA) monolith was prepared inside PEEK tubing and applied as a nanobiocatalyst microreactor for online/flow-through digestion proteins. Meanwhile, the trypsin-immobilized poly-(GMA-co-TRIM) monolith used as the chiral stationary phase (CSP) was synthesized inside silicosteel tubing. During the immobilization process, the amino groups of trypsin reacted with the epoxide groups of poly-(GMA-co-EDMA) and poly-(GMA-co-TRIM), which were stabilized in benzamidine hydrochloride and carbonate buffer.

In our previous works [13, 30, 39], some parameters for the preparation of the poly-(GMA-co-EDMA) and poly-(GMA-co-TRIM) monoliths were investigated. It was found that the back pressure increased with increasing total monomer (%T) and crosslinker (%C). Similarly, an increase in back-pressure was also observed at a higher ratio of 1-propanol in the ternary porogen mixture of 1-propanol/1,4-butanediol/H_2O. In this work, we optimized the immobilization time of trypsin onto the poly-(GMA-co-EDMA) and poly-(GMA-co-TRIM) monoliths. The low flow-resistance characteristics of the trypsin-immobilized monolithic columns make it attainable to use a high flow rate, enabling high-speed operation. Thus, the mechanical stability of the monolith needs to be evaluated by testing the effect of increasing the flow rate on the back pressure. Figure 3 demonstrated the back-pressure-flow rate curves of the trypsin-immobilized poly-(GMA-co-EDMA) and the trypsin-immobilized poly-(GMA-co-TRIM) monoliths. The linear relationship of the flow rate against the back-pressure with correlation coefficients (R^2) in the range of 0.9785–0.9975 indicated good mechanical stability of the columns without significant deterioration as the flow rate increased. Additionally, a longer immobilization time of trypsin tends to increase back-pressures, as noted in the higher slope of the linear curves; there may be a change in the morphological structure of the monoliths to be more dense and rigid. There is also a tendency that the use of TRIM as a crosslinker increases the slope of the curve, which may be due to the formation of a crosslinker network that is more rigid than EDMA. We also examined a column-to-column reproducibility prepared from the same batch ($n = 5$), demonstrating an acceptable result since the relative standard deviation was within 10%.

Table 1 showed the permeability test of trypsin-immobilized poly-(GMA-co-EDMA) and trypsin-immobilized poly-(GMA-co-TRIM) monoliths at various trypsin immobilization times with constant flow rate using the mobile phase of ACN: water (50: 50, v/v). The permeability of the column is strongly influenced by the immobilization time of trypsin on the monolith surface. The permeability tends to decrease as the immobilization time longer. This may be due to the increasing amount of trypsin on the monolith surface, reaching a maximum of 5 h immobilization time. Taking into account the relatively low back pressure, the permeability of all monolithic columns at various times of trypsin immobilization made in this study was relatively high.

While the optimized conditions for preparation of poly-(GMA-co-EDMA) [13, 30, 31] and poly-(GMA-co-TRIM) [39] monoliths were adopted from previous works, we also optimized the immobilization time of trypsin for surface modification and obtaining the appropriate amount of trypsin covalently attached to the monolith. The elemental composition of the trypsin-immobilized monolith was detected by energy dispersive X-ray analysis (EDX). The result showed that the nitrogen (N) content (proportional to trypsin amount) increased with increasing the trypsin immobilization time. The nitrogen amounts for 1, 3, and 5 h immobilization time were 6.54, 7.64, and 12.40%, respectively. Therefore, the immobilization time strongly affects the amount of trypsin that reacts with epoxide groups existing in monoliths, and 5 h was selected for further experiment due to the highest nitrogen content, which corresponds to the highest immobilized trypsin on the surface of monoliths. The immobilization time of 7 h resulted in decreased nitrogen content (10.79%) because there might be a decrease in the trypsin's catalytic activity (autoproteolysis).

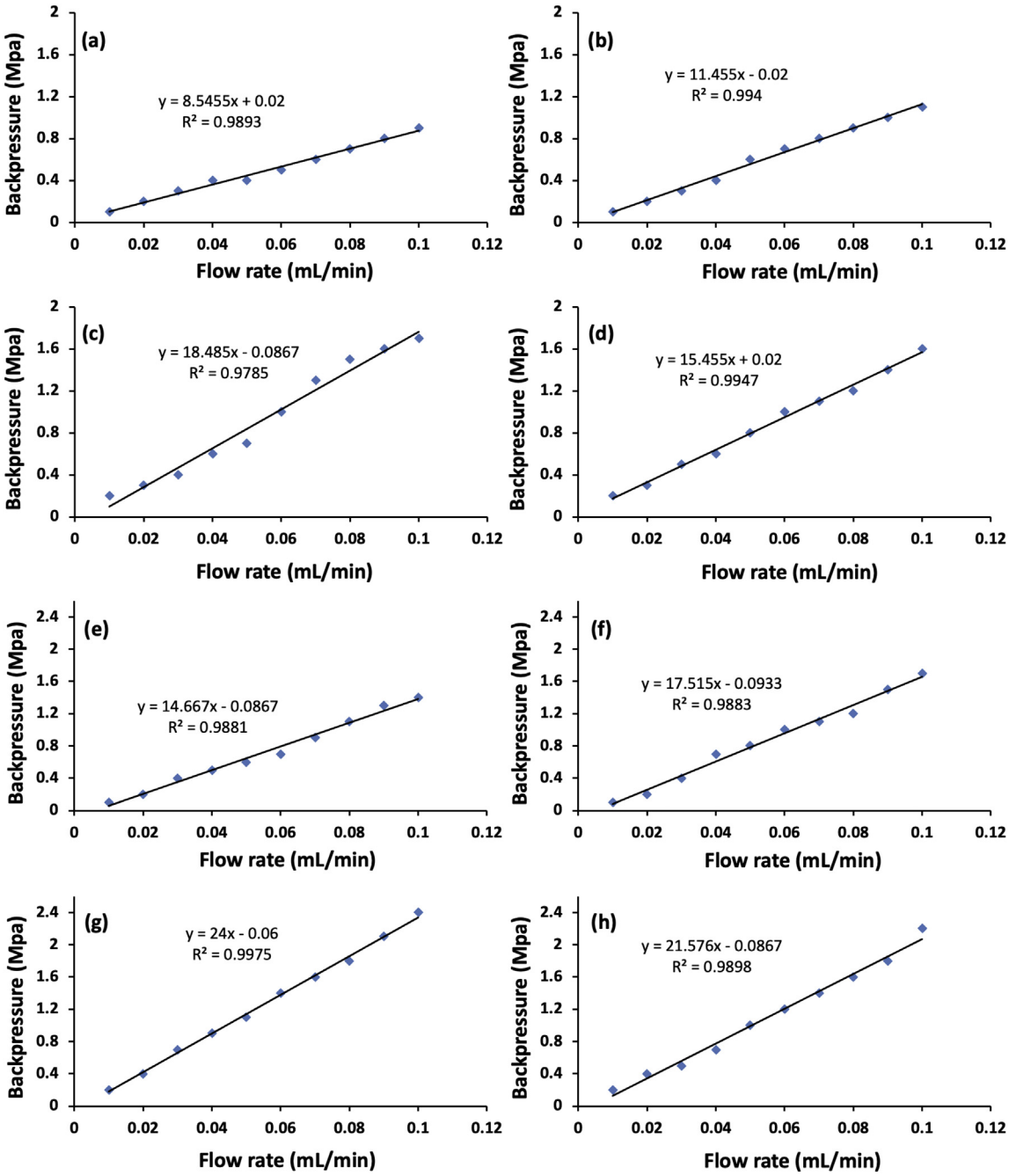

Figure 3. The curves of flow rate againts backpressure of the trypsin-immobilized poly-(GMA-co-EDMA) monolithic columns inside PEEK tubing at trypsin immobilization time of 1h (a), 3h (b), 5h (c), 7h (d) and the trypsin-immobilized poly-(GMA-co-TRIM) monolithic columns in silicosteel tubing at trypsin immobilization time of 1h (e), 3h (f), 5h (g), 7h (h). Column dimension: 1 mm i.d × 100 mm.

Figure 4(a) and Figure 4(d) showed SEM photos of the cross-sections of the monoliths inside PEEK and silicosteel tubing, respectively. It was found that no significant gaps were observed between the inner wall surface and the solid monoliths, confirming that the monoliths were tightly attached to the tubings. All monoliths with TRIM and EDMA as crosslinkers exhibited interconnected microglobules network to form continuous porous material with relatively good homogeneity (Figure 4b-c and 4e-f), indicating uniform surface area which is required for consistent flow patter, chromatographic separation, and other interactions [38]. Additionally, the space between the globules (flow--through pores), that allows convective flow of the mobile phase, can be seen in all the resulting monoliths. TRIM crosslinker has the potential to produce a better crosslinking network than EDMA because it has more methacrylate groups so that functional monomer of GMA may be more abundant on the monolith surface [40].

3.2. Trypsin-immobilized poly-(GMA-co-EDMA) monolith as nanobiocatalyst microreactor for online digestion of protein

The poly-(GMA-co-EDMA) monolith columns were made by the polymerization time of 12 h before their surfaces were further modified through the chemical immobilization of trypsin. The performance of the resulted trypsin-immobilized monoliths was examined by online digestion of protein as shown in Figure 5. For this purpose, two columns, such

Table 1. Permeability of the trypsin-immobilized poly-(GMA-co-EDMA) and the trypsin-immobilized poly-(GMA-co-TRIM) monolithic columns.

Monolith	Flow rate (mL/min)	Trypsin immobilization time (h)	Back-pressure (MPa)	Permeability	
				($m^2 \times 10^{-16}$)	(Darcy$\times 10^{-4}$)
Trypsin-immobilized poly-(GMA-co-EDMA) inside PEEK tubing 1.0 mm i.d × 100 mm	0.05*[1]	1	0.4	3.18	3.22
		3	0.6	2.21	2.14
		5	0.7	1.82	1.84
		7	0.7	1.82	1.84
Trypsin-immobilized poly-(GMA-co-TRIM) inside silicosteel tubing 1.0 mm i.d × 100 mm	0.05*[1]	1	0.6	2.21	2.14
		3	0.8	1.59	1.61
		5	1.1	1.16	1.17
		7	1.0	1.27	1.29

*1 Normal flow rate of 1 mm i.d column, solvent: ACN/water = 50/50 (v/v).

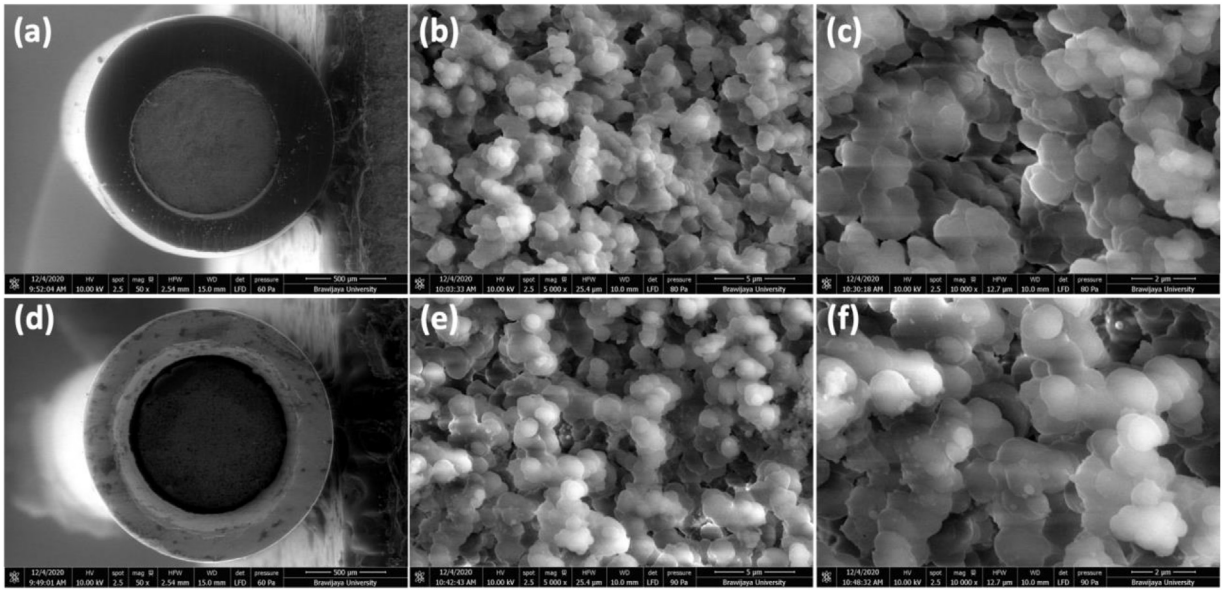

Figure 4. SEM images of trypsin-immobilized poly-(GMA-co-EDMA) monolith inside PEEK tubing with 50× (a), 5000× (b), and 10000× (c) magnifications, and trypsin-immobilized poly-(GMA-co-TRIM) monolith inside silicosteel tubing with 50× (d), 5000× (e), and 10000× (f) magnifications. Immobilization time of trypsin: 5h.

as (1) the trypsin-immobilized monolithic column for flow-through and high speed digestion of β-casein to produce their peptides fragments and (2) the reverse-phase C12 monolithic column [27] for separation of resulted peptides, were applied.

The effect of the digestion flow rate of β-casein passing through the trypsin-immobilized poly-(GMA-co-EDMA) monolithic column was investigated to obtain the optimum interaction between the protein with the immobilized trypsin. This procedure is essential to confirm the trypsin enzyme's effectiveness inside the monolithic column in detecting lysine and arginine amino acid residues from β-casein, producing a complete conversion of protein into its peptides. As shown in Figure 5, the number of appeared peaks as well as the peak intensities at the digestion flow rate of 1 μL/min, which corresponds to the residence time of 80 min, was higher than that in a flow rate of 5 μL/min (residence time ≈16 min), and 10 μL/min (residence time ≈8 min). This result indicated that the longer residence time of β-casein inside the trypsin-immobilized monolithic column generated higher conversion of the protein into its peptides. Accordingly, the digestion flow rate of 1 μL/min was selected for further experiment to obtain the optimal β-casein cleavage into its peptide sequences.

Figure 6 showed the effects of column temperature for online/flow-through digestion of β-casein using the trypsin-immobilized poly-(GMA-co-EDMA) monolith. It was found that the digestion temperature of 45 °C–50 °C exhibited better conversion of the protein into its peptides than the temperature of 37 °C, as indicated by higher peak intensities as well as a higher number of identified peaks. Although in a batch-wise method, the optimal operating temperature of trypsin for protein digestion is 37 °C [41], a higher temperature is recommended for flow-through digestion of a protein due to the temperature difference between inside and outside of the trypsin-immobilized monolithic column. Digestion temperature >50 °C is not examined to avoid denaturation of the protein samples as well as the immobilized enzyme.

Figure 7 showed the peptide profiles obtained by online/flow-through and off-line/batch-wise digestion methods. According to the theoretical tryptic cleavage sites of the β-casein primary structure, it is expected that there are 14 peptide sequences with two free amino acids, arginine and, lysine [42]. As shown in Figure 7(a), it seems that β-casein could not be completely digested by batch-wise method within 15 h, as indicated by very high peak intensity at the retention time of 11 min. Additionally, the number of appeared peaks was less than that of the flow-through digestion method. As can be seen in Figure 7(b), approximately there are 14–15 peptide peaks that can be detected after the online digestion of β-casein by passing the sample through the trypsin-immobilized poly-(GMA-co-EDMA) monolithic column with a residence time of 80 min (digestion flow rate ≈1 μL/min. This result is almost similar to the theoretical tryptic cleavage sites of β-casein,

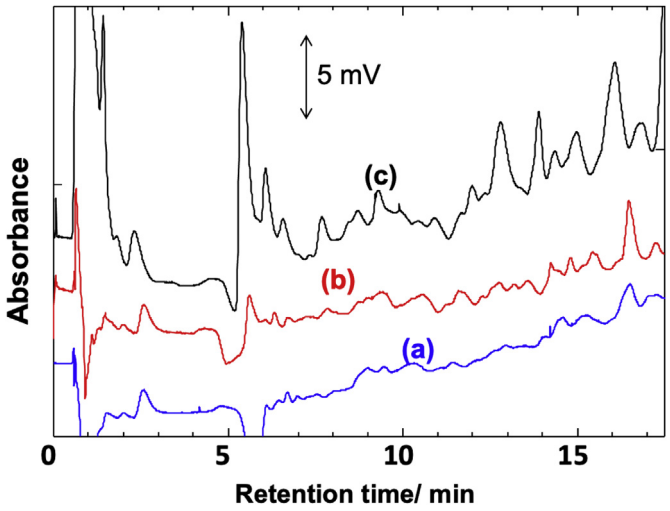

Figure 5. Separation of peptides after on-line/flow-through digestion at residence time of 8 min (a), 16 min (b), and 80 min (c) using the trypsin-immobilized poly-(GMA-co-EDMA) monolithic column at temperature of 37 °C. Separation column: reverse phase C12 monolith (1.0 mm i.d × 100 mm) [27], mobile phase A: 0.05% TFA in water, mobile phase B: acetonitrile/water (80/20, v/v) containing 0.05% TFA, gradient elution: 0–53% B in 30 min, flow rate: 0.10 mL/min, wavelength: 214 nm, sample volume: 2 μL.

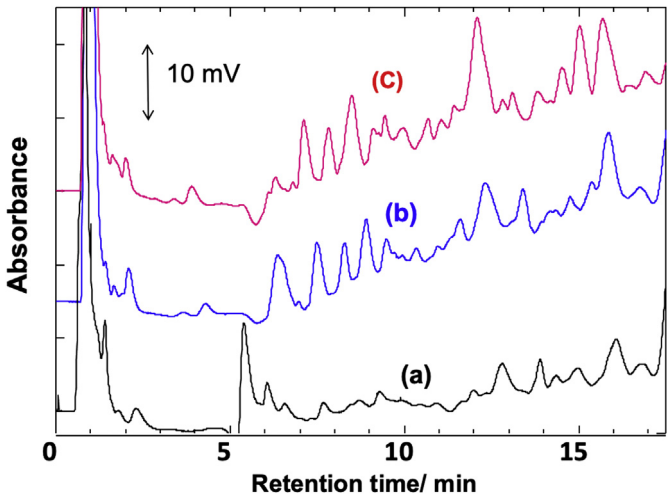

Figure 6. Separation of peptides after on-line/flow-through digestion at temperature of 37 °C (a), 45 °C (b), and 50 °C (c) using the trypsin-immobilized poly-(GMA-co-EDMA) monolithic column at residence time of 80 min. The separation column and other operating conditions are similar to Figure 5.

indicating potential application of the monolith for online peptide digestion and analysis. Since similar peaks were obtained after 25 injections (online digestion), we supposed that the trypsin-immobilized monolithic column can be used for the long term.

Such results confirm that the flow-through digestion method by passing the protein sample through the trypsin-immobilized monolithic column is very effective in reducing digestion time compared to the batch method which usually takes about 15–20 h. We suppose that all active sites of trypsin are existing on the monolith's surface when this enzyme is covalently immobilized onto the monolith, resulting in quick protein digestion. According to theoretical cleavage, trypsin cuts peptide chains mainly at the carboxyl side of the amino acid lysine or arginine residues [16, 43, 44].

Confirmation of each peptide fragment using molecular mass profiling is not carried out in this work because of the limitation of

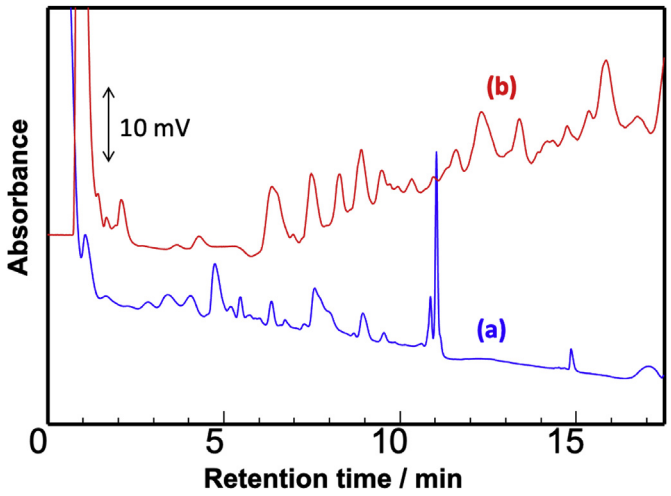

Figure 7. Separation of peptides after off-line/batch-wise digestion at the temperature of 37 °C and incubation time of 15 h (a) and online/flow-through digestion at the temperature of 45 °C and residence time of 80 min using the trypsin-immobilized poly-(GMA-co-EDMA) monolithic column (b). The separation column and other operating conditions are similar to Figure 5.

instrumentation and peptide standards availability. Although it is not a straightforward approach, we compared the hydrolysis pattern of β-casein peptides obtained by the trypsin-immobilized monolith in this work (Figures 6b–c and 7b) with the patterns studied by Bonneil et al. [42] and Che et al. [45]. It was found that the peptides profile patterns showed similar peaks absorption, confirming the accuracy of our results.

3.3. Trypsin-immobilized poly-(GMA-co-TRIM) monolith as chiral stationary phase (CSP)

Like other proteins, trypsin is actually a single enantiomer, even though it contains many chiral centers originating from its amino acid building blocks. Therefore, trypsin has chiral recognition sites, which can be applied as a chiral stationary phase (CSP) when immobilized into material supports [18, 20, 46]. In this work, the trypsin-immobilized poly-(GMA-co-TRIM) monolith column is used as the CSP for separation of natural products of R/S-citronellal compounds using isocratic elution (Figure 8) and gradient elution (Figure 9) of the liquid chromatography. As shown in Table 2, flow rates did not significantly affect the enantioselectivity (α) of R/S-citronellal but influence separation efficiency as reflected by resolution values (Rs). It seems that higher flow rates in both gradient and isocratic elution modes provided better resolutions. Probably, it is because the larger dispersion of analytes into the mobile phase occurred at a lower flow rate, resulting in a decrease in resolution. The flow rate of 0.1 mL/min using the gradient elution method provides a comparable resolution (Rs) as in the flow rate of 0.02 mL/min for the isocratic method. Although a higher flow rate is applied, variation of eluent composition as in the gradient elution causes a longer residence time of analytes than the isocratic elution for separation of R/S-citronellal using trypsin-immobilized monolithic column. Moreover, the gradient elution method causes stronger interaction of analytes with the stationary phase as indicated by increasing capacity factor ($k^{'}$). Such results imply that the gradient elution method with a flow rate of 0.1 mL/min and the isocratic method with a flow rate of 0.02 mL/min are the best option for separating the R/S-citronellal enantiomer since maximum theoretical plate numbers (N) reached 4392 and 3472 per-meter column at these conditions. However, further effort is still required to improve enantioselectivity and resolution.

All chromatographic data, including $k^{'}$, Rs, α, and theoretical plate number (N), were summarized in Table 2. Although the baseline resolved was not reached, but split peaks with different retention times could be

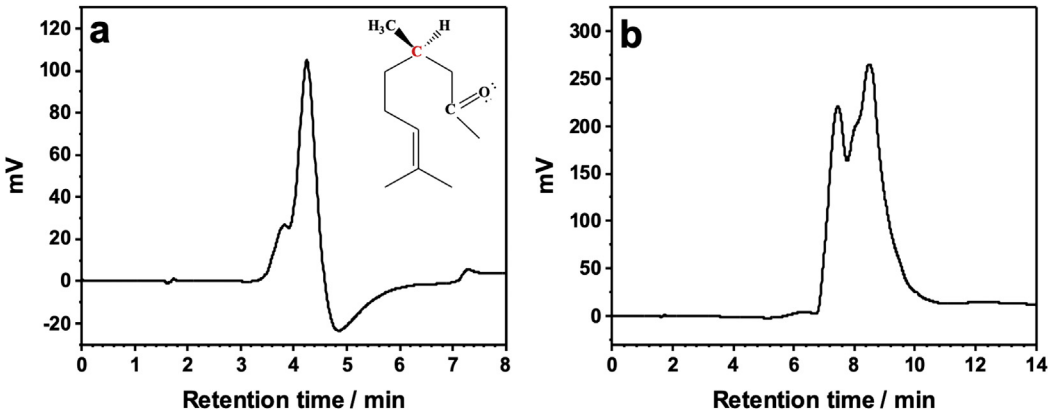

Figure 8. Enantioseparation of R/S-citronellal using the trypsin-immobilized poly-(GMA-co-TRIM) monolithic column at flow rates of 0.04 mL/min (a) and 0.02 mL/min (b) by isocratic elution method. Mobile phase: ACN/H_2O (50:50 v/v), sample volume: 2 μL, and detection wavelength: 290 nm.

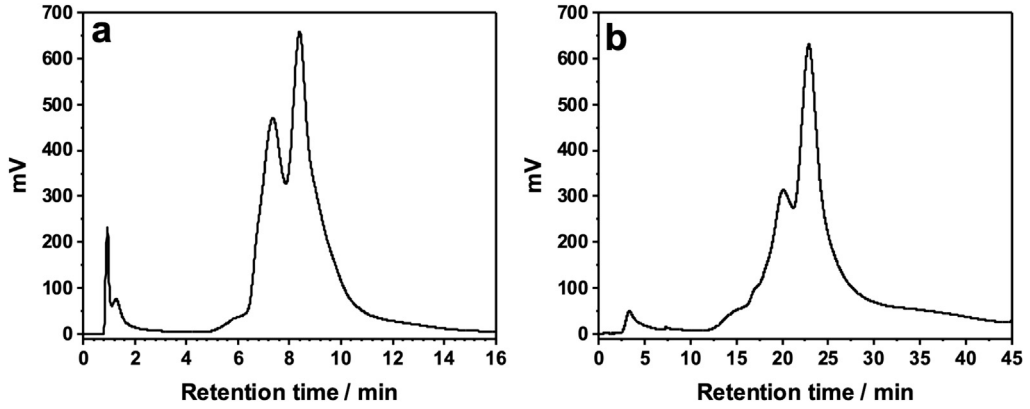

Figure 9. Enantioseparation of R/S-citronellal using the trypsin-immobilized poly-(GMA-co-TRIM) monolithic column at flow rates 0.1 mL/min (a) and 0.04 mL/min (b) by gradient elution method. Mobile phase A: ACN, mobile phase B: water, gradient elution: 50% B in 5 min, 50–55% B in 3 min, 55–60% B in 2 min, 60–65% B in 10 min, 65–70% B in 10 min, 70-50% B in 15 min, sample volume: 2 μL, detection wavelength: 290 nm.

Table 2. Chromatographic data for enantioseparation of R/S-citronellal using trypsin-immobilized poly-(GMA-co-TRIM) monolithic column.

Elution	Figure	Flow Rate (mL/min)	tR (min)	W (min)	N*[3]	k_1'	k_2'	α	Rs
Isocratic*[1]	8(a)	0.04	tR_1: 3.82	W_1: 1.5	1038	1.22	1.47	1.20	0.34
			tR_2: 4.24	W_2: 1.0	2882				
	8(b)	0.02	tR_1: 7.45	W_1: 1.6	3472	3.33	3.93	1.18	0.54
			tR_2: 8.48	W_2: 2.2	2375				
Gradient*[2]	9(a)	0.10	tR_1: 7.34	W_1: 2.3	1632	7.02	8.15	1.16	0.53
			tR_2: 8.38	W_2: 1.6	4392				
	9(b)	0.04	tR_1: 20.08	W_1: 9.0	797	27.41	31.38	1.15	0.40
			tR_2: 22.89	W_2: 5.0	3353				

[1]see Figure 8 for operating conditions, [2] see Figure 9 for operating conditions, tR: retention time, W: peak width, N: theoretical plate number per-meter column, k': capacity factor, α: selectivity factor, Rs: resolution.

identified, showing that trypsin-immobilized poly-(GMA-co-EDMA) monolith has shown potential to separate citronellal enantiomers. Capillary-scales column (i.e., 100 μm i.d) may provide a more efficient separation of an analyte. However, incomplete separation results of R/S-citronellal in this work were not caused by the use of a larger diameter of column (1.0 mm i.d). In our previous work [29], we could separate R/S-citronellal (Rs > 1.5) using Poly-(GMA-co-TRIM) modified with ethylenediamine-β-cyclodextrin (EDA-β-CD) inside 1.0 mm i.d x 100 mm tubing. Although this monolith exhibited its potential for enantioseparation of R/S citronellal, its chemical stability still needs improvement due to loss of EDA-β-CD after 250 h usage. Additionally, the synthesis procedure of EDA-β-CD was also complicated. To the best of our knowledge, reports on R/S-citronellal enantioseparation using a monolithic liquid chromatography are scarce [29, 39].

The ability of the chiral stationary phase to interact differently with each enantiomer to form transient-diastereomeric complexes requires a minimum of 3 interactions through H-bonding, π-π interactions, dipole stacking, inclusion complexing, and steric bulk. Proteins contain large numbers of chiral centers and may interact strongly with chiral analytes. However, the reports dealing with proteins as chiral selectors are quite limited [20, 21]. Calleri et al. [2, 22] modified commercial silica monolith with trypsin for peptide analysis. From the previous works reported so far [2, 20, 21, 22], we supposed that proteins as chiral selectors are more suitable for an ionizable group of enantiomers (i.e., amine,

carboxyl, acid). Therefore, the separation of aldehyde enantiomers like R/S-citronellal is still challenging using a chiral protein selector.

The mechanism of R/S-citronellal separation using a trypsin-immobilized monolithic column may rely on a combination of hydrogen bonding, hydrophobic and polar interactions. The functional groups of R/S-citronellal will change their position when they are mirrored. Thus, the only active group with an appropriate configuration with an amino acid of trypsin will retain on the column. For example, the citronellal hydrophilic group (-CH_2-CHO) interacts specifically with a hydrophilic amino acid of trypsin (i.e., tyrosine). Inversely, the hydrophobic group of the citronellal (–CH_2-CH_2-CH = C(CH_3)–CH_3) will not interact with another hydrophilic amino acid of trypsin (i.e., serine). Hydrogen atoms located in the chiral carbon center of the citronellal can interact via hydrogen bonding with tyrosine. This interaction strongly affects the residence time of the R/S-citronellal in the trypsin-immobilized poly-(GMA-co-TRIM) monolith column.

4. Conclusions

A good linear relationship was observed between the flow rate and back-pressure, indicating good mechanical stability of the prepared trypsin-immobilized poly-(GMA-co-EDMA) and the trypsin-immobilized poly-(GMA-co-TRIM) monolith columns. Morphological analysis using SEM shows globules dense formation with interconnected pores to form a continuous porous material. The trypsin-immobilized poly-(GMA-co-EDMA) monolith column has shown potential for flow-through/online and high-speed digestion of proteins for proteomic analysis. Additionally, the trypsin-immobilized poly-(GMA-co-TRIM) monolithic column is promising in the separation of chiral compounds.

Declarations

Author contribution statement

Suci Amalia: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Stevin Carolius Angga, Dias Septiana, Baiq Octaviana D. Anggraeny: Performed the experiments.

Elvina Dhiaul Iftitah, Warsito, Aliya Nur Hasanah: Conceived and designed the experiments; Analyzed and interpreted the data.

Akhmad Sabarudin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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