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Stop-flow reversed phase liquid chromatography × size-exclusion chromatography for separation of peptides

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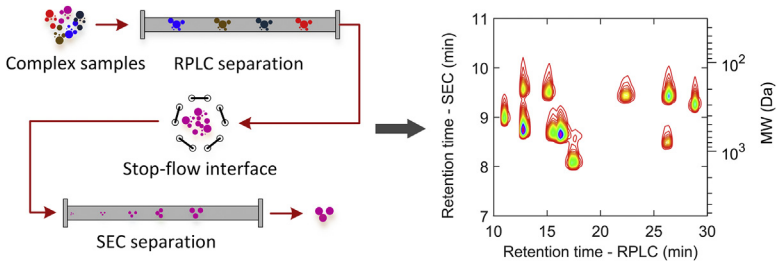
HIGHLIGHTS

- A stop-flow RPLC × SEC system was established for peptide analysis.
- The additional band broadening of peptides in RPLC was quantitatively evaluated.
- D_{eff} value of analytes with long retention time was determined to be low in RPLC.
- The SEC analysis was evidently improved by the first dimension RPLC separation.

GRAPHICAL ABSTRACT



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ABSTRACT

Size-exclusion chromatography (SEC) with wide application in peptide analysis presents challenges in determination of molecular weight distribution due to the relatively low resolution. In this study, a stop-flow reversed phase liquid chromatography (RPLC) × SEC system was constructed, aiming at improving the peptide separation in SEC. As the chromatographic dispersion during stop-flow operation might contribute to the band broadening in the first dimension (1st D) RPLC, the effects of different stop-flow operational parameters on the additional band broadening were quantitatively evaluated. Unlike analytes of large molecular size or long retention time with low effective diffusion coefficient (D_{eff}), additional band broadening was evidently observed for analytes of small molecular size and short retention time (high D_{eff}). Therefore, optimal flow rate, low column temperature and short stop-flow time were suggested for analyzing small molecules of short retention time. The established stop-flow two-dimensional liquid chromatography (2D-LC) was further tested on protein hydrolysates. The resolution was evidently improved for both heart-cutting and comprehensive 2D-LC analysis (despite additional band broadening in RPLC). Compared with heart-cutting analysis with higher 1st D resolution for selective fractions, comprehensive analysis could provide more complete information about the molecular weight distribution of the eluting solutes along RPLC.

1. Introduction

Conventional one-dimensional liquid chromatography (1D-LC) is usually unable to provide sufficient resolving power for analysis

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of complex samples though with the progress in column technology [1,2]. This motivates the development of combination of different liquid chromatography (LC) technologies (two-dimensional liquid chromatography, 2D-LC) [3]. In 2D-LC, fractions from the first dimension liquid chromatography (1st D LC) are collected and then re-injected to the second dimension liquid chromatography (2nd D LC) [4,5]. Higher resolving power can then be gained through the two different separations [6].

Two-dimensional liquid chromatography systems are mainly performed in three approaches, namely as off-line, on-line and stop-flow techniques [7]. Off-line 2D-LC is the simplest approach but with the highest resolving power [8]. However, quantitative analysis is very difficult in off-line 2D-LC due to the off-line operation [9]. In comparison, the on-line and stop-flow 2D-LC perform much better in quantitative analysis by using an automatic switching valve for fraction transfer [10,11]. In on-line 2D-LC, the first dimension (1st D) fractions are transferred sequentially to second dimension (2nd D) and thereby the 2nd D separation of each fraction is required to be finished before the transfer of next fraction. On the contrary, stop-flow 2D-LC employs an interrupted fraction transfer between the two dimensions and the 1st D flow is stopped during the 2nd D analysis of each fraction [12]. Therefore, there are no time constraints for 2nd D separation in stop-flow 2D-LC. The separation performance by stop-flow 2D-LC is much better than that by on-line 2D-LC [12,13].

While on-line 2D-LC has been widely applied in analysis of various samples [14,15], stop-flow 2D-LC is not so popular due to the concern of the possible additional band broadening resulting from stop-flow operation. Bedani et al. [16] studied the additional band broadening of peptides in stop-flow size-exclusion chromatography (SEC), but found no significant additional band broadening. In our previous research [17], a more detailed research was performed on the effects of different stop-flow operational parameters on the additional band broadening in SEC. In contrast, the additional band broadening was evidently observed while analyzing small molecules. This necessitates a detailed research on additional band broadening of analytes in the 1st D separation before the use of a stop-flow 2D-LC system. As a highly efficient separation method for peptide analysis, reversed phase liquid chromatography (RPLC) can play an important role in the 1st D separation of stop-flow 2D-LC [18]. However, the possible additional band broadening of peptides in the 1st D RPLC of stop-flow 2D-LC has not been studied yet. Since the effective diffusion coefficient (D_{eff}) of analytes in RPLC may be different from that in SEC due to the hydrophobic interaction between solutes and the stationary phase, a detailed research is needed.

SEC plays an important role in peptide analysis for molecular weight distribution [19,20]. Practically, the single separation in SEC is usually unable to provide accurate or complete information due to the low resolving power [21]. Besides, conventional SEC columns are usually not allowed in the 2nd D of on-line comprehensive 2D-LC due to the relatively low analysis speed [21]. Therefore, the coupling of RPLC and conventional SEC in stop-flow mode will be an attractive method, which can help to provide more accurate and complete information about the molecular weight distribution of peptides.

In the present study, a stop-flow RPLC × SEC system for peptide analysis was designed and constructed in-house. The effects of molecular weight (MW) and retention time of the analyte, stop-flow time, flow rate and column temperature in the 1st D LC on the additional band broadening were quantitatively evaluated using commercially available small peptides. The established stop-flow RPLC × SEC system was then utilized for analysis of complex protein hydrolysates. Two different separation methods, comprehensive analysis and heart-cutting analysis, were performed to demonstrate the utility of stop-flow RPLC × SEC system in analysis of complex protein hydrolysates using the same LC equipment.

2. Materials and methods

2.1. Reagents and materials

High performance liquid chromatography (HPLC) grade bovine serum albumin (BSA, 66,463 Da), cytochrome C (CTC, 12,384 Da), aprotinin (APR, 6512 Da), Gly-Gly-Tyr-Arg (GGYR, 451 Da) and Gly-Gly-Gly (GGG, 189 Da) were purchased from Sigma-Aldrich (Steinheim, Germany). Defatted peanut meal was purchased from Shandong Luhua Group Co., Ltd. (Shandong, China). HPLC grade Trp-Ser-Arg-Glu-Glu-Gln-Glu-Arg-Glu-Glu (WSREEQEREE, 1377 Da), Ala-Asp-Ile-Tyr-Thr-Glu-Glu-Ala-Gly-Arg (ADIYTEEAGR, 1124 Da), Trp-Ser-Arg-Glu-Glu-Gln-Gln-Glu (WSREEQE, 963 Da), Trp-Ser-Arg-Glu-Glu-Gln (WSREEQ, 834 Da), Glu-Gln-Gln-Gln-Gln (EQQQQ, 660 Da), Ala-Leu-Pro-Glu-Glu-Val (ALPEEV, 657 Da), Tyr-Ala (YA, 252 Da), Pro-Tyr (PY, 278 Da), Tyr-Val (YV, 280 Da), Leu-Tyr (LY, 294 Da) and Tyr-Leu (YL, 294 Da) were purchased from GL Biochem Ltd (Shanghai, China). HPLC grade His-Gly (HG), trifluoroacetic acid (TFA), and acetonitrile (ACN) were purchased from Aladdin Bio-chem Technology Co., Ltd (Shanghai, China). Alcalase 2.4 L for protein hydrolysis was purchased from Novo Industry A/S (Bagsvaerd, Denmark). Ultra-pure water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA).

Standard solutions of BSA (2 mg mL^{-1}), CTC (2 mg mL^{-1}) and APR (1 mg mL^{-1}) were prepared in 60% water/40% acetonitrile (v/v) with 0.1% TFA. Standard solutions of GGYR (2 mg mL^{-1}), GGG (8 mg mL^{-1}), WSREEQEREE (1 mg mL^{-1}), ADIYTEEAGR (2 mg mL^{-1}), WSREEQE (2 mg mL^{-1}), WSREEQ (2 mg mL^{-1}), EQQQQ (2 mg mL^{-1}), ALPEEV (2 mg mL^{-1}), YA (2 mg mL^{-1}), PY (4 mg mL^{-1}), YV (4 mg mL^{-1}), LY (2 mg mL^{-1}), YL (2 mg mL^{-1}) and HG (1 mg mL^{-1}) were prepared in 88% water/12% acetonitrile (v/v) with 0.1% TFA.

2.2. Sample preparation

Soybean protein hydrolysates were prepared through enzymatic hydrolysis by the previously reported method [22]. Insoluble elastin was purified from aortas according to Sionkowska et al. [23], and then dispersed in deionized water (elastin: water = 1:10, w/w). The hydrolysis of elastin was carried out with Alcalase 2.4 L (2% w/w, protein basis) for 10 h under optimal conditions (pH 8.0, 55 °C) and then terminated by heating in boiling water for 15 min. The obtained mixture was then centrifuged in a CR22N refrigerated centrifuge (Hitachi Co. Ltd., Japan) at 12000 *g* for 20 min to obtain the elastin hydrolysates.

2.3. Instrumentation and chromatographic methods

The stop-flow RPLC × SEC system was constructed in-house using a Dionex Ultimate 3000 Standard performance liquid chromatography system (Thermo Fisher Scientific Inc., Shanghai, China). The instrument was equipped with two pumps (pump 1 and pump 2), an autosampler, a column oven, a DAD detector, a ten-port switching valve and a Rheodyne 7725i manual injector. A schematic of the system is shown in Fig. 1. The flow from C18 column was split using a 'tee' connector (Agilent Technologies, Guangzhou, China) at a ratio of 2:1 to waste and the sample loop (200 μL), respectively.

A 4.6 × 250 mm SinoChrom ODS-AP C18 column packed with 5 μm particles (300 Å, Dalian Elite Analytical Instruments Co.,Ltd, Dalian, China) was used as the 1st D column. The 2nd D column was a 7.8 × 300 mm G2000 SWXL (LOT. 501 Y) column (Tosoh