View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Carolina Digital Repository

HHS Public Access

Author manuscript

J Chromatogr A. Author manuscript; available in PMC 2017 July 05.

Published in final edited form as:

J Chromatogr A. 2017 March 24; 1490: 126–132. doi:10.1016/j.chroma.2017.02.024.

Performance comparison of three trypsin columns used in liquid chromatography*

Tereza Šlechtová[a], **Martin Gilar**[b,*], **Kv ta Kalíková**[a], **Stephanie M. Moore**[c], **James W. Jorgenson**[c], and **Eva Tesa ová**[a]

[a]Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Hlavova 8, 128 43, Prague, Czechia

[b]Waters Corporation, 34 Maple Street, Milford, MA 01757, USA

[c]Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290, USA

Abstract

Trypsin is the most widely used enzyme in proteomic research due to its high specificity. Although the in-solution digestion is predominantly used, it has several drawbacks, such as long digestion times, autolysis, and intolerance to high temperatures or organic solvents. To overcome these shortcomings trypsin was covalently immobilized on solid support and tested for its proteolytic activity. Trypsin was immobilized on bridge-ethyl hybrid silica sorbent with 300 Å pores, packed in 2.1 × 30 mm column and compared with Perfinity and Poroszyme trypsin columns. Catalytic efficiency of enzymatic reactors was tested using N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride as a substrate. The impact of buffer pH, mobile phase flow rate, and temperature on enzymatic activity was investigated. Digestion speed generally increased with the temperature from 20 to 37 °C. Digestion speed also increased with pH from 7.0 to 9.0; the activity of prototype enzyme reactor was highest at pH 9.0, when it activity exceeded both commercial reactors. Preliminary data for fast protein digestion are presented.

Keywords

Digestion efficiency; Immobilized enzymatic reactor; Trypsin; Activity

1. Introduction

Enzymatic proteolysis is a common sample preparation method for LC–MS identification, and characterization of proteins. The most common choice of enzyme is trypsin, a highly specific protease, which cleaves peptide bonds only after arginine and lysine residues [1]. The enzymatic in-solution protein digestion is associated with a number of difficulties. It can be time consuming (up to 24 h), trypsin autolysis peptides are observed, and the enzymatic

*Selected paper from 31st International Symposium on Chromatography (ISC2016), 28 August–1 September 2016, Cork, Ireland.

*Corresponding author. Martin_Gilar@waters.com (M. Gilar).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

activity can be impaired at elevated temperatures [2] or in the presence of organic solvents [3].

Some of these limitations can be overcome by enzyme immobilization on solid support. Immobilized enzymes can be used in either batch mode digestion or as flow through reactors. Both approaches have been investigated over the past two decades and several commercial products have been introduced. However, adoption of immobilized enzyme reactors (IMERs) for protein analysis has been slow due to their price, limited stability, and in some cases by an incorrect application. While the IMERs can be highly active, the speed of proteolysis can be reduced by secondary/tertiary protein sequence (inaccessible digestion sites). Presence of chaotropic agents used to denature proteins may inactivate immobilized enzyme, while the presence of primary sequence motifs with slow digestion kinetics may result in production of missed cleavage peptides. The dependence of tryptic digestion kinetics on primary protein sequence was studied recently [4–6].

Despite these shortcomings, IMERs have been shown to perform protein digestion within minutes [7,8], or even seconds [9–11]. Since the intermolecular collisions of trypsin are minimal, high enzyme concentration can be used in immobilized reactors, improving speed of digestion, while minimizing enzyme autolysis. Packed IMERs can be readily used with LC instrumentation in flow through mode, reducing the sample manipulations and possible contamination. Trypsin has been immobilized using a wide variety of carriers; monoliths [8,12], silica particles, nano particles, or membranes [9,13,14]. The nature of the carrier is the most important factor in determining activity stability, and capacity of enzymatic reactors; stationary phase can either stabilize or denature the enzyme [9,15]. As suggested in a recent review [13], the development of carriers with superior characteristics, such as mechanical strength, large surface area, low back pressure, high enzyme loading capacity, and good biocompatibility, is the main goals of current IMER research.

The commercially available IMERs are reportedly stable in organic solvents, high flow rates, elevated temperatures, and compatible with moderate pressures up to 2500 psi. The most frequently used is Poroszyme® Immobilized Trypsin Cartridge from Applied Biosystems® based on polystyrene divinylbenzene (PDVB) particles available since 1995 [11,16–34]. The manufacturer states, that this column provides enhanced biomolecule access *via* large pores, fast 1–5 min digestion, high sample recovery, and chemical stability [35]. A similar product StyrosZyme™ TPCK-Trypsin column manufactured by OraChrom was applied to on-line BSA digestion [36] and compared to other trypsin columns [37]. Perfinity Biosciences offer trypsin immobilized on sorbent for batch digestion in vial [38] or in column format. Perfinity Biosciences IMERs have been used for automated protein digestion –LC/MS analysis and applied for identification of transferrin peptides [39], characterizing the post-translational modifications of human serum albumin [40] and for analysis of therapeutic proteins [41].

Abundance of IMER research reports have been published in last two decades including useful reviews [15,39,42,43]. While the research is very active in microscale proteomic LC–MS applications [9,44], where the ability to process small samples without protein losses is important, the IMER technology is not widely applied to routine protein bioanalysis or

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

peptide mapping [43]. The reason that the conventional in-solution trypsin digestion remains the workhorse may be attributed to several factors. (i) Commercially available IMERs are expensive. (ii) Although the trypsin autolysis is reportedly limited, it is still present. Acetylation has been shown to reduce the immobilized trypsin autolysis and improve its proteolytic activity [10]. (iii) IMER and in-solution tryptic digestions provide consistent, but different results [39,43]. The presence of incompletely cleaved peptides have been reported in IMER digest [42]; this may be related to the short digestion times chosen for digestion. (iv) Immobilized trypsin is reportedly stable [11] and reasonably resistant to mildly denaturing conditions such as elevated digestion temperature [38] or the presence of organic solvents [3]. However, some loss of digestion performance is expected over long term use, posing problem for the digestion repeatability. (v) Immobilized trypsin should not be exposed to reduction agents (to avoid enzyme activity loss). This forces the changes of traditional digestion protocol. The reduction/alkylation step can either be omitted (resulting in generation of different peptide map) or performed pre or post digestion. Performing reduction/alkylation step on certain protein classes (antibodies) often lead to their denaturation. This may improve the success of tryptic digestion. On the other hand, denaturation may lead to protein precipitation out of solution [45], posing a challenge for sample handling and on-line IMER digestion. In such case the elevated temperature, organic solvents and mild denaturants [46] may have to be used to aid the solubilization of sample, with potential adverse effects on IMER columns longevity.

Many immobilized enzymatic reactors have been prepared in past years; however, their performance is rarely compared to the commercial IMERS [33,37]. In this work we prepared a prototype immobilized trypsin reactor based on high pressure compatible 5 μm bridged ethyl hybrid (BEH) silica particles with 300 Å pores packed into ultra performance chromatography column hardware withstanding operational pressures up to 15 000 psi [47]. The prototype IMER was compared to Poroszyme and Perfinity IMERs available in similar column dimensions (Table 1). The goal of the work was to benchmark the digestion activity and repeatability of three IMERs at various temperatures, flow rates (residence time) and buffer pH. N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride, a common digestion substrate. Preliminary digestion test of the prototype IMER with model protein was performed using Cytochrome C and BSA. Further evaluation of prototype IMER with more complex protein samples was described in a separate report [7].

2. Materials and methods

2.1. Chemicals and reagents

N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, 99%), N_{α}-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), acetonitrile (ACN, gradient grade), chloroacetic acid (ACS reagent, 37%), calcium chloride (Dri™, 97%), Trizma® base (Tris, 99.9%), ethanolamine (98%), formic acic (>99%), bovine cytochrome C (molecular weight 12230.9 Da), BSA, dithiothreitol (DTT, >99%), iodoacetamide (IAM, >99%), and trypsin (T1426, TPCK Treated, essentially salt-free, lyophilized powder, 10,000 BAEE units/mg protein) were supplied by Sigma Aldrich (St. Louis, MO, USA). Micro BCA Protein Assay Reagent Kit and trifluoroacetic acid (99.5%) were supplied by Thermo Fisher Scientific (Waltham,

Author Manuscript

MA, USA). ALD Coupling Solution which contained 1 M $NaCNBH_3$ was obtained from Sterogene (Carlsbad, CA, USA), triethoxysilyl butyraldehyde (90%) from Gelest Inc. (Morrisville, PA, USA), ammonium bicarbonate (99.5%) from Fluka (Buchs, Switzerland), anhydrous ethanol (99.5%) from Acros Organics (Belgium, NJ, USA). Non-bonded BEH silica particles (5 μm, 300 Å) were supplied by Waters Corporation (Milford, MA, USA). Deionized water was purified with a Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). 50 mM ammonium bicarbonate buffer was made by dissolving the appropriate amount of ammonium bicarbonate in deionized water and adjusted to pH 9.0 with 1 M NaOH. Tris digestion buffer composed of 0.05 M Tris, 0.01 M calcium chloride titrated to desired pH by 2 M chloroacetic acid.

2.2. Preparation of IMER prototype

The trypsin immobilization was carried out at the University of North Carolina at Chapel Hill (UNC) using protocol adapted from Ahn et al. [47]. Approximately 0.6 g of BEH silica particles supplied by Waters Corporation were added to a 25 mL round bottom flask and placed in an oven (110 °C) overnight prior to immobilization. When cooled, the flask and its contents were kept under a constant flow of nitrogen. Then 220 μL of triethoxysilyl butyraldehyde in 4 mL of anhydrous ethanol was added to the flask. The mixture underwent magnetic stirring for 2 h at room temperature. Approximately 10 mL of 50 mM ammonium bicarbonate buffer (pH 9.0) was added to the reaction and the mixture was transferred into a 50 mL centrifuge tube. The particles were washed three times *via* centrifugation with 20–30 mL of 50 mM ammonium bicarbonate buffer to remove unreacted silane. After the final decant, particles were slurried in 4 mL of buffer in a round bottom flask with magnetic stirrer. 80 mg of trypsin dissolved in 4 mL of 50 mM ammonium bicarbonate buffer, followed by the addition of 2 mL of ALD Coupling Solution (containing 1 M $NaCNBH_3$). Reaction was performed for 2 h at room temperature. Afterwards, 2 mL of 1 M ethanolamine was added to quench the reaction for another 30 min at room temperature. Particles were washed three times with 50 mM ammonium bicarbonate buffer to remove excess of trypsin and ethanolamine using the above described protocol. Scheme of covalent bond can be found in Ahn et al. [47]; basic chemistry of aldehyde reductive amination reaction is described in the following textbook [48]. Bonded BEH particles with immobilized trypsin were slurried in water with trifluoroacetic acid (pH 4.0) and packed into the 2.1 × 30 mm column hardware.

Before column packing, the amount of immobilized trypsin on BEH particles was estimated using Micro BCA Protein Assay Reagent Kit. Presence of protein was measured as a color reaction at 562 nm using UV–vis μQuant microplate spectrophotometer (BioTek, Winooski, VT, USA). BSA was used to construct the calibration plot of absorbance versus mg of protein. The amount of immobilized trypsin per 1 g of BEH sorbent was approximately 84 mg. This value indicates the amount of immobilized protein, but not its activity, which could be impaired by immobilization procedure.

To determine the activity of immobilized trypsin, BAEE was used as substrate. BAEE digestion was monitored as change in UV absorbance at 253 nm using Labda35 UV/VIS spectrometer (Perkin Elmer, Waltham, MA, USA). Slope of the UV absorbance change

Author Manuscript

versus time obtained for trypsin in-solution digestion was compared to equal amount of trypsin immobilized on BEH particles dispersed in the reaction buffer. The retained activity of immobilized trypsin was ~35–40% compared to freshly prepared trypsin solution. Prototype IMER was further tested in flow-through mode LC/MS using direct injection of Cytochrome C (the protein was neither reduced nor alkylated).

2.3. Instrumentation and methods

The activity of immobilized trypsin (IMERs) was determined by HPLC using a Waters Alliance System composed of Waters 2690 Separation Module, Waters 2487 UV–vis 2-channel detector, 717 Plus autosampler and Waters Alliance Series column heater and cooler (Waters Corp., Milford, MA, USA). Instrument control and data collection were performed by Empower 2 software. For enzymatic digestion, three different trypsin columns were used, *i.e.* Poroszyme® (Applied Biosystems, Foster City, CA, USA), Perfinity (Perfinity Biosciences Inc., West Lafayette, IN, USA) and prototype IMER synthesized at University of North Carolina at Chapel Hill using a modified method described previously [47]. Separation of digestion products was carried out using an XTerra® RP18 column (Waters, Milford, MA, USA), 150 × 3.0 mm, 3.5 μm. In Table 1 we list IMER data provided by manufacturers. Although the operating temperature range is up to 67 °C, we restricted digestion temperatures 20–37 °C in order to maintain IMER's activity.

Digestion experiments were carried out using BAPNA as a substrate. BAPNA was prepared at concentration 6 mg/mL in mixture of 25% ACN, 25% water and 50% digestion buffer (*v/v/v*). 100 μL of BAPNA substrate was injected on IMER connected in series to separation column (mobile phase gradient passed through both IMER and separation column). Substrate BAPNA and its yellow digestion product *p*-nitroaniline were identified and quantified using UV–vis detector set at 254 and 410 nm detection wavelengths. Mobile phase consisted of Tris buffer (pH 7.0, 8.0 or 9.0) and ACN. Three different gradients and flow rates were used. The rationale for changing flow rate was to alter the residence time of substrate on the IMER column. The completeness of digestion is expected to decrease at elevated flow rates. Mobile phase gradient started at 95/5 Tris/ACN volume ratio held for 1 min; it was raised to 50/50 in 110, 70 and 50 min for flow rates 0.1, 0.2, and 0.3 mL/min, respectively. Given the physical dimension of IMERs and volume fraction of reactor containing mobile phase ($\varepsilon_t \sim 0.7$), the estimated residence times of substrate on the IMERs were 44, 22 and 15 s for flow rates 0.1, 0.2, and 0.3 mL/min, respectively. Separation and digestion temperature was 20, 25, 30 and 37 °C. The autosampler temperature was set to 25 °C.

The IMER experiments started at pH 8.0 (recommended pH for tryptic digestion) followed by pH 7.0 and pH 9.0. The order of temperature experiments for each IMER at given pH was 20, 25, 30, and 37 °C; each digestion condition was tested at 0.3, 0.2 and 0.1 mL/min flow rate, respectively. Initial mobile phase composition, *i.e.* 5/95 (*v/v*) ACN/Tris buffer, is recommended by column manufacturers. Since the presence of organic modifier in the buffers affects the activity of hydronium ions [49], we report pH of aqueous buffer before the addition of acetonitrile. On-line digestion measurements were performed in eight repetitions for each separation conditions; the calculated relative standard deviation (RSD)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

values are reported (Supplemental Table S3). All experiments were reported using a single IMER column of each type. Enzyme in all columns was treated by *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) by manufacturer to minimize the undesired chymotrypsin activity.

Activity of prototype BEH IMER column was subsequently evaluated for digestion of bovine cytochrome C protein. 11.5–115 μg of protein (1–10 nmol) was injected onto prototype IMER at room temperature at 1 mL/min flow rate. Sample solvent and IMER mobile phase was 50 mM ammonium bicarbonate, pH 7.8. Digested peptides were trapped on 3 × 250 mm CSH XSelect C18, 5 μm column (Waters Corp., Milford, MA, USA). Then the IMER was taken off-line and peptides were eluted with gradient of acetonitrile in water (both containing 0.1% formic acid). Gradient was held from 0 to 0.6 min at 0.5% acetonitrile content, followed with 0.5–70% of acetonitrile in 0.6–5.6 min. ACQUITY UPLC system with TQ triple quadrupole MS (Waters Corp., Milford, MA, USA) in full scan mode were used for analysis of cytochrome C digest. Digested peptides data were analyzed manually using Masslynx v 4.1 (Waters Corp., Milford, MA, USA) using Biolynx Protein/Peptide editor autodigest simulation function. TQ MS was tuned to resolution 1800 Da; average mass error for peptides was 240 ppm. Cytochrome C and BSA data analysis is provided as Supplemental information.

3. Results and discussion

For proteomic research, fast, repeatable and more reliable protein digestions are desirable. With the on-line digestion setup the IMER is connected in series with chromatographic column. In such case, protein sample is introduced by HPLC injector, transported by the stream of mobile phase through the IMER, digested, and the resulting peptides trapped onto an HPLC column prior to analysis. In this experimental setup the IMER is exposed to elevated working pressures. This was the motivation for creation of in-house prototype IMER capable of withstanding pressures up to 15 000 psi produced by UPLC instrument. This prototype IMER was compared to two commercially available IMERs.

One drawback of the described experimental setup is that the mobile phase gradient eluting digestion products from the column passes *via* IMERs (serially connected to column). Enzyme was repeatedly exposed to high concentration of acetonitrile in our experiment, and a slight decrease in trypsin catalytic activity was noted. To insure meaningful comparison we performed the pH, flow rates and temperature experiments in the same order for all three tested IMERs.

To evaluate the LC-UV–VIS analysis repeatability we first performed analysis of BAPNA substrate without the IMER in the flow path and compared it with the setup where the IMER was placed between the injector and a RP18 HPLC column for all the digestion conditions tested. Obtained RSD values of eight repetitive analyses are summarized in Table 2. The LC analysis repeatability (no IMER) was 1.4%; the variability with IMER was several time higher (see Table 2). The increased variability is in part due to the digestion process, and in part caused by integration error of substantially smaller peak (BAPNA substrate is partially

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

or nearly completely converted by the IMER to digestion product). Average RSD obtained for trypsin digestion in tandem with separation on RP18 column was 5.4%.

IMER activity results (percent of BAPNA substrate remaining) are summarized in Table 3; the measurements repeatability is provided in Supplemental Table S3. Several common trends were observed for all three IMERs: (i) Digestion speed increases with pH of the digestion buffer. This is most obvious for the UNC prototype, which had significantly higher activity at pH 9.0 than pH 8.0 or 7.0. (ii) With exception of a few experiments at pH 7.0 (suboptimal pH) the IMERs activity increases with temperature. The most complete digestion was observed at 37 °C. (iii) Digestion improves at lower flow rate (longer residence time of substrate on IMER). Notable exceptions from this trend are the Poroszyme IMER at pH 8.0 and experiments at the suboptimal pH 7.0.

The optimal pH range for tryptic digestion is 7–9 according to Sigma Aldrich [50] or 7.8–8.7 according to Worthington Biochemical Corporation [51]. Our results indicate great loss of trypsin activity at pH 7.0 for all IMERs used in the study. Interestingly, the UNC prototype (prepared using TPCK treated bovine trypsin from Sigma Aldrich) exhibited significantly higher activity at pH 9.0; at flow rate 0.1 mL/min no undigested BAPNA substrate remained.

Given that 2.1 × 30 mm IMER hardware has ~0.1 mL volume, packing density of BEH is 0.7 g/mL, and amount of trypsin immobilized on UNC prototype is ~78 mg/mL, the amount of trypsin in the prototype IMER is approximately 5.7 mg. Even if only 35–40% remains active, the amount of trypsin available for digestion is three orders of magnitude higher than the amount typically used for in-solution protein digestion (microgram amounts of trypsin). This explains why IMER's has a potential to perform faster digestions compared to in-solution proteolysis typically practiced today.

Out of the three IMERs tested in this study, the UNC prototype outperformed the Poroszyme and Perfinity at pH 9.0 and performed comparably well at pH 7.0 and 8.0. It should be noted that BEH sorbent is stable in such pH range.

We selected UNC prototype IMER for further experiments with bovine cytochrome C sample, a protein often selected as model protein substrate for IMER activity evaluation [3,8,11,12]. To investigate the utility of IMER for accelerated digestion we utilized 1 mL/min digestion flow rate which resulted in 4.2 s protein residential time on IMER (calculated from the void volume of packed 2.1 × 30 mm IMER). Cytochrome C was prepared at concentration of 2.3 mg/mL in ammonium bicarbonate buffer, pH 7.8 and digested in the same running mobile phase.

Fig. 1A shows a control experiment for 11.5 μg cytochrome C injection with no IMER present. The protein elutes from the CSH XSelect C18 column undigested and its intact mass was detected by MS instrument (experimental deconvoluted mass was 12226.7 Da, expected mass is 12230.9 Da). Fig. 1A shows MS chromatogram generated by extracting three dominant ions of cytochrome C (14+, 15+, and 16+ charged states) for mass spectrum of protein (see inset in Fig. 1A).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In the subsequent experiment the IMER was included in the flow path, and identical amount of protein was injected. Only 0.5% of intact cytochrome C peak area was observed by MS compared to control experiment (data not shown). Fig. 1B shows the experiment with ten-fold increased protein load (115 μg); we detected approximately 2% of undigested protein (Fig. 1B). It appears that IMER is capable of digesting significant mass loads of proteins in reaction time of 4.2 s.

The experimental data from 115 μg cytochrome C injection were used to investigate the masses of tryptic peptides formed during the passage of cytochrome C through the IMER. Total ion MS chromatogram in Fig. 2 show signal of multiple tryptic peptides (along with a small amount of undigested cytochrome C, and 6557 Da protein clip). Intriguingly, many of the observed peptides appear to be so-called missed cleavage peptides, i.e. incompletely digested peptides. Selected ion current MS chromatogram in Fig. 2B highlights the signals of selected peptides. The dominant signals are related to T8, T10, T12, and T19 peptides. Apparently, the 4.2 s digestion time is not a sufficient to achieve complete proteolysis of cytochrome C. The full set of MS identified peptides is listed in Fig. 3. Sequence coverage (Fig. 3B) illustrates that only several short hydrophilic peptides and T5 peptide with HEME group were not detected in the experiment.

Further inspection of sequences in Fig. 3 reveals missed-cleaved motifs that are known to be digested to final tryptic peptides with slow kinetics [4]. Among those are peptides with arginine/lysine cleavage site surrounded with acetic amino acids or miss-cleaved peptides with arginine/lysine residue at N- or C- termini [4]. We believe this is the main reason for the incomplete on-line digestion. This hypothesis is supported by presence of multiple variants of T19 peptide (Fig. 3B). The cleavage site between T18/T19 peptides GER/EDLIAYLK consists of arginine surrounded by three acidic amino acids. This is likely to make the cleavage site more resistant to digestion.

We performed digestion at similar conditions as described above with 10 fold lower mass load of cytochrome C (11.5 μg load). Smaller mass load did not result in more complete digestion (see Supplemental information, Fig. S1). Missed cleaved peptides were still present; their relative quantity compared to fully digested peptides (zero missed cleavages) did not decrease significantly.

To further investigate the missed-cleavages phenomenon we performed digestion of BSA (alkylated and reduced) using UNC IMER prototype. 200 μL of 100 nM BSA solution (1.3 μg mass) was loaded on IMER at flow rate of 1000, 500, 100, and 10 μL/min. This resulted in digestion/residential time of 5, 10, 48, and 480 s (8 min); respectively. The flow through fractions were collected and analyzed by LC MS(E) experiment as described previously [7]. The IMER digestions were compared to 15 h long in-solution digestion (Supplemental information, Tables S4A–C, and Fig. S4A–B). The results support the previous cytochrome C observations. Abundant missed-cleaved BSA peptides were observed in IMER digestions (supplemental Fig. S4A). When extending the IMER digestion time, some of the BSA missed-cleaved peptides were more readily digested to final products. However, the digestion of peptides with cleavage resistant motifs [4] is not accomplished neither in IMER, nor 15 h long in-solution digestion experiments (supplemental Fig. S4B). The digestion of

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

such missed-cleaved peptides is hindered by their 2–3 orders of magnitude slower digestion kinetics (compared to digestion of arginine site [4]). The implication of this phenomenon to IMER based digestions have not been studied yet.

One of the reviewers of this manuscript proposed additional investigation of the digestion specificity of the three IMERS using protein substrates. According to him/her, both activity and selectivity of trypsin may be affected by the immobilization procedure or environment (solid support). While we believe the LC–MS investigation of protein digestion selectivity, bleed of trypsin peptides from IMERs, and digestion efficiency of proteolytic-resistant proteins could be useful [45], the proposed study exceeds the scope of this manuscript. Such study may become a subject of the future work.

4. Concluding remarks

We evaluated a novel prototype IMER with high amount of trypsin immobilized on high pressure resistant BEH 300 Å particles. Trypsin activity was not significantly affected at elevated pressure (up to 2600 psi) used in the initial study even with mobile phases containing high acetonitrile concentrations. We compared the activity of UNC prototype IMER with commercially available devices. The activity of all the IMERs was suboptimal at pH 7.0; several fold faster digestion was observed at pH 8.0 and 9.0. The highest activity of the UNC prototype was measured at pH 9.0 for all temperatures and flow rates. At this pH, the UNC prototype IMER activity surpassed the commercial enzymatic reactors used in this study. Temperature had moderate effect on digestion speed; the IMERs activity improved 2–3 fold when increasing the digestion temperature from 20 to 37 °C.

The UNC prototype IMER compatible with operational pressure up to 15,000 psi was tested for preliminary experiments with cytochrome C at 8000 psi. The 115 μg protein injection on IMER was nearly completely digested in 4.2 s in flow-through mode. Analysis of generated peptides revealed the presence of missed-cleaved tryptic peptides, often as the dominant products. Slower digestion speed is required to achieve more complete proteolysis.

Further optimization of trypsin immobilization procedure, selection of particle pore size, enhancement of activity and IMER stability at elevated pressure, temperature, and organic solvent conditions is desirable in order to design enzymatic reactor with superior performance to existing products. Few reports have been published on commercially available IMERs activity [33,37], autolysis rate, longevity, and digestion selectivity (completeness) compared to in-solution digestion. These attributes of IMERs are important for rational selection of digestion method.

Immobilizing trypsin onto a high pressure compatible media opens a possibility of high pressure and high speed digestions with IMER serially connected to long LC columns or to columns packed with sub-two micron particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acknowledgments

The authors thank Dr. Beatrice Muriithi, Oksana D. Tchoul, and Thomas S McDonald for their support in selected experiments. The authors would like to express their gratitude to the Grant Agency of the Charles University in Prague, project No. 254214, and the Czech Science Foundation, Grant No. 16-05942S for the financial support. The work at UNC was supported by National Institute of Health Grants #5U24DK097153 and #1R01DK101473-01A1. The authors also express their gratitude to the Waters Corporation for technical support and the gift of XTerra RP18 and Perfinity columns.

References

1. Keil, B. Specificity of Proteolysis. Springer-Verlag; New York: 1992.
2. Stefansson B, Helgadottir L, Olafsdottir S, Gudmundsdottir A, Bjarnason JB. Characterization of cold-adapted Atlantic cod (Gadus morhua) trypsin I-kinetic parameters, autolysis and thermal stability. Comp Biochem Physiol B Biochem Mol Biol. 2010; 155:186–194. [PubMed: 19913635]
3. Slysz GW, Schriemer DC. On-column digestion of proteins in aqueous-organic solvents. Rapid Commun Mass Spectrom. 2003; 17:1044–1050. [PubMed: 12720284]
4. Slechtova T, Gilar M, Kalikova K, Tesarova E. Insight into trypsin miscleavage: comparison of kinetic constants of problematic peptide sequences. Anal Chem. 2015; 87:7636–7643. [PubMed: 26158323]
5. Monigatti F, Berndt P. Algorithm for accurate similarity measurements of peptide mass fingerprints and its application. J Am Soc Mass Spectrom. 2005; 16:13–21. [PubMed: 15653359]
6. Yen CY, Russell S, Mendoza AM, Meyer-Arendt K, Sun S, Cios KJ, Ahn NG, Resing KA. Improving sensitivity in shotgun proteomics using a peptide-centric database with reduced complexity: protease cleavage and SCX elution rules from data mining of MS/MS spectra. Anal Chem. 2006; 78:1071–1084. [PubMed: 16478097]
7. Moore S, Hess S, Jorgenson J. Characterization of an immobilized enzyme reactor for on-line protein digestion. J Chromatogr A. 2016; 1476:1–8. [PubMed: 27876348]
8. Krenkova J, Lacher NA, Svec F. Highly efficient enzyme reactors containing trypsin and endoproteinase LysC immobilized on porous polymer monolith coupled to MS suitable for analysis of antibodies. Anal Chem. 2009; 81:2004–2012. [PubMed: 19186936]
9. Safdar M, Spross J, Janis J. Microscale immobilized enzyme reactors in proteomics: latest developments. J Chromatogr A. 2014; 1324:1–10. [PubMed: 24360812]
10. Freije JR, Mulder PP, Werkman W, Rieux L, Niederlander HA, Verpoorte E, Bischoff R. Chemically modified, immobilized trypsin reactor with improved digestion efficiency. J Proteome Res. 2005; 4:1805–1813. [PubMed: 16212436]
11. Hsieh YL, Wang H, Elicone C, Mark J, Martin SA, Regnier F. Automated analytical system for the examination of protein primary structure. Anal Chem. 1996; 68:455–462. [PubMed: 8712355]
12. Ruan G, Wei M, Chen Z, Su R, Du F, Zheng Y. Novel regenerative large-volume immobilized enzyme reactor: preparation, characterization and applicatio. J Chromatogr B Anal Technol Biomed Life Sci. 2014; 967:13–20.
13. Ma J, Zhang L, Liang Z, Zhang W, Zhang Y. Recent advances in immobilized enzymatic reactors and their applications in proteome analysis. Anal Chim Acta. 2009; 632:1–8. [PubMed: 19100875]
14. Qiao J, Kim JY, Wang YY, Qi L, Wang FY, Moon MH. Trypsin immobilization in ordered porous polymer membranes for effective protein digestion. Anal Chim Acta. 2016; 906:156–164. [PubMed: 26772135]
15. Savino R, Casadonte F, Terracciano R. In mesopore protein digestion: a new forthcoming strategy in proteomics. Molecules. 2011; 16:5938–5962. [PubMed: 21765391]
16. Lombardo CR, Consler TG, Kassel DB. In vitro phosphorylation of the epidermal growth factor receptor autophosphorylation domain by c-src: identification of phosphorylation sites and c-src SH2 domain binding sites. Biochemistry. 1995; 34:16456–16466. [PubMed: 8845374]
17. Boerner RJ, Kassel DB, Barker SC, Ellis B, DeLacy P, Knight WB. Correlation of the phosphorylation states of pp60c-src with tyrosine kinase activity: the intramolecular pY530-SH2 complex retains significant activity if Y419 is phosphorylated. Biochemistry. 1996; 35:9519–9525. [PubMed: 8755732]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

18. Nadler T, Blackburn C, Mark J, Gordon N, Regnier FE. Automated proteolytic mapping of proteins. J Chromatogr A. 1996; 743:91–98.
19. van der Donk WA, Zeng C, Biemann K, Stubbe J, Hanlon A, Kyte J. Identification of an active site residue of the R1 subunit of ribonucleotide reductase from Escherichia coli: characterization of substrate-induced polypeptide cleavage by C225SR1. Biochemistry. 1996; 35:10058–10067. [PubMed: 8756468]
20. Lebrun B, Romi-Lebrun R, Martin-Eauclaire MF, Yasuda A, Ishiguro M, Oyama Y, Pongs O, Nakajima T. A four-disulphide-bridged toxin, with high affinity towards voltage-gated K+ channels, isolated from Heterometrus spinnifer (Scorpionidae) venom. Biochem J. 1997; 328(Pt 1):321–327. [PubMed: 9359871]
21. Blackburn RK, Anderegg RJ. Characterization of femtomole levels of proteins in solution using rapid proteolysis and nanoelectrospray ionization mass spectrometry. J Am Soc Mass Spectrom. 1997; 8:483–494.
22. Richards GP, Liang YM, Chao J, Chao L. Purification, characterization and activation of fish muscle prokallikrein. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1997; 118:39–48. [PubMed: 9366034]
23. Fukuda H, Irie K, Nakahara A, Ohigashi H, Wender PA. Solid-phase synthesis mass spectrometric analysis of the zinc-folding, and phorbol ester-binding studies of the 116-mer peptide containing the tandem cysteine-rich C1 domains of protein kinase C gamma. Bioorg Med Chem. 1999; 7:1213–1221. [PubMed: 10428394]
24. Rocklin RD, Ramsey RS, Ramsey JM. A microfabricated fluidic device for performing two-dimensional liquid-phase separations. Anal Chem. 2000; 72:5244–5249. [PubMed: 11080871]
25. Hara S, Katta V, Lu HS. Peptide map procedure using immobilized protease cartridges in tandem for disulfide linkage identification of neu differentiation factor epidermal growth factor domain. J Chromatogr A. 2000; 867:151–160. [PubMed: 10670717]
26. Riggs L, Sioma C, Regnier FE. Automated signature peptide approach for proteomics. J Chromatogr A. 2001; 924:359–368. [PubMed: 11521885]
27. Vecchione G, Casetta B, Santacroce R, Margaglione M. A comprehensive on-line digestion-liquid chromatography/mass spectrometry/collision-induced dissociation mass spectrometry approach for the characterization of human fibrinogen. Rapid Commun Mass Spectrom. 2001; 15:1383–1390. [PubMed: 11507749]
28. Canarelli S, Fisch I, Freitag R. Hyphenation of multi-dimensional chromatography and mass spectrometry for the at-line-analysis of the integrity of recombinant protein drugs. J Chromatogr B. 2002; 775:27–35.
29. Samskog J, Bylund D, Jacobsson SP, Markides KE. Miniaturized on-line proteolysis-capillary liquid chromatography-mass spectrometry for peptide mapping of lactate dehydrogenase. J Chromatogr A. 2003; 998:83–91. [PubMed: 12862374]
30. Carol J, Gorseling MC, de Jong CF, Lingeman H, Kientz CE, van Baar BL, Irth H. Determination of denaturated proteins and biotoxins by on-line size-exclusion chromatography-digestion-liquid chromatography-electrospray mass spectrometry. Anal Biochem. 2005; 346:150–157. [PubMed: 16185643]
31. Nicoli R, Gaud N, Stella C, Rudaz S, Veuthey JL. Trypsin immobilization on three monolithic disks for on-line protein digestion. J Pharm Biomed Anal. 2008; 48:398–407. [PubMed: 18242915]
32. Hoehenwarter W, van Dongen JT, Wienkoop S, Steinfath M, Hummel J, Erban A, Sulpice R, Regierer B, Kopka J, Geigenberger P, Weckwerth W. A rapid approach for phenotype-screening and database independent detection of cSNP/protein polymorphism using mass accuracy precursor alignment. Proteomics. 2008; 8:4214–4225. [PubMed: 18924179]
33. Nicoli R, Rudaz S, Stella C, Veuthey JL. Trypsin immobilization on an ethylenediamine-based monolithic minidisk for rapid on-line peptide mass fingerprinting studies. J Chromatogr A. 2009; 1216:2695–2699. [PubMed: 18962647]
34. Schneiderheinze J, Walden Z, Dufield R, Demarest C. Rapid online proteolytic mapping of PEGylated rhGH for identity confirmation, quantitation of methionine oxidation and quantitation

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

of UnPEGylated N-terminus using HPLC with UV detection. J Chromatogr B Anal Technol Biomed Life Sci. 2009; 877:4065–4070.

35. [Accessed 30 June 2016] General Product Information. www.thermofisher.com/order/catalog/product/2312800
36. Hoos JS, Sudergat H, Hoelck JP, Stahl M, de Vlieger JS, Niessen WM, Lingeman H, Irth H. Selective quantitative bioanalysis of proteins in biological fluids by on-line immunoaffinity chromatography-protein digestion-liquid chromatography-mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2006; 830:262–269.
37. Cingoz A, Hugon-Chapuis F, Pichon V. Evaluation of various immobilized enzymatic microreactors coupled on-line with liquid chromatography and mass spectrometry detection for quantitative analysis of cytochrome c. J Chromatogr A. 2008; 1209:95–103. [PubMed: 18823630]
38. Shah V, Lassman ME, Chen Y, Zhou H, Laterza OF. Achieving efficient digestion faster with Flash Digest: potential alternative to multi-step detergent assisted in-solution digestion in quantitative proteomics experiments. Rapid Commun Mass Spectrom. 2017; 31:193–199. [PubMed: 27794205]
39. Rivera-Burgos D, Regnier FE. Disparities between immobilized enzyme and solution based digestion of transferrin with trypsin. J Sep Sci. 2013; 36:454–460. [PubMed: 23281316]
40. Jelinek, CA. Characterizing the Post-translational Modifications of Human Serum Albumin as They Correlate to Cardiac Ischemia, Chapter IV: Sample Preparation Strategies for Use in Clinical MS Assays. Johns Hopkins University; Baltimore, Maryland: 2012. http://gradworks.umi.com/35/28/3528552.html [Accessed 30 June 2016]
41. Lewis, E., Meyer, K., Hao, Z., Herold, N., Bennett, P. [Accessed 30 June 2016] Rapid peptide mapping via automated integration of on-line digestion, separation and mass spectrometry for the analysis of therapeutic proteins Thermo Scientific Poster Note, 06/13S. http://apps.thermoscientific.com/media/cmd/ASMS-TNG-Roadshow/TNG/resouces/805_PN_ASMS13_WP24_ELewis.pdf
42. Massolini G, Calleri E. Immobilized trypsin systems coupled on-line to separation methods: recent developments and analytical applications. J Sep Sci. 2005; 28:7–21. [PubMed: 15688626]
43. Regnier FE, Kim J. Accelerating trypsin digestion: the immobilized enzyme reactor. Bioanalysis. 2014; 6:2685–2698. [PubMed: 25411709]
44. Yamaguchi H, Miyazaki M. Enzyme-immobilized reactors for rapid and efficient sample preparation in MS-based proteomic studies. Proteomics. 2013; 13:457–466. [PubMed: 23255229]
45. Yu YQ, Gilar M, Gebler JC. A complete peptide mapping of membrane proteins: a novel surfactant aiding the enzymatic digestion of bacteriorhodopsin. Rapid Commun Mass Spectrom. 2004; 18:711–715. [PubMed: 15052583]
46. Yu YQ, Gilar M, Lee PJ, Bouvier ES, Gebler JC. Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. Anal Chem. 2003; 75:6023–6028. [PubMed: 14588046]
47. Ahn J, Jung MC, Wyndham K, Yu YQ, Engen JR. Pepsin immobilized on high-strength hybrid particles for continuous flow online digestion at 10,000 psi. Anal Chem. 2012; 84:7256–7262. [PubMed: 22856522]
48. Hermanson, GT. Bioconjugate Techniques. Academic Press; San Diego, California: 1996.
49. Subirats X, Rosés M, Bosch E. On the effect of organic solvent composition on the pH of buffered HPLC mobile phases and the pKa of analytes—a review. Sep Purif Rev. 2007; 36:231–255.
50. [Accessed 30 June 2016] Trypsin, general informations. www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/trypsin.html
51. Introduction to Enzymes. Worthington Biochemical Corporation; 2015. www.worthington-biochem.com/introbiochem/Enzymes.pdf [Accessed 30 June 2016]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.02.024.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

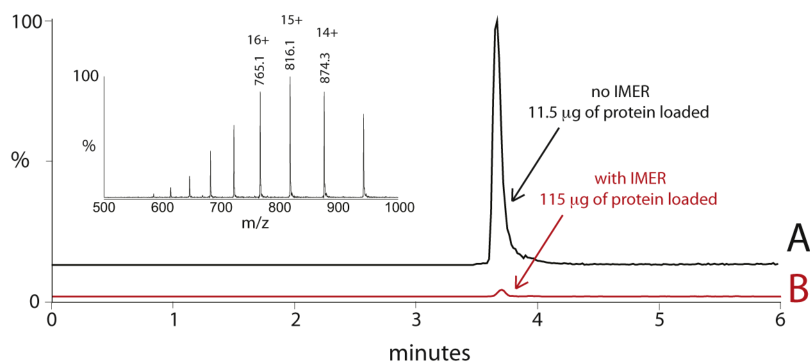

Fig. 1.

MS chromatograms of cytochrome C. (A) Injection of 11.5 μg of protein without enzymatic reactor; (B) injection of 115 μg (10 fold greater mass) with IMER on-line. About 2% of protein was left undigested. The MS chromatograms were generated by summing signals for three most dominant 14–16+ charge states indicated in the MS spectrum inset.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

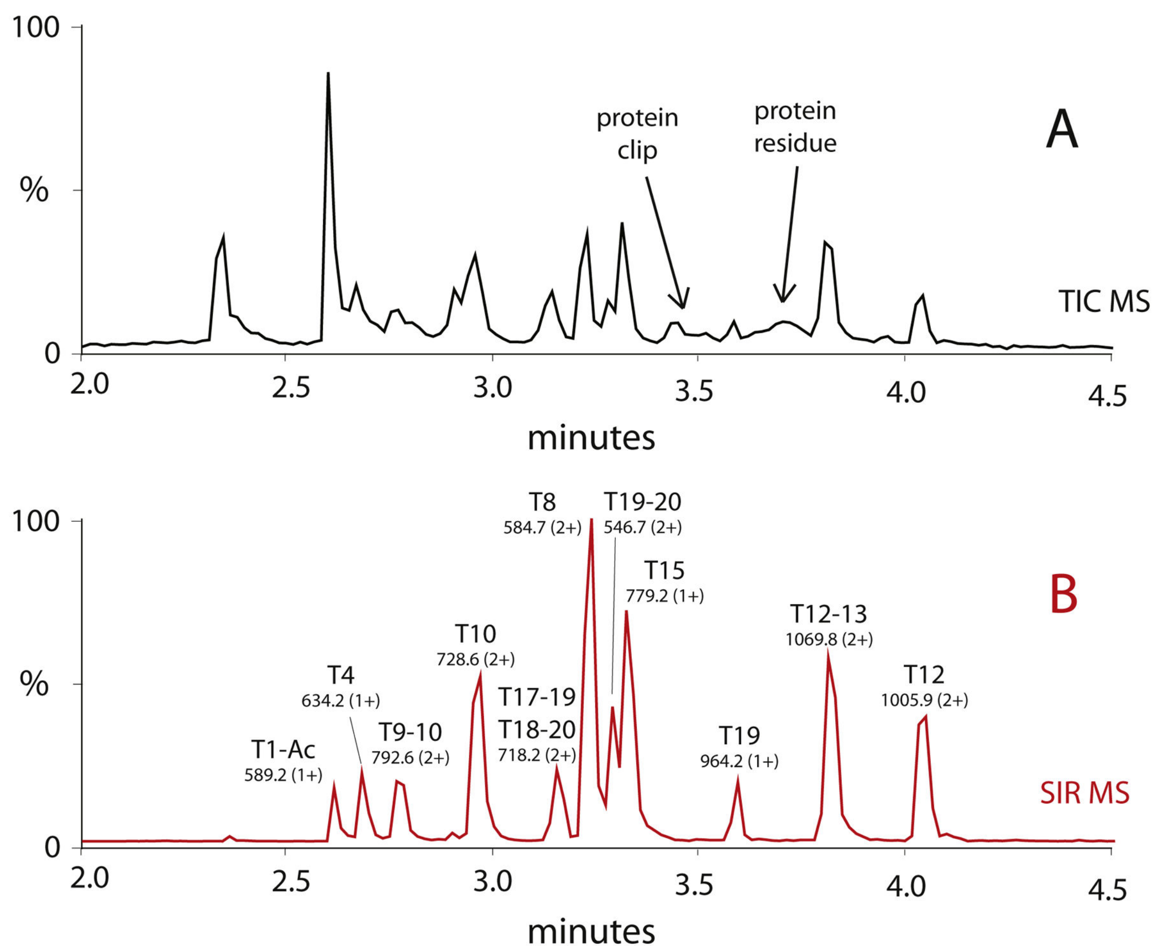

Fig. 2.

Analysis of on-line digested cytochrome C; injection of 115 μg of protein. (A) Total ion current MS chromatogram of tryptic peptides and residual protein; (B) selected ions MS chromatogram of the dominant peptides. The *m*/*z* and charge state of peptides are indicated along with the peptide assignment. For additional data see Supplemental information, Table S1, S2, and Fig. S1.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

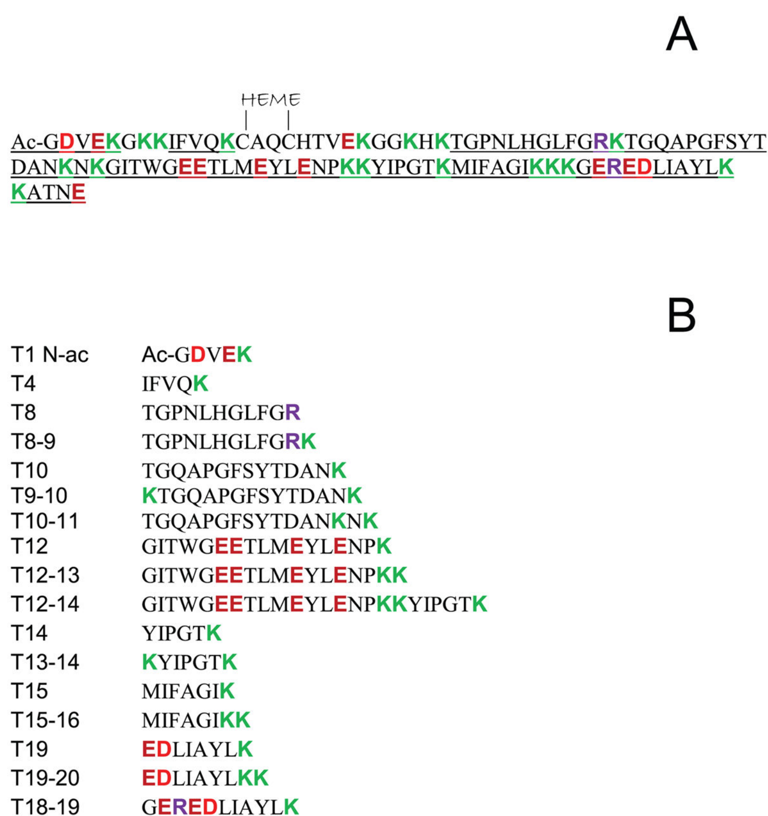

Fig. 3.

(A) Bovine cytochrome C sequence with acetylated N-terminus and covalently attached HEME group. Arginine, R, lysine, K (basic cleavage sites), glutamic acid, E, and aspartic acid, D (acidic amino acids) are highlighted. The underlining indicates the detected sequences. (B) List of LC/MS observed peptides generated by on-line digestion with UNC IMER prototype (115 μg protein injection). For additional data see Supplemental information, Table S1, S2, and Fig. S1.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Table 1

General parameters of tested trypsin IMERs.

	Poroszyme	Perfinity	UNC Prototype
Dimensions, particle size	2.1 × 30 mm, 20 μm	2.1 × 33 mm, 20 μm	2.1 × 30 mm, 5 μm
Pore size	500–10000 Å	»1000 Å	300 Å
Maximum pressure	2500 psi	2500 psi	15000 psi
Operating temperature	25–67 °C	25–67 °C	20–60 °C
Sorbent	PDVB	PDVB	BEH
Trypsin origin	Bovine	Porcine	Bovine

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

RSD values of injections obtained with XTerra RP18 column only and trypsin reactors connected in series with XTerra RP18 column for various IMER's and pH conditions. RSDs were calculated from substrate peak area. All experiments were performed in eight replicates (n = 8).

	Perfinity	**Poroszyme**	**UNC Prototype**	**RP18**
	RSD [%]	**RSD [%]**	**RSD [%]**	**RSD [%]**
pH 7.0	2.6	3.7	4.8	1.3
pH 8.0	6.1	6.7	3.1	1.4
pH 9.0	5.4	9.2	6.7	1.3

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 3

Activity of Perfinity, Poroszyme and UNC prototype IMERs measured as percent of undigested peak area of BAPNA substrate at indicated pH, temperatures, and flow rates. All experiments were performed in eight replicates (n = 8); RSDs were comparable with values shown in Table 2. Complete RSD data are provided in Supplemental information, Table S3.

	Perfinity				Poroszyme				UNC prototype		
	pH 7.0				pH 7.0				pH 7.0		
	0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	58.2%	62.8%	59.9%	20 °C	63.7%	55.9%	58.0%	20 °C	62.5%	66.9%	53.1%
25 °C	59.2%	61.0%	62.0%	25 °C	61.1%	53.6%	55.4%	25 °C	61.5%	55.7%	50.1%
30 °C	59.4%	58.0%	58.0%	30 °C	60.6%	53.2%	52.1%	30 °C	58.7%	55.7%	47.2%
37 °C	58.7%	63.1%	54.2%	37 °C	59.5%	58.5%	56.5%	37 °C	54.0%	53.6%	52.4%
	Perfinity				Poroszyme				UNC prototype		
	pH 8.0				pH 8.0				pH 8.0		
	0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	35.1%	29.2%	17.6%	20 °C	40.7%	21.4%	23.5%	20 °C	40.4%	17.3%	10.3%
25 °C	26.0%	20.3%	11.1%	25 °C	32.4%	13.8%	16.8%	25 °C	33.9%	13.7%	7.8%
30 °C	18.2%	11.9%	8.4%	30 °C	22.4%	10.7%	12.1%	30 °C	27.3%	8.2%	5.2%
37 °C	11.0%	6.0%	6.7%	37 °C	16.1%	7.7%	12.1%	37 °C	21.7%	5.7%	3.0%
	Perfinity				Poroszyme				UNC prototype		
	pH 9.0				pH 9.0				pH 9.0		
	0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min		0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	32.4%	25.2%	19.1%	20 °C	41.0%	34.0%	16.6%	20 °C	7.8%	1.3%	0.0%
25 °C	25.6%	18.4%	13.9%	25 °C	32.8%	25.5%	9.3%	25 °C	6.5%	1.5%	0.0%
30 °C	20.1%	11.0%	5.2%	30 °C	25.8%	16.0%	4.7%	30 °C	6.0%	2.1%	0.0%
37 °C	11.8%	3.2%	0.4%	37 °C	16.3%	5.3%	0.5%	37 °C	4.0%	0.3%	0.0%