Published in final edited form as:

Proteomics. 2011 January ; 11(2): 309–318. doi:10.1002/pmic.201000378.

Rapid and Efficient Protein Digestion using Trypsin Coated Magnetic Nanoparticles under Pressure Cycles

Byoungsoo Lee^{1,†}, Daniel Lopez-Ferrer^{2,†}, Byoung Chan Kim³, Hyon Bin Na⁴, Yong Il Park⁴, Karl K. Weitz², Marvin G. Warner², Taeghwan Hyeon⁴, Sang-Won Lee⁵, Richard D. Smith^{2,*}, and Jungbae Kim^{1,*}

¹ Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Korea

² Pacific Northwest National Laboratory, Richland, WA 99352, USA

³ Environment Division, Korea Institute of Science and Technology, Seoul 136-791, Korea

⁴ National Creative Research Initiative Center for Oxide Nanocrystalline Materials and School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Korea

⁵ Department of Chemistry, Korea University, Seoul 136-701, Korea

Abstract

Trypsin-coated magnetic nanoparticles (EC-TR/NPs), prepared via a simple multilayer random crosslinking of the trypsin molecules onto magnetic nanoparticles, were highly stable and could be easily captured using a magnet after the digestion was complete. EC-TR/NPs showed a negligible loss of trypsin activity after multiple uses and continuous shaking, while the conventional immobilization of covalently-attached trypsin on NPs resulted in a rapid inactivation under the same conditions due to the denaturation and autolysis of trypsin. A single model protein, a five protein mixture, and a whole mouse brain proteome were digested at atmospheric pressure and 37 °C for 12 h or in combination with pressure cycling technology (PCT) at room temperature for 1 min. In all cases, EC-TR/NPs performed equally to or better than free trypsin in terms of both identified peptide/protein number and the digestion reproducibility. In addition, the concomitant use of EC-TR/NPs and PCT resulted in very rapid (~1 min) and efficient digestions with more reproducible digestion results.

Keywords

enzyme coatings; magnetic nanoparticles; pressure cycling technology; protein digestion

1 Introduction

In recent years, proteomics has enormously expanded its applicability to many different scientific fields, such as biomedicine [1], bioterrorism [2], bioenergy [3], forensics [4,5], and food science [6,7]. Since proteins play a key role in all areas, their identification, quantification and characterization are of great importance for a better understanding of biological systems. Though several strategies could be employed for this kind of proteomic analysis, the so-called “bottom-up” approach is the most widely used [8]. This analytical

* **Corresponding Authors** Jungbae Kim: Department of Chemical and Biological Engineering, Korea University, Seoul, Republic of Korea, Tel: +82-2-3290-4850, Fax: +82-2-926-6102, jbkim3@korea.ac.kr Richard D. Smith: Pacific Northwest National Laboratory, Richland, WA 99352, USA, Tel: +1-509-371-6576, Fax: +1-509-371-6546, rds@pnl.gov.

[†]Both authors have equally contributed to this work

path is based on the digestion of proteins into peptides, using trypsin as the protease of choice, and the resulting peptide mixture is then analyzed by LC-MS(/MS). However, as the number of applications increase, the demand for higher throughput also increases. Several efforts, in addition of developing faster mass spectrometers [9,10], have aimed to increase the throughput in LC-MS analyses. For example, LC separations have been performed in parallel with continuous data acquisition, which maximizes the use of the mass spectrometer[11]. In addition, great efforts have been put forth to develop a computational platform for efficient storage and management of proteomic data that enables more effective proteomic research[12]. Of all steps in the proteome analysis workflow, the sample preparation step is one of the most time consuming steps[13]. Digesting proteins into peptides usually takes several hours to overnight, and approaches have also been developed to ensure a higher proteolytic activity and avoid autolytic side reactions. One such promising effort involves the use of immobilized proteases. Immobilized proteases possess the inherent advantage of increased enzyme-substrate ratios and enhancement of the enzyme stability.

Enzyme immobilization can be performed using a variety of solid supports, and recent developments in the area of nanobiocatalysis have demonstrated effective enzyme stabilization and have proven its potential applications in various fields, including protein digestion for proteomic applications[14,15]. Recently, highly active and stable enzyme immobilized nanobiocomposites have been developed based on various nanobiocatalytic approaches[16-23]. Various nanostructured materials, such as mesoporous materials, carbon nanotubes, nanofibers, and nanoparticles, have been used as solid supports for enzyme immobilization. One key advantage in using nanostructured materials is that their fundamental properties offer higher surface area to volume ratios[15]. Current state of the art in nanomaterials sciences offers the possibility of producing silica-modified nanoparticles (NPs) with a large number of functional groups and chemistries. From a proteomic point of view, silica-coated magnetic nanoparticles are attractive since their magnetic core made up of Fe_3O_4 enables magnetic capturing while the silica surface allows a great variety of chemical modifications that make them adaptable to many different chemistries and applications [24]. Our study explores the use of magnetic NPs as enzyme immobilization agents for proteomic applications by applying an enzyme coating protocol [14,25] that can provide a highly stable, active and reusable nanobiocatalytic system [26]. To expedite the protein digestion, we have used enzyme-coated magnetic nanoparticles (EC-NPs) in combination with pressure cycling technology (PCT) [27,28]. In the previous work, we demonstrated that applying PCT above 0.5 kpsi accelerated enzyme kinetics when compared with a traditional digestion carried out at 37 °C for several hours [29]. This procedure has recently also been successfully employed by other groups [30-32]. Here we describe a novel technology platform that combines PCT and EC-NPs for rapid and efficient protein digestions. This platform was evaluated using digestions of a single protein or complex protein mixtures, and its digestion performance was compared with traditional in-solution digestions using free trypsin. We characterized the digestions in terms of reproducibility, protein coverage in terms of the number of unique peptides identified, stability across a long period of time, and tentative reusability.

2 Materials and methods

2.1 Materials

Trypsin, sodium phosphate monobasic, sodium phosphate dibasic, Tris-HCl, Trizma base, glutaraldehyde (GA), N- α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), N,N-dimethylformamide (DMF), ammonium bicarbonate, urea, five proteins (bovine serum albumin, ovalbumin, myoglobin, carbonic anhydrase and lactoglobulin), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA). Sodium dodecylbenzenesulfonate, xylene, hydrazine, 3-aminopropylsilane, and TEOS were

purchased from Aldrich (Milwaukee, WI, USA). Bond-breaker tris(2-carboxyethyl)phosphine (TCEP) solution was purchased from Thermo scientific (Rockford, IL, USA).

2.2 Synthesis of amino functionalized silica-coated magnetic nanoparticles

Amino functionalized silica-coated magnetic nanoparticles were synthesized using a modification of a recently reported procedure [26]. Briefly, a microemulsion was prepared by dissolving 1.5 g of sodium dodecylbenzenesulfonate in 15 mL of xylene at room temperature for 2 h. 0.5 g of FeCl_3 dissolved in 1 mL of deionized water was added to the microemulsion and the solution was stirred at room temperature for 12 h. The stabilized reverse-micelle solution was then slowly heated up to 90 °C under argon gas at a rate of 2 mL/min for an hour. After this, 1 mL of hydrazine (34 wt% aqueous solution) was injected into the solution. Particles were then aged at 90 °C for 3 h, and cooled down to 40 °C within 1 h. Once the particles were stabilized, the surface modification step was performed. A mixture of 3-aminopropylsilane (1 mL) and TEOS (1 mL) was added to the solution to form amorphous silica shells on the surface, and were aged at 40 °C for 3 h. The particles were then washed with acetone and dispersed in water. The dispersed particles were further purified by using a centrifugal filter (MWCO 100,000) and PD-10 desalting column (GE healthcare, Piscataway, NJ, USA).

2.3 Enzyme coating on magnetic nanoparticles

Trypsin coated magnetic nanoparticles were prepared as follows. 1 mg of Trypsin was mixed with 8.3 mg of magnetic nanoparticles in 1.5 mL of aqueous buffer (100 mM sodium phosphate buffer, pH 7.9), and then the mixture was shaken at 200 rpm for 30 min at room temperature. 1.5 mL of 0.2% GA solution was added, and the mixture was incubated at 4 °C on a rocker (50 rpm) overnight. GA-treated samples were excessively washed by using 100 mM sodium phosphate buffer (pH 7.9), and unreacted aldehyde groups were capped by using Tris-HCl buffer (pH 7.9). EC-TR/NPs were finally washed with 10 mM sodium phosphate buffer (pH 7.9), and stored in the refrigerator until use. Covalently-attached trypsin on magnetic nanoparticles (CA-TR/NPs) were prepared as a control sample by treating NPs with 0.1% GA solution, followed by excessive washing of the GA-treated NPs, and a final incubation in the trypsin solution.

2.4 Measurements of loading, activity, and stability

Elemental analysis was performed to measure the amount of C, H, O, and N in the samples. The enzyme loadings of CA-TR and EC-TR/NPs were estimated from the measured amounts of N, most of which originate from trypsin. Free trypsin was used to get the standard curve between the trypsin weight and the measured N amount under elemental analysis. The activities of CA and EC-TR/NP were measured by the hydrolysis of BAPNA in 10 mM sodium phosphate buffer (pH 7.9). The generation of product, *p*-nitroaniline, was monitored by measuring the increase in the absorbance at 410 nm (A_{410}) with a UV-vis spectrophotometer (Shimadzu, Kyoto, Japan), and the enzyme activity was calculated from the time-dependent increase of A_{410} . The stabilities of CA-TR and EC-TR/NPs were checked under recycled uses and incubation in an aqueous buffer under rigorous shaking (200 rpm) with vials on a horizontal position. The residual activity at each time point was measured by the hydrolysis of BAPNA as described above. After each measurement of residual activity, the sample was washed three times to remove substrate and products. Excessively-washed samples were incubated in an aqueous buffer under shaking until the measurement of residual activity at the next time point. The relative activity was defined as the ratio of the residual activity to the initial activity at each time point.

2.5 BSA digestion by EC-TR/NP

A standard solution of reduced and alkylated BSA was prepared in 50 mM ammonium bicarbonate (pH 8.3) at a concentration of 1 mg/mL. The 100 μ L of the standard solution was mixed with the EC-TR/NP in a glass vial. Prior to digestion, the nanoparticles were extensively washed with 50 mM ammonium bicarbonate (pH 8.3). The solution was incubated in a 96 well-plate at 300 rpm for 16 hours. Once the digestions had finished, EC-TR/NPs were captured using a magnet, and the clear solution was transfer to a new reaction tube and acidified with formic acid to a final concentration of 0.1% (v/v). The samples were stored at -20 °C until the MS/MS analysis. EC-TR/NPs were washed with ammonium bicarbonate and stored at room temperature for further experiments.

2.6 Digestion of a five protein mixture

An equimolar mixture of five proteins (bovine serum albumin, ovalbumin, myoglobin, carbonic anhydrase and lactoglobulin) were digested by using either EC-TR/NPs or free trypsin as described previously [33]. Briefly, 400 μ L of five protein solution at a 1 mg/mL concentration of total protein was reduced and alkylated with 5 mM TCEP and 50 mM IAA, and sonicated for 3 min at 50% power using a sonoreactor in the presence of 8 M urea. To prepare the sample digested with free trypsin, the reduced and alkylated protein solution was diluted four times with 50 mM ammonium bicarbonate (pH 8.0), and then trypsin was added in a 1:25 trypsin:protein (wt/wt) ratio. For the samples that were to be digested with EC-TR/NPs, a 50 μ L aliquot of NP solution was placed into a Teflon reactor tube (3 mm i.d.), EC-TR/NPs were captured using a magnet and the buffer solution was removed. Then, the sample was mixed with the reduced and alkylated protein solution, which was diluted four times with 50 mM ammonium bicarbonate (pH 8.0) in a Teflon tube yielding a volume of 150 μ L. The reaction tubes were sealed, and placed either in a thermo-mixer at 37 °C overnight and 500 rpm, or into the pressure cycling device (Barocycler NEP 2320, Pressure Biosciences Inc., South Easton, MA, USA) for protein digestion. The pressure program cycles were performed at 25 kpsi for 20 sec and at ambient pressure for 5 sec. A total of 5 cycles (from 25 kpsi to ambient pressure) over a total time of 1 min under pressure was carried out. Once the samples were digested, the reaction was quenched by removing the EC-TR/NPs using a magnet, and then formic acid was added to the solution to inhibit any residual trypsin activity. The samples were then analyzed by LC-MS/MS [34,35].

2.7 Digestion of mouse brain protein extracts

A mouse brain was obtained from an 8 week old C57BL/6J male mouse as described elsewhere [36]. The whole frozen brain was placed in a Shredder Pulse tube (Pressure Biosciences Inc., South Easton, MA, USA), and homogenized mechanically using the PCT Shredder from Pressure Biosciences Inc. 8 M urea was added, and the sample was submitted to PCT for 5 min. The whole homogenized tissue was centrifuged and the supernatant was transfer to a new reaction tube. Protein reduction and alkylation as well as digestion steps were done as described above.

2.8 LC-MS-MS analysis

For the analyses of BSA digests, 500 fM of protein digest were analyzed by LC-MS/MS using an Agilent HPLC-Chip system coupled to a MSD XCT Ultra ion trap (Agilent Technologies, Santa Clara, CA, USA). The ion trap mass spectrometer was operating in positive ion mode with the capillary voltage set at 2500 V and in data-depend acquisition mode, where a full scan (m/z 500-1600) MS analysis was performed. Then, the five most abundant ions were isolated for fragmentation. Separations were carried out on a chip with a 40-nL enrichment column and a 43 mm $\times$ 75 μ m analytical column, both packed with 5 μ m ZORBAX 300SB C18 particles. A flow rate of 1 μ L/min was employed for loading and

enriching the sample. Peptides were eluted at 600 nL/min, using for some analyses a 5 min gradient from 5% to 90% B (Solvent B: 0.5% formic acid in water:acetonitrile of 10:90; & Solvent A: 0.5% formic acid in water:acetonitrile of 97:3) with a separation window of 2 min and total analysis time of 12 min.

The rest of the samples were analyzed using an in-house developed capillary LC system, described elsewhere [11], coupled online to either an LTQ (brain samples) or an LTQ-FTICR (protein standards) mass spectrometer (Thermo-Fisher, San Jose, CA, USA). Data were collected in a data-dependent mode where the ten most abundant ions were selected for tandem MS after a full scan MS (m/z 400-2000). A dynamic exclusion time of 1 min and a normalized collision energy of 35% were used for the entire run. In the case of the LTQ-FTICR, the full scan was performed in the FTICR analyzer and the resolution was set to 100,000. In both cases, the capillary voltage was set to 2200 V.

2.9 Protein identification and validation

Peptide identifications was performed using SEQUEST [37] to search MS/MS spectra against a *Shewanella oneidensis* protein database containing the target protein sequences or against an 2006-IPI *Mus musculus* database in the case of the mouse brain proteomes. Database search parameters included a dynamic modification for Met oxidation and for carbamidomethylation of Cys. Peptide identification statistics, including estimating random match probabilities and false discovery rates, were performed using a two-variable Gaussian method [38] with some modifications as described elsewhere [29].

3 Results and discussion

3.1 Preparation and characterization of EC-TR/NPs

Figure 1a shows a schematic flowchart for the preparation of covalently-attached trypsin (CA-TR) and trypsin coating (EC-TR) on magnetic nanoparticles (NPs). The critical difference between these two different approaches is the order of GA and enzyme additions. The preparation of CA-TR started with the GA treatment of NPs, followed by the incubation in the enzyme solution after excessive washing of GA-treated NPs, while EC-TR is based on the early work done by Persichetti *et al.* [39] developing cross-linked enzyme crystals (CLECs) with GA. The EC-TR was prepared by the GA treatment after mixing NPs and trypsin in an aqueous buffer solution. Because NPs were functionalized with amino groups, the GA treatment of NPs end up becoming aldehyde-functionalized NPs, which are then able to form a covalent linkage with the amino groups on the surface of the trypsin molecules, leading to CA-TR/NPs. As a result, CA-TR has a mono-layered coverage of trypsin on the NPs. On the other hand, the addition of GA into the well-mixed NP and trypsin solution results in crosslinks of enzyme with the NPs in a form that is referred to here as EC-TR/NPs. These multilayered EC-TR/NPs have a lot more covalent linkages between the enzyme molecules and the trypsin on the NPs surface. In other words, the EC-TR/NPs evaluated here consisted of thick multilayered trypsin coatings instead of a monolayer of covalently attached trypsin on each nanoparticle.

The enzyme coating approach improved several properties for the practical applications of EC-TR/NPs in various fields, including protein digestion for proteomic analysis. First, as shown in Figure 1b, EC-TR/NPs could be captured very rapidly using a magnet, enabling an easy separation of EC-TR/NPs from the aqueous solution. This rapid magnetic capture is most likely due to the dense large packed matrix of crosslinked NPs having a greater attraction for the magnetic field than when the NPs are not crosslinked together. The difference in magnetic attraction is revealed in the TEM image of EC-TR/NPs (Figure 1c).

Typically, the enzyme loading of EC-TR/NPs cannot be obtained via conventional protein assays due to the generation of insoluble crosslinked enzymes in solution after the GA treatment, which prevents the correct measurement of protein concentration. In the present work, we employed a direct measurement of enzyme loading by using elemental analyses. The measured amounts of nitrogen (N) using elemental analysis was used to calculate the amounts of enzyme in the samples of CA-TR and EC-TR/NPs. Elemental analyses were also performed with various amounts of free trypsin as a control to obtain a standard curve converting the measured nitrogen amount into the amount of trypsin in the samples. Directly-measured enzyme loadings of CA-TR and EC-TR/NPs were 23.12 % (w/w) and 26.10 % (w/w), respectively, suggesting that the enzyme loading of EC-TR was marginally improved when compared to CA-TR.

The activities of CA-TR and EC-TR/NPs were measured by the trypsin-catalyzed hydrolysis of BAPNA in an aqueous solution. Once BAPNA is hydrolyzed, *p*-nitroaniline is released as one of hydrolytic products (Figure 2a). *p*-Nitroaniline is highly chromophoric displaying a characteristic yellow color with a maximal absorbance at 410 nm, which can be easily measured in a spectrophotometer. The measured apparent activities of CA-TR and EC-TR/NPs were 2.73 and 3.19 $\mu\text{mole/min per mg}$ of NPs, and the specific activities were calculated to be 9.08 and 8.70 $\mu\text{mole/min per mg}$ of trypsin, respectively. The difference in the apparent activities of CA-TR and EC-TR/NPs correlates well with the difference of enzyme loadings, resulting in a similar level of specific activities of CA-TR and EC-TR/NPs. However, this result should be interpreted with the consideration of two factors. One is the number of covalent linkages, and the other is the trypsin inactivation via denaturation and autolysis. The former factor would decrease the activity of EC-TR/NPs with a higher number of covalent linkages on the surface of the enzyme molecules, while the latter factor would lower the activity of CA-TR/NPs due to the trypsin molecules becoming more vulnerable to denaturation and autolysis.

The stabilities of CA-TR and EC-TR/NPs were checked in an aqueous buffer at room temperature under recycled uses. Residual activities were measured time dependently. Then, the samples were excessively washed and incubated under rigorous shaking (200 rpm) until the next time measurements of residual activities were made. CA-TR/NPs showed a rapid decrease of TR activity while EC-TR/NPs showed a negligible activity loss after multiple uses for 27 days under rigorous and continuous shaking conditions (Figure 2b). The inactivation of CA-TR/NPs can be explained by the denaturation and/or autolysis of trypsin under rigorous shaking. The better stability of EC-TR/NPs suggests that there is a synergy of multiple stabilization processes. First, it is well known that the attachment of enzymes to a solid support prevents protein unfolding because of the multipoint attachment which locks the protein in place. In addition to this, the stabilization effects correlate with the number of contacts involved [40]. This effect was observed and characterized in thermolysin. This protein has 11 lysine residues capable of attachment, that 8 residues are enough to significantly improve its stability and enzymatic activity [39]. Second, Islam and Weaver described that when the overall freedom in the structure of a protein is reduced its stability increases dramatically, because of the hydrophobic interactions and electrostatic forces that held together enzyme molecules [42]. In a trypsin molecule there are 11 lysines that could tentatively react with GA through the ϵ -amino group of the lysine residues [41]. Some of those lysines will attach to the nanoparticles while the others, due to the enzyme coating process will undergo through covalent cross-linking of enzyme molecules and intramolecular cross-linking. This also contributes to the prevention of dissociation, unfolding or aggregation and holds the enzyme in an extremely stable conformation which can withstand temperature and chaotrophic agents. Finally, a third source of stabilization would come from the use of GA. The reaction with GA is not fully understood, but it is well known that GA improves the thermostabilization of proteins by preventing their unfolding.

It is also known that GA tends to undergo a polymerization reaction forming a mixture of oligomers of various lengths and structures [43] and so it is possible that these varied GA species are able to custom link the multiple length sites between different lysine residues.

3.2 Digestion of a model protein using EC-TR/NPs

To demonstrate the feasible application of stable EC-TR/NPs in the protein digestion for proteomic analysis, multiple digestions of a model protein (BSA) were performed using the same EC-TR/NPs over a period of 253 days. Two different batches of EC-TR/NPs were simultaneously tested to prove the robustness of the method. For each digestion, reduced and alkylated BSA was mixed with EC-TR/NPs, and the mixture was incubated under shaking (200 rpm) at 37 °C for 12 h. After each digestion, excessively-washed EC-TR/NPs were stored in an ammonium bicarbonate buffer at room temperature until the next digestion. Figure 3 shows the digestion results from the LC-MS/MS analysis, represented by the number of identified peptides and the protein coverage (%). A marginal decrease in the digestion performance was observed over a period of 8 months in terms of the number of unique identified peptides and protein coverage, but the model protein (BSA) could still be successfully and easily identified by using 8 month old EC-TR/NPs for the protein digestion. No remaining intact protein was observed at the end of the gradient, indicating that the digestion was carried out to completion.

3.3 Pressure-assisted digestion of a five protein mixture using EC-TR/NPs

After the successful digestion of a model protein using EC-TR/NPs, PCT and EC-TR/NPs were combined together to develop a rapid and efficient digestion method. Previous work has shown that a successful digestion is achieved by coupling immobilized enzymes with different energy inputs [44-47]. It has been reported before that pressure accelerates enzyme reactions and PCT is readily applicable to any proteomic analysis [29,33]. We hypothesized that the introduction of PCT in the protein digestion workflow would not only allow a better mixing between EC-TR/NPs and the target proteins in a solution, but also accelerate the digestion reaction. To evaluate the use of EC-TR/NPs in combination with PCT, an equimolar mixture of five proteins was digested. Overnight digestions were performed either with free trypsin as a control or with EC-TR/NPs. In addition, we also explored if the mixing efficiency of EC-TR/NPs and substrate proteins have an effect on the digestion process. It is known that most of immobilized enzyme systems present a challenge by decreasing the conversion rates due to the mass transfer limitation. To test whether or not it has an influence on the protein digestion, the total reaction volume was doubled with a fixed amount of EC-TR/NPs and substrate proteins. Finally, one of the samples was digested using EC-TR/NPs under 5 pressure cycles, with each cycle consisted of 20 s at 25 kpsi and 10 s at atmospheric pressure. All the experiments were carried out in triplicate to evaluate the reproducibility of the digestion efficiency.

Figure 4a summarizes the results of the LC-MS/MS analyses in terms of the number of unique identified peptides and protein coverage. For all the different protocols, little or no variation was observed with the four protocols roughly yielding the same results. Due to the quality of the trypsin used some chymotryptic activity was observed. For this reason, other quality control parameters such as reproducibility in the peptide identifications and number of missed cleavages were studied in detail, as well as carried out a control experiment using the same trypsin for the in-solution digestions. Looking at the reproducibility on the peptide identifications across technical replicates in all cases, ~50% of the peptides were identified in the three replicates except in the case for of EC-TR/NPs combined with PCT where the number of peptides observed across all the technical replicates ramps up to over 60% (Figure 4b). High reproducibility in the case of the PCT digestion in 1 min can be explained by the extreme accuracy of the pressure controller on the Barocycler™, which enables an

extremely efficient mixing and denaturation of the sample proteins under the pressure cycles. The number of missed cleavages of identified peptides was also evaluated. Figure 4c shows the pie charts summarizing the amount of missed cleavages for each sample. In all cases where digestions were carried out using EC-TR/NPs, the percentage of peptides with no missed cleavages was equal or significantly higher than that of the free trypsin digestion. For the pressure assisted digestions the higher number of missed cleavages suggest that the reaction was not fully complete and increasing slightly the reaction time will help to achieve the same results as the overnight digestions. Nevertheless, must be point out that the pressure digestion in 1 min yields equivalent results to the overnight experiment using free trypsin. Overall, these results can be explained by the highly stable activity of EC-TR/NPs when compared to free trypsin due to their good stability under the rigorous pressure cycles. These results suggest that the performances of protein digestions using EC-TR/NPs either overnight or in 1 min using PCT are better than the ones obtained from the traditional protocol of in-solution digestion using free trypsin. It should also be noted that free trypsin could not be reused while EC-TR/NPs can be reused multiple times over many days due to the high stability of TR activity.

3.4 Application of pressure-assisted digestion with EC-TR/NPs for proteome analysis

Since the ultimate goal is to confirm the feasibility of EC-TR/NP for use in digesting complex proteome mixtures, a whole mouse brain proteome was chosen to test this novel procedure. Digesting this sample has several challenges mainly due to the challenge in protein extraction from the tissue and also because of the high-lipid content yielding a high number of membrane proteins. To overcome these challenges, the whole brain was shredded in 8 M urea followed by PCT treatment as it is shown in Figure 5a. After reduction and alkylation, three equal aliquots were made and subjected to proteolytic digestion. Peptide digests were analyzed using gas-phase fractionation to overcome the under sampling in the mass spectrometer. Figure 5b shows the chromatograms obtained for the different m/z ranges. The gas-phase separation, even though it is not a completely orthogonal separation to the LC dimension, clearly showed an improvement over running the same sample three times by yielding a higher number of identifications [48, 49]. MS/MS results were analyzed as previously described by using a mixed model that combined the two main scores of SEQUEST [38]. Figure 5c shows the contour plots obtained for the different charge states and the clear division between true and false hits. Histograms were built containing the number of MS/MS spectra passing the FDR-cut off filter as well as the unique peptides and proteins (Figure 5d). These results clearly show that this novel approach noticeably outperformed the in-solution digestion using free trypsin and few differences were observed between doing the digestion overnight or in 1 min under pressure cycles using EC-TR/NPs. The number of identified proteins that overlap among the three protocols is shown in figure 5e. Noticeable is the improved number of proteins identifications for each of the experiments where the nanoparticles were used compared to the experiment with free trypsin. Finally, the number of peptide identifications was somewhat higher for the PCT digestions although the inverse trend was observed at the protein level, but it is reasonably expected that both trends would converge if more fractionation would be performed. Previously we have shown that PCT assisted protein digestions show a higher reproducibility and can be effectively performed in less than 10 min. Here we demonstrate that PCT assisted digestions can also be successful, reproducible and robust with the inclusion of immobilized EC-TR/NPs. This combined technology may lead the way to robust, rapid and reusable automatable protein digestion systems for proteomic research.

4. Concluding remarks

The combination of PCT and EC-TR/NPs appears effective for a fast and efficient protein digestion. The trypsin coating aggregated and crosslinked onto magnetic NPs represents a

promising strategy for high-throughput platforms. The high stability obtained allows for an easy storage and continuous use at room temperature for an extended period, and the magnetic capture of EC-TR/NPs is extremely useful for their easy recovery when using robotic stations in a 96-well plate format. Furthermore, by using EC-TR/NPs under pressure cycles, the protein digestion is significantly accelerated, eliminating the need for long incubation times, and the excellent mixing translates into higher digestion reproducibility. These developments provide a basis for fast, robust and automated sample preparation in support of e.g. high-throughput proteomic analyses.

Acknowledgments

Portions of this work were supported by the NIH National Center for Research Resources (RR018522), NIH National Cancer Institute (R21 CA12619-01), and the Pacific Northwest National Laboratory's (PNNL) Laboratory Directed Research and Development Program. Portions of this work were supported by grants from the National Research Foundation (NRF) funded by the Korean Ministry of Education, Science & Technology (MEST) (No. 2009-0082314, No. 2009-0059861, and No. 2009-0075638). Part of this work was performed in the Environmental Molecular Science Laboratory, a DOE national scientific user facility located on the campus of PNNL in Richland, Washington. PNNL is a multi-program national laboratory operated by Battelle for the DOE under Contract DE-AC05-76RLO1830. The authors thank Ron Moore and Dr. Mikhail E. Belov for helpful suggestions and technical support. Authors also want to express their gratitude to Dr. Vlad E. Petyuk for providing the mouse brain sample and the script for counting the number of missed cleavages. The authors would like to thank Kim Hixson for her helpful suggestions during the writing of the manuscript.

REFERENCES

1. Ruiz-Romero C, Blanco FJ. Mitochondrial proteomics and its application in biomedical research. *Mol. Biosyst.* 2009; 5:1130–1142. [PubMed: 19756302]
2. McGarvey PB, Huang HZ, Mazumder R, Zhang J, et al. Systems Integration of Biodefense Omics Data for Analysis of Pathogen-Host Interactions and Identification of Potential Targets. *PLoS One.* 2009; 4:11.
3. Kalluri UC, Hurst GB, Lankford PK, Ranjan P, Pelletier DA. Shotgun proteome profile of *Populus* developing xylem. *Proteomics.* 2009; 9:4871–4880. [PubMed: 19743414]
4. Guan FY, Uboh CE, Soma LR, Birks E, et al. LC-MS/MS method for confirmation of recombinant human erythropoietin and darbepoetin alpha in equine plasma. *Anal. Chem.* 2007; 79:4627–4635. [PubMed: 17500535]
5. Guan FY, Uboh CE, Soma LR, Birks E, et al. Differentiation and identification of recombinant human erythropoietin and darbepoetin alfa in equine plasma by LC-MS/MS for doping control. *Anal. Chem.* 2008; 80:3811–3817. [PubMed: 18380469]
6. Carrera M, Canas B, Pineiro C, Vazquez J, Gallardo JM. Identification of commercial hake and grenadier species by proteomic analysis of the parvalbumin fraction. *Proteomics.* 2006; 6:5278–5287. [PubMed: 16927426]
7. Carrera M, Canas B, Pineiro C, Vazquez J, Gallardo JM. De novo mass spectrometry sequencing and characterization of species-specific peptides from nucleoside diphosphate kinase B for the classification of commercial fish species belonging to the family Merlucciidae. *J. Proteome Res.* 2007; 6:3070–3080. [PubMed: 17622168]
8. Canas B, Lopez-Ferrer D, Ramos-Fernandez A, Camafeita E, Calvo E. Mass spectrometry technologies for proteomics. *Brief Funct Genomic Proteomic.* 2006; 4:295–320. [PubMed: 17202122]
9. Baker ES, Livesay EA, Orton DJ, Moore RJ, et al. An LC-IMS-MS Platform Providing Increased Dynamic Range for High-Throughput Proteomic Studies. *J. Proteome Res.* 2010; 9:997–1006. [PubMed: 20000344]
10. Olsen JV, Schwartz JC, Griep-Raming J, Nielsen ML, et al. A Dual Pressure Linear Ion Trap Orbitrap Instrument with Very High Sequencing Speed. *Mol. Cell. Proteomics.* 2009; 8:2759–2769. [PubMed: 19828875]

11. Livesay EA, Tang KQ, Taylor BK, Buschbach MA, et al. Fully automated four-column capillary LC-MS system for maximizing throughput in proteomic analyses. *Anal. Chem.* 2008; 80:294–302. [PubMed: 18044960]
12. Kiebel GR, Auberry KJ, Jaitly N, Clark DA, et al. PRISM: A data management system for high-throughput proteomics. *Proteomics.* 2006; 6:1783–1790. [PubMed: 16470653]
13. Canas B, Pineiro C, Calvo E, Lopez-Ferrer D, Gallardo JM. Trends in sample preparation for classical and second generation proteomics. *J. Chromatogr. A.* 2007; 1153:235–258. [PubMed: 17276441]
14. Kim BC, Lopez-Ferrer D, Lee SM, Ahn HK, et al. Highly stable trypsin-aggregate coatings on polymer nanofibers for repeated protein digestion. *Proteomics.* 2009; 9:1893–1900. [PubMed: 19288524]
15. Kim JB, Grate JW, Wang P. Nanobiocatalysis and its potential applications. *Trends Biotechnol.* 2008; 26:639–646. [PubMed: 18804884]
16. Heyman A, Levy I, Altman A, Shoseyov O. SP1 as a novel scaffold building block for self-assembly nanofabrication of submicron enzymatic structures. *Nano Lett.* 2007; 7:1575–1579. [PubMed: 17530810]
17. Koblenz TS, Wassenaar J, Reek JNH. Reactivity within a confined self-assembled nanospace. *Chem. Soc. Rev.* 2008; 37:247–262. [PubMed: 18197342]
18. Munge B, Liu GD, Collins G, Wang J. Multiple enzyme layers on carbon nanotubes for electrochemical detection down to 80 DNA copies. *Anal. Chem.* 2005; 77:4662–4666. [PubMed: 16013886]
19. Wang J, Liu GD, Jan MR. Ultrasensitive electrical biosensing of proteins and DNA: Carbon-nanotube derived amplification of the recognition and transduction events. *J. Am. Chem. Soc.* 2004; 126:3010–3011. [PubMed: 15012105]
20. Wang P. Nanoscale biocatalyst systems. *Curr. Opin. Biotechnol.* 2006; 17:574–579. [PubMed: 17084611]
21. Wang QG, Yang ZM, Ma ML, Chang CK, Xu B. High catalytic activities of artificial peroxidases based on supramolecular hydrogels that contain heme models. *Chem.-Eur. J.* 2008; 14:5073–5078.
22. Willner B, Katz E, Willner I. Electrical contacting of redox proteins by nanotechnological means. *Curr. Opin. Biotechnol.* 2006; 17:589–596. [PubMed: 17084610]
23. Willner I, Basnar B, Willner B. Nanoparticle-enzyme hybrid systems for nanobiotechnology. *Febs J.* 2007; 274:302–309. [PubMed: 17181543]
24. Palani A, Lee JS, Huh J, Kim M, et al. Selective enrichment of cysteine-containing peptides using SPDP-Functionalized superparamagnetic Fe₃O₄@SiO₂ nanoparticles: Application to comprehensive proteomic profiling. *J. Proteome Res.* 2008; 7:3591–3596. [PubMed: 18563925]
25. Kim BC, Nair S, Kim J, Kwak JH, et al. Preparation of biocatalytic nanofibres with high activity and stability via enzyme aggregate coating on polymer nanofibres. *Nanotechnology.* 2005; 16:S382–S388.
26. Lee J, Lee Y, Youn JK, Bin Na H, et al. Simple synthesis of functionalized superparamagnetic magnetite/silica core/shell nanoparticles and their application as magnetically separable high-performance biocatalysts. *Small.* 2008; 4:143–152. [PubMed: 18189246]
27. Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA. Application of pressure cycling technology to tissue sample preparation for 2-DE. *Electrophoresis.* 2007; 28:1022–1024. [PubMed: 17300130]
28. Smejkal GB, Robinson MH, Lawrence NP, Tao F, et al. Increased protein yields from *Escherichia coli* using pressure-cycling technology. *J Biomol Tech.* 2006; 17:173–175. [PubMed: 16741245]
29. Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, et al. Application of pressurized solvents for ultrafast trypsin hydrolysis in proteomics: Proteomics on the fly. *J. Proteome Res.* 2008; 7:3276–3281. [PubMed: 18605748]
30. Jones LM, Zhang H, Vidavsky I, Gross ML. Online, High-Pressure Digestion System for Protein Characterization by Hydrogen/Deuterium Exchange and Mass Spectrometry. *Anal. Chem.* 2010; 82:1171–1174. [PubMed: 20095571]
31. Szabo Z, Guttman A, Karger BL. Rapid Release of N-Linked Glycans from Glycoproteins by Pressure-Cycling Technology. *Anal. Chem.* 2010; 82:2588–2593. [PubMed: 20170179]

32. Yang HJ, Hong J, Lee S, Shin S, Kim J. Pressure-assisted tryptic digestion using a syringe. *Rapid Commun. Mass Spectrom.* 2010; 24:901–908. [PubMed: 20196188]
33. Lopez-Ferrer D, Petritis K, Lourette NM, Clowers B, et al. On-line Digestion System for Protein Characterization and Proteome Analysis. *Anal. Chem.* 2008; 80:8930–8936. [PubMed: 19551971]
34. Lopez-Ferrer D, Heibeck TH, Petritis K, Hixson KK, et al. Rapid sample processing for LC-MS-based quantitative proteomics using high intensity focused ultrasound. *J. Proteome Res.* 2008; 7:3860–3867. [PubMed: 18686986]
35. Shen Y, Tolic N, Masselon C, Pasa-Tolic L, et al. Ultrasensitive proteomics using high-efficiency on-line micro-SPE-NanoLC-NanoESI MS and MS/MS. *Anal. Chem.* 2004; 76:144–154. [PubMed: 14697044]
36. Petyuk VA, Qian WJ, Chin MH, Wang HX, et al. Spatial mapping of protein abundances in the mouse brain by voxelation integrated with high-throughput liquid chromatography-mass spectrometry. *Genome Res.* 2007; 17:328–336. [PubMed: 17255552]
37. Eng JK, McCormack AL, Yates JR. AN APPROACH TO CORRELATE TANDEM MASS-SPECTRAL DATA OF PEPTIDES WITH AMINO-ACID-SEQUENCES IN A PROTEIN DATABASE. *Journal of the American Society for Mass Spectrometry.* 1994; 5:976–989.
38. Lopez-Ferrer D, Martinez-Bartolome S, Villar M, Campillos M, et al. Statistical model for large-scale peptide identification in databases from tandem mass spectra using SEQUEST. *Anal. Chem.* 2004; 76:6853–6860. [PubMed: 15571333]
39. Persichetti RA, Stclair NL, Griffith JP, Navia MA, Margolin AL. Cross-Linked Enzyme Crystals (ClecS) of Thermolysin in the Synthesis of Peptides. *Journal of the American Chemical Society.* 1995; 117:2732–2737.
40. Mozhaev VV. Mechanism-Based Strategies for Protein Thermostabilization. *Trends in Biotechnology.* 1993; 11:88–95. [PubMed: 7763513]
41. Hardy PM, Nicholls AC, Rydon HN. Hydration and Polymerization of Succinaldehyde, Glutaraldehyde, and Adipaldehyde. *Journal of the Chemical Society-Perkin Transactions 2.* 1972:2270–2278.
42. Islam SA, Weaver DL. Molecular-Interactions in Protein Crystals - Solvent Accessible Surface and Stability. *Proteins-Structure Function and Genetics.* 1990; 8:1–5.
43. Tashima T, Imai M, Kuroda Y, Yagi S, Nakagawa T. Structure of a New Oligomer of Glutaraldehyde Produced by Aldol Condensation Reaction. *Journal of Organic Chemistry.* 1991; 56:694–697.
44. Hahn HW, Rainer M, Ringer T, Huck CW, Bonn GK. Ultrafast Microwave-Assisted In-Tip Digestion of Proteins. *J. Proteome Res.* 2009; 8:4225–4230. [PubMed: 19639939]
45. Lin S, Yao GP, Qi DW, Li Y, et al. Fast and efficient proteolysis by microwave-assisted protein digestion using trypsin-immobilized magnetic silica microspheres. *Anal. Chem.* 2008; 80:3655–3665. [PubMed: 18407620]
46. Lin S, Yun D, Qi DW, Deng CH, et al. Novel microwave-assisted digestion by trypsin-immobilized magnetic nanoparticles for proteomic analysis. *J. Proteome Res.* 2008; 7:1297–1307. [PubMed: 18257514]
47. Lopez-Ferrer D, Hixson KK, Smallwood H, Squier TC, et al. Evaluation of a High-Intensity Focused Ultrasound-Immobilized Trypsin Digestion and O-18-Labeling Method for Quantitative Proteomics. *Anal. Chem.* 2009; 81:6272–6277. [PubMed: 19555078]
48. Scherl A, Shaffer SA, Taylor GK, Kulasekara HD, et al. Genome-specific gas-phase fractionation strategy for improved shotgun proteomic profiling of proteotypic peptides. *Anal. Chem.* 2008; 80:1182–1191. [PubMed: 18211032]
49. Yi EC, Marelli M, Lee H, Purvine SO, et al. Approaching complete peroxisome characterization by gas-phase fractionation. *Electrophoresis.* 2002; 23:3205–3216. [PubMed: 12298092]

Figure 1.

(a) Schematic illustrations for the preparation of trypsin coating onto magnetic nanoparticles. (b) Magnetic capture of EC-TR/NPs. (c) Transmission electron microscopy (TEM) image of EC-TR/NPs (scale bar, 100 nm).

Figure 2.

(a) Schematic for the trypsin-catalyzed hydrolysis of BAPNA. (b) Enzyme stabilities of CA-TR/NP (empty circles), EC-TR/NP (filled circles) and Free trypsin (empty triangles) under recycled uses and shaking at 200 rpm at room temperature. The activity was measured by the hydrolysis of BAPNA at each time point, and the relative activity was calculated from the ratio of residual activity at each time point to the initial activity of each sample.

Figure 3. Time course analyses of iterative BSA digestions using EC-TR/NPs as a function of unique identified peptides (empty circles) or protein coverage (filled circles).

Figure 4. Comparison of digestion performances for EC-TR/NPs and free trypsin overnight, and EC-TR/NPs pressure assisted digestion. A five standard protein mixture consisting of BSA, ovalbumin, myoglobin, carbonic anhydrase and lactoglobulin were used for the experiment. (a) Histograms comparing number of unique peptides and percentage of protein coverage for three technical replicates. (b) Venn diagrams showing the peptide overlap between three technical digestion replicates. (c) Pie charts showing the percentage of missed cleavages for the different digestion methods.

Figure 5.

(a) The workflow of analyzing whole mouse brain proteome utilizing EC-TR/NP. After reduction and alkylation in 3 min using a sonoreactor, the proteome samples are subjected to three different digestions: conventional overnight digestion using free enzyme, overnight digestion using EC-TR/NP, and 1 min digestion using EC-TR/NP with PCT treatment. (b) Representative chromatograms obtained from EC-TR/NPs-PCT digestion of mouse brain samples after gas-phase fractionations. While imperfect, gas-phase fractionations clearly helped alleviating under sampling of whole proteome analyses. (c) Scatter plots of the corrected scores for charge states +2 and +3 obtained after SEQUEST database search from the generated MS/MS spectra using the three samples using different digestion methods. (d) Histograms comparing the number of unique identified peptides (empty bars), total identified peptides (diagonal filled bars) and percentage of protein coverage for the three different digestion protocols. (e) Venn diagram showing the protein overlap among the three different protocols.