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Improving digestion efficiency under H/D exchange conditions with activated pepsinogen coupled columns

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Abstract

The ability to analyze localized amide hydrogen/deuterium (H/D) exchange kinetics of a protein is highly dependent on the digestion efficiency of the enzyme being used to generate a collection of labeled peptides. Pepsin is one of the most commonly used acid proteases in amide H/D exchange experiments due to its broad specificity and robustness at "slow exchange" conditions of low temperature and pH. The chemistry used to immobilize pepsin to POROS AL-20 beads is optimal under neutral pH conditions but acid proteases such as pepsin are irreversibly denatured above pH 5 so coupling must be performed under suboptimal conditions. Thus, in the current study we report a technique to improve the digestion efficiency for amide H/D exchange by immobilizing pepsinogen to POROS AL-20 support under optimal coupling conditions of pH 6.7 where pepsinogen is stable and subsequently converting the coupled pepsinogen into active pepsin. This activated pepsinogen column demonstrated a higher specific activity at both 25 °C and 0 °C than an identically coupled pepsin column and gave better peptide coverage for cytochrome *C* and manganese superoxide dismutase (MnSOD) improving our amide H/D exchange data for these proteins. These results were reproducible for three independently coupled and activated pepsinogen columns. Protein assays demonstrated that more enzyme was bound to the POROS AL-20 resin coupled with pepsinogen at pH 6.7 than pepsin at pH 5.

Keywords: H/D exchange; Pepsin; Pepsinogen; Mass spectrometry

1. Introduction

Amide hydrogen/deuterium (H/D) exchange is a powerful biophysical technique used to study protein dynamics [1–5]. The rate at which an amide hydrogen undergoes isotope exchange with solvent deuterium can reveal a great deal about the structure and dynamics of a protein. NMR has been the preferred method to measure amide H/D exchange kinetics in proteins due to its ability to measure exchange rates of individual amide hydrogens. However, such experiments require a large quantity of protein and are limited to the study of proteins or protein complexes that are less than ~30,000 Da [6]. Over a decade ago it was demonstrated that amide H/D exchange of polypeptides could be measured using mass spectrometry [7,8]. Compared to amide H/D exchange as measured by NMR, mass spectrometry is much more sensitive, requiring only picomoles of sample, and can be used to measure proteins and protein complexes of theoretical unlimited size and complexity [9–14]. As a result of this pioneering work, amide H/D exchange mass spectrometry has been used to measure protein folding, protein aggregation, protein oligomerization, and protein–ligand interactions.

Current methods used in amide H/D exchange are based on the initial work described by Rosa and Richards [15] and Englander et al. [4]. In these experiments, native proteins were labeled with tritium and subsequently digested enzymatically into fragments that were chromatographically separated. Following separation, the degree of exchange of amide hydrogens with tritium was quantified by measuring the amount of radiolabel incorporated in the peptides. Zhang and Smith refined the experiment by substituting tritium with deuterium, digesting the protein under "slow exchange" conditions, and measuring the percent deuterium incorporation (in-exchange or on-exchange) on polypeptide fragments using LC–MS [16]. The key to this approach is the ability to process the sample under "slow exchange" conditions to prevent back exchange or loss of incorporated deuterium during the sample processing and

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analysis steps. The exchange rates of peptide amide hydrogens are highly dependent on pH and temperature [17,18]. At pH 7 and 25 °C, the half-life of an amide hydrogen is approximately 10–50 ms, whereas at pH 2.5 and 0 °C, the half-life is between 1 h and 2 h. Therefore, to minimize back exchange and maximize the ability to measure deuterium incorporation, most H/D exchange reactions are quenched after a specified time period with a low pH and low temperature buffer and the labeled protein is digested by acid proteases such as pepsin. Pepsin is the enzyme of choice as it functions well under these "slow exchange" conditions however; other acid stable proteases such as fungal acid protease XIII and XVIII have been used [2].

Two different approaches have been used to digest labeled proteins under "slow" hydrogen exchange quench conditions. The first involves the use of soluble pepsin added to the deuterium labeled protein and subsequently incubated at 0 °C. This approach has been described extensively in published amide H/D exchange reports but is not ideal due to the fact that the high concentrations of pepsin used to obtain a rapid digest in solution can block HPLC columns and interfere with the ESI process [19]. Consequently, Smith and coworkers developed an on-column digestion approach involving the use of pepsin immobilized to POROS AL-20 resin. In this approach, the deuterium labeled protein is passed through the immobilized pepsin column that cleaves it into peptides which are subsequently concentrated on a trapping column followed by HPLC separation and analysis by ESI-MS. This approach allows for shorter digestion periods resulting in less deuterium loss or back exchange with non-deuterated solvent as well as increased identification of peptides from labeled protein due to the absence of excessive amounts of pepsin peptides in the sample. For these reasons, many amide H/D exchange studies have incorporated immobilized acid protease columns coupled to the POROS AL-20 support [19].

The ability to analyze localized amide H/D exchange kinetics of a protein is highly dependent on the digestion efficiency of the enzyme being used to generate a collection of labeled peptides. In the absence of enzymatic digestion exchange kinetics can be determined only as the average of all amides in the protein. Digestion of the target protein into a collection of smaller peptides allows some degree of localization of exchange kinetics and the larger the pool of peptides generated the greater the degree of localization. Pepsin is one of the most commonly used acid proteases in amide H/D exchange experiments due to its broad specificity and robustness at "slow exchange" conditions of low temperature and pH. However, there are proteins that immobilized pepsin will not efficiently cleave under "slow exchange" conditions which consequently limit the amount of localized amide H/D exchange data that can be collected on these proteins. Therefore, improving the digestion efficiency of immobilized pepsin can vastly increase the number of proteins being analyzed by amide H/D exchange and the quality of the data being collected.

The procedure used to couple proteins to POROS AL-20 beads involves forming a secondary amine linkage by reducing a Schiff's base between the support and the coupled protein [19]. The reaction kinetics are optimal at pH 6.9 conditions but acid proteases are irreversibly inactivated above pH 5.5. Thus, the coupling of acid proteases to POROS AL-20 must be done under suboptimal reaction conditions to prevent inactivation of enzyme. Interestingly, the inactive precursor of pepsin, pepsinogen, can be exposed to pH above 5.5 and retain the ability to be activated at lower pH [20]. Thus, in the current study we report a technique to improve the digestion efficiency for amide H/D exchange by immobilizing pepsinogen to POROS AL-20 support at the optimal pH 7 and subsequently converting the coupled pepsinogen into active pepsin by lowering the pH to 2.5 and incubating the column with soluble pepsin. This activated pepsinogen column demonstrated a higher specific activity at both 25 °C and 0 °C than an identically coupled pepsin column and gave better peptide coverage for both cytochrome *C* and manganese superoxide dismutase (MnSOD) that improved the sequence coverage of our amide H/D exchange data for these proteins.

2. Experimental methods

2.1. Generation of immobilized pepsin column

The immobilized pepsin column was generated as previously described [18] with the following modifications. Porcine pepsin A (Sigma, P-6887) was immobilized to POROS AL-20 support in several steps using a 25 mL beaker at room temperature. Eighty milligrams of pepsin was dissolved in 2 mL of 50 mM sodium citrate (pH 5.0) (Sigma). The coupling reaction was then started by adding 20 mg of sodium cyanoborohydride (Sigma) to the pepsin solution followed by slow addition 1 mL of 2 M Na_2SO_4 (Sigma). The mixture was mixed on a rocker/shaker for 10 min prior to addition of 600 mg of POROS AL-20 resin (Applied Biosystems). Finally, 2.13 mL of Na_2SO_4 was added drop wise to the reaction in 500 μL aliquots over a 30 min time span. The coupling reaction was incubated for 16 h at room temperature with gentle mixing on a rocker/shaker. Following overnight incubation, the coupling reaction was quenched by addition of 1 mL of 1 M ethanolamine (Sigma) (2 h at room temperature). After quenching the reaction, the immobilized pepsin was transferred to a sintered glass funnel and carefully washed with 50 mL of sodium citrate buffer (pH 5.0) followed by 25 mL of 1 M sodium chloride (pH 5.0) (Sigma) and a final wash of 50 mL of sodium citrate buffer (pH 5.0). After the last rinse, a 50% slurry of immobilized pepsin was generated by mixing the support with an equal volume of 0.08% TFA (pH 2.0) (Sigma). Immobilized pepsin column was generated by using 600 μL of 50% enzyme slurry and packing it into 2 mm × 2 cm guard column holder (C-130B, Upchurch Scientific). Pepsin columns were rinsed with 10 column volumes of 0.08% TFA (pH 2.5) prior to use.

2.2. Generation and activation of immobilized pepsinogen column

The immobilized pepsinogen column was generated as previously described [18] with the following modifications. Porcine pepsinogen (Sigma, P4656) was immobilized to

POROS AL-20 support in several steps using a 25 mL beaker at room temperature. Eighty milligrams of pepsinogen was dissolved in 2 mL of 50 mM sodium citrate (pH 6.7). The coupling reaction was then started by adding 20 mg of sodium cyanoborohydride to the pepsinogen solution followed by slow addition 1 mL of 2 M Na_2SO_4 (pH 6.7). The mixture was mixed on a rocker for 10 min prior to addition of 600 mg of POROS AL-20 powder. Finally, 2.13 mL of Na_2SO_4 (pH 6.7) was added to the reaction drop wise in 500 μL aliquots over a 30 min time span. The coupling reaction was then incubated for 16 h at room temperature while gently mixing on a rocker. Following overnight incubation, the coupling reaction was quenched by addition of 1 mL of 1 M ethanolamine and incubating for 2 h at room temperature. After quenching the reaction, the immobilized pepsin was transferred to a sintered glass funnel and carefully washed with 50 mL of 50 mM sodium citrate buffer pH 6.9 followed by 25 mL of 1 M sodium chloride pH 6.9 and a final wash of 50 mL of 20 mM Tris–HCl (pH 7.5). After the last rinse, a 50% slurry of immobilized pepsinogen was generated by mixing the support with an equal volume of 20 mM Tris–HCl (pH 7.5). The immobilized pepsinogen column was generated by using 600 μL of 50% enzyme slurry and packing it into 2 mm × 2 cm guard column holder (C-130B, Upchurch Scientific).

The immobilized pepsinogen column was activated as previously described. Briefly, 10 column volumes of 0.8% TFA (pH 2.5) were passed through the column prior to addition of 2 mg/mL soluble pepsin [20]. The pepsinogen column was incubated in the presence of 2 mg/mL soluble porcine pepsin (Sigma, P6887) for 16 h at 37 °C. Following incubation, the activated pepsinogen column was rinsed with 50 column volumes of 0.8% TFA (pH 2.5) to remove all soluble pepsin prior to use.

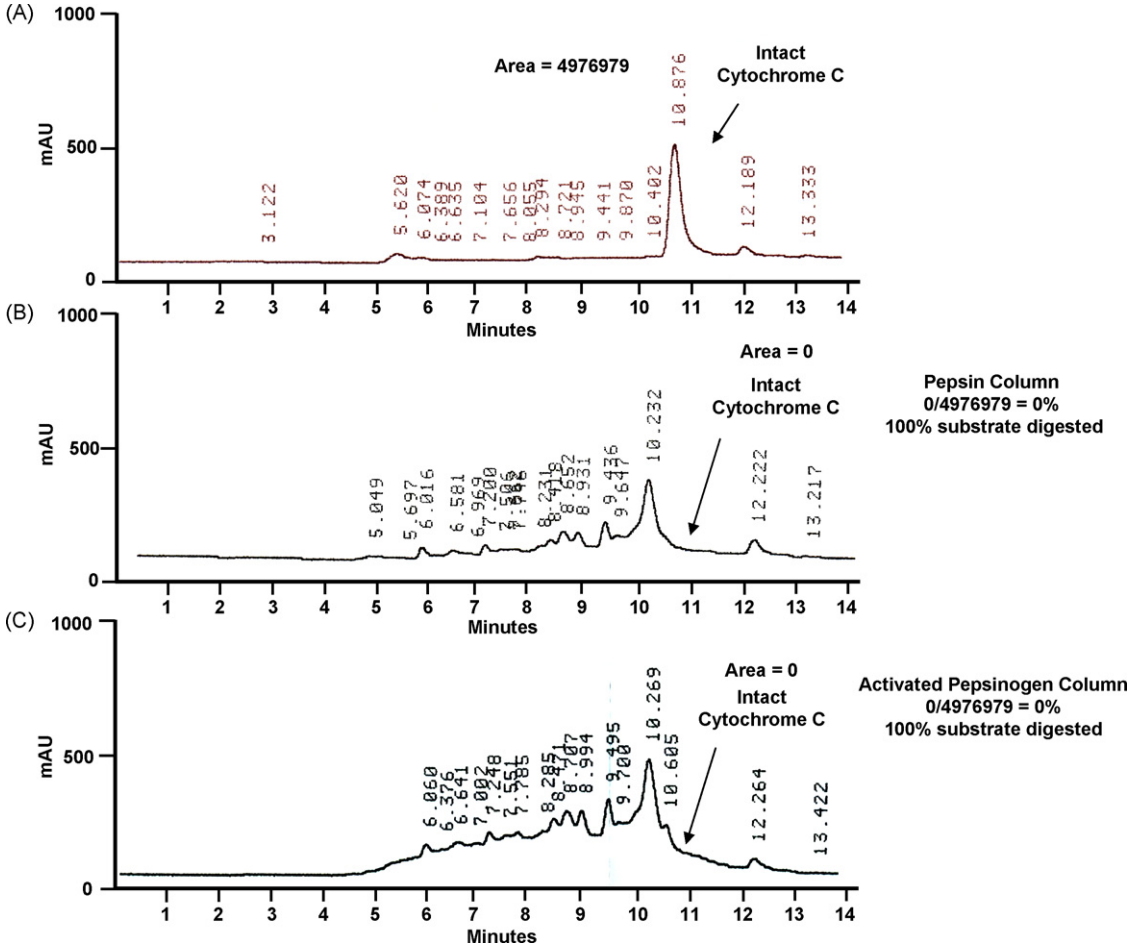

Fig. 1. Comparing the specific activity of pepsin and activated pepsinogen columns at 25 °C using cytochrome *C*: (A) 200 pmol of cytochrome *C* was injected onto C_{18} analytical column and eluted with a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated; (B) 200 pmol of cytochrome *C* was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 25 °C. The resulting peptides were eluted over a 20 min reverse-phase gradient and the absorbance of the peaks was monitored at 280 nm and the areas under the curves were calculated. The specific activity of the column was calculated by taking the area under the curve for the intact protein and dividing it by the area under the curve for the undigested protein; (C) 200 pmol of cytochrome *C* was injected onto an on-line activated pepsinogen column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 25 °C. The resulting peptides were then eluted over a 20 min reverse-phase gradient and the absorbance of the peaks was monitored at 280 nm and the areas under the curves were calculated. The specific activity of the column was calculated by taking the area under the curve for the intact protein and dividing it by the area under the curve for the undigested protein. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows mark the retention time of intact cytochrome *C*.

2.3. Determination of the amount of enzyme bound to POROS AL-20 by BCA protein assay

After coupling pepsin and pepsinogen onto POROS AL-20 support, we evaluated the amount of protein immobilized onto the resin using the BCA protein assay kit per manufacturer's instructions (Pierce). Briefly, we generated a standard curve using BSA in concentrations from 125 μg to 1 mg. We then prepared 30 μL of immobilized pepsin beads and 30 μL of immobilized pepsinogen beads from three independent coupling reactions. The BCA working reagent was added to all samples and allowed to incubate for 30 min at 37 °C. Following incubation, the absorbance of the standards and unknowns were measured at 562 nm. As a negative control, POROS AL-20 beads with no protein coupled onto them were included in the assay. All samples were analyzed in triplicate and the average values were used in the calculation of total protein.

2.4. Evaluating the specific activity of the immobilized columns using RP-HPLC

The specific activities of the immobilized pepsin and activated pepsinogen columns were evaluated by comparing the on-column digestion efficiencies of cytochrome *C*, manganese superoxide dismutase and glyceraldehyde-3-phosphate dehydrogenase using an Agilent 1100 series HPLC at 25 °C and 0 °C. The substrates were passed through the immobilized enzyme columns for 3 min at a flow rate of 200 μL/min (0.1% TFA) across a 5 μM C_{18} guard column (Higgins Analytical) where the peptides were trapped. Following the 3 min digestion, the enzyme column was taken offline and the peptides were gradient eluted from the C_{18} guard column using a gradient of 0% CH_3CN containing 0.1% TFA to 80% CH_3CN containing 0.1% TFA in 20 min. The flow rate was kept constant at 200 μL/min and eluting peaks were detected by their absorbance at 280 nm. The areas under the peaks were integrated by an Agilent 3396

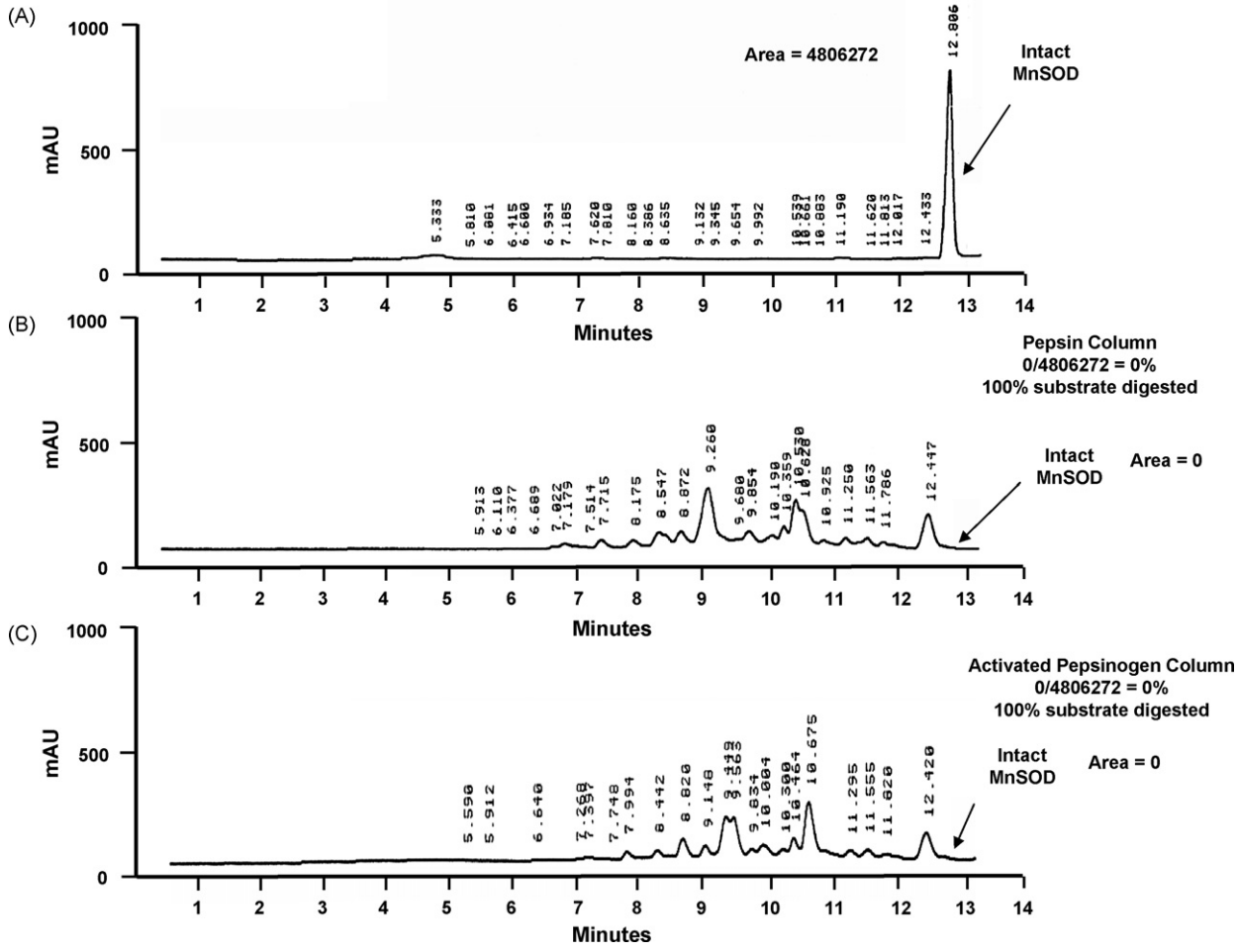

Fig. 2. Comparing the specific activity of pepsin and activated pepsinogen columns at 25 °C using MnSOD: (A) 200 pmol of MnSOD was injected onto C_{18} analytical column and eluted with a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated; (B) 200 pmol of MnSOD was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at 25 °C prior to reverse-phase elution and specific activity calculation; (C) 200 pmol of MnSOD analyzed the same as above except with the use of an activated pepsinogen column. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows mark the retention time of MnSOD.

series III integrator. As positive controls equivalent amounts of each substrate were loaded onto and gradient eluted from the C_{18} guard column without being digested. The specific activity of each column was determined as a ratio of the area of the intact substrate peak in each digested sample to the area of the intact substrate peak in each undigested control.

2.5. Evaluation of the peptide coverage of digested substrates from immobilized pepsin and immobilized activated pepsinogen columns using ESI-LCMS-MS

The peptide coverage of cytochrome *C* and MnSOD generated by on-column digestion with either immobilized pepsin or immobilized activated pepsinogen were evaluated by our automated H/D exchange MS platform as described elsewhere [1]. Briefly, 40 pmol of substrate was subjected to on-column digestion with either the pepsin or activated pepsinogen columns and the resultant peptides were collected on a C_{18} trap column. At a constant flow rate of 60 μL/min the peptides were gradient eluted from a POROS 20R2 column (1 mm i.d. × 1 cm) into a linear ion trap mass spectrometer (Thermo-Electron) using a gradient of 4% CH_3CN containing 0.3% formic acid to 40% CH_3CN containing 0.3% formic acid in 18 min. MS spectra were acquired in data dependent mode over a range of $300 < m/z < 1700$ where the top five most abundant ions were isolated and fragmented in the linear ion trap. Peptide identification was performed using Bioworks 3.1 and the SEQUEST searching algorithm (Thermo-Electron) by searching the MS/MS data files against a database of the corresponding substrate protein. Peptide coverage maps were plotted using software developed in-house.

3. Results and discussion

3.1. Comparing the specific activity of the immobilized pepsin and activated pepsinogen columns using cytochrome C and MnSOD at 25 °C

Immobilized enzyme columns are a key component of the automated amide H/D exchange MS platform we have developed to study changes in protein dynamics upon binding of

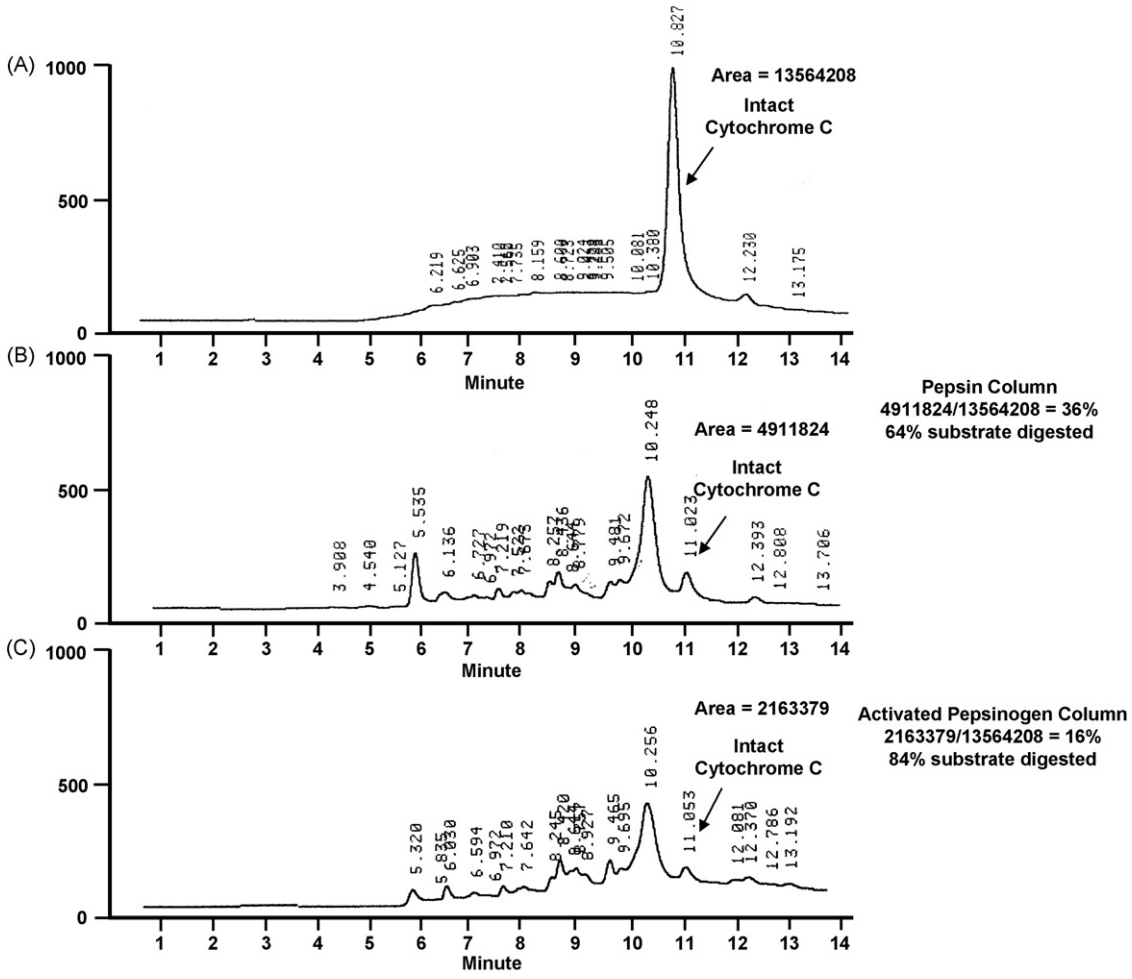

Fig. 3. Comparing the specific activity of pepsin and activated pepsinogen columns at 0 °C using cytochrome *C*: (A) 500 pmol of cytochrome *C* was injected onto C_{18} analytical column and eluted with a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated; (B) 500 pmol of cytochrome *C* was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at 0 °C prior to reverse-phase elution and specific activity calculation; (C) 500 pmol of cytochrome *C* was analyzed the same as above except with the use of an activated pepsinogen column. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows indicate the retention time of intact cytochrome *C*.

small molecules to nuclear receptor proteins [1]. The quality of the amide H/D exchange data obtained is dependent largely on the ability of the immobilized enzyme to generate a large pool of fragment peptides that afford as complete a digestion profile of the substrate protein as possible. In our system, the deuterium labeled protein is passed through the immobilized enzyme column in 3 min at a flow rate of 200 μL/min at 0 °C and pH 2.5 (slow exchange conditions). Only acid proteases such as pepsin are useful at these acidic conditions but the efficiency of the enzyme is hampered by the low temperatures and short digestion times required for the experiment. In addition, the Schiff-base chemistry used in coupling the enzymes to the POROS AL-20 support is optimal at pH 6–7 but since acid proteases are irreversibly inactivated above pH 5.5, suboptimal coupling conditions have to be used to generate the pepsin column which should lead to lower loading of enzyme on the resin. In an effort to overcome these limitations, improve the specific activity of the resin, and improve the digestion profile of substrate proteins in our H/D exchange experiments, we generated an immobilized pepsinogen column. Pepsinogen, the precursor of pepsin, is stable at pH 7 and therefore can be coupled to POROS AL-20 support under the optimal coupling pH of 6–7 and subsequently activated by subjecting it to acidic conditions and soluble pepsin [20]. By coupling pepsinogen to POROS AL-20 under more optimal coupling conditions than can be employed for coupling pepsin, we could increase the specific activity of the column due to the greater amount of enzyme immobilized per gram of resin. To test this hypothesis, we generated immobilized pepsin and pepsinogen columns as described in Section 2 and evaluated the ability of each column to cleave substrates at different temperatures using RP-HPLC. We evaluated the ability of the pepsin and activated pepsinogen columns to cleave 200 pmol of cytochrome *C* at 25 °C. As a control experiment, 200 pmol of cytochrome *C* was injected onto a C_{18} analytical column and gradient eluted over the course of 20 min. The absorbance was followed at 280 nm and the area under the peak was calculated (Fig. 1A). The immobilized pepsin column was then connected in-line with the analytical column and 200 pmol of cytochrome *C* was injected onto it and allowed to flow through the column for 3 min at 25 °C. The peptides were collected on the analytical C_{18} column and gradient eluted by RP-HPLC. The chromatogram shown in Fig. 1B demonstrates that the peak corresponding to

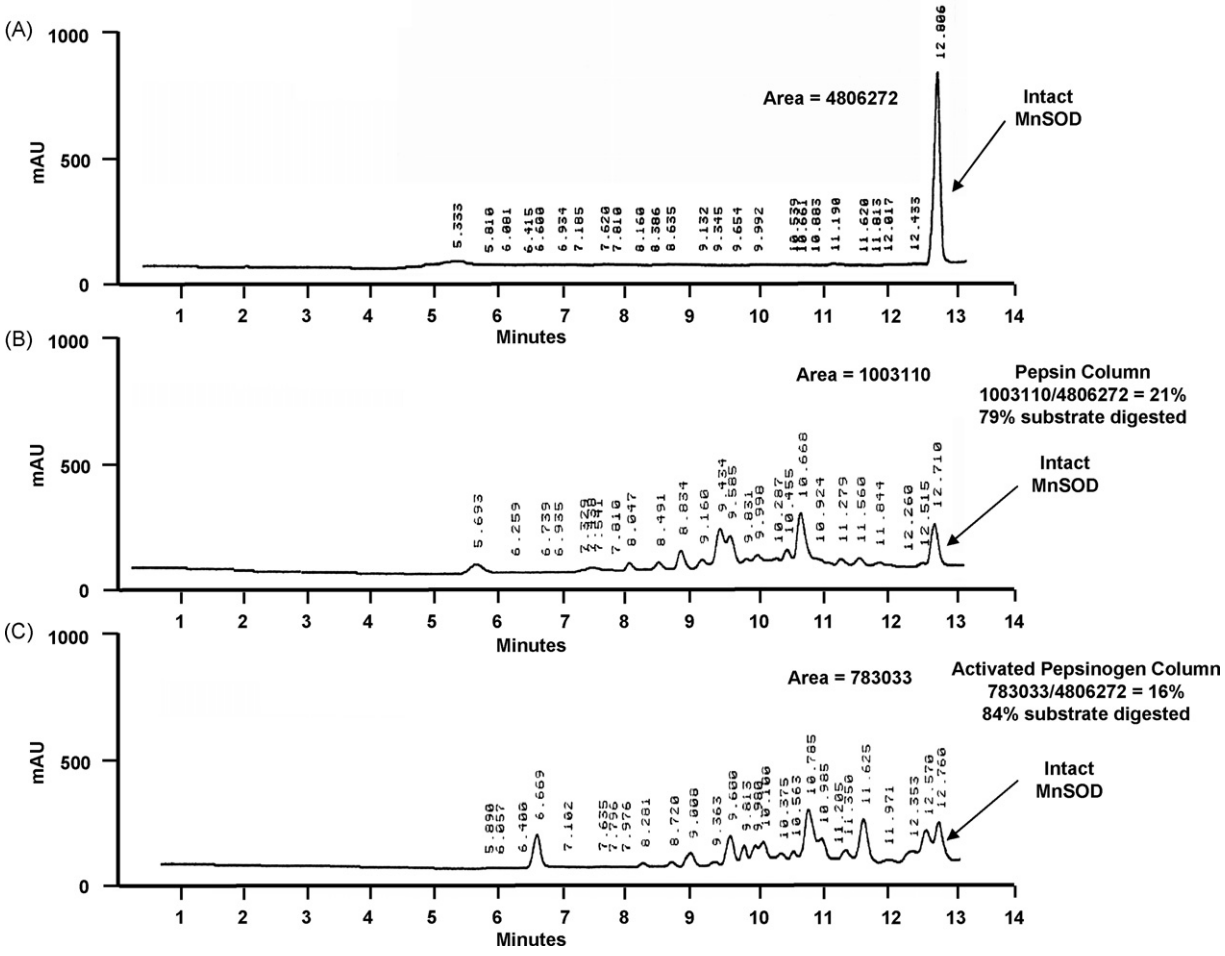

Fig. 4. Comparing the specific activity of pepsin and activated pepsinogen columns at 0 °C using MnSOD: (A) 200 pmol of MnSOD was injected onto C_{18} analytical column and eluted over a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated; (B) 200 pmol of MnSOD was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at 0 °C prior to reverse-phase elution and specific activity calculation; (C) 200 pmol of MnSOD analyzed the same as above except with the use of an activated pepsinogen column. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows mark the retention time of intact MnSOD.

the undigested cytochrome *C* peak had completely disappeared during the digestion at 25 °C. The experiment was repeated using the activated pepsinogen column and again complete digestion of the cytochrome *C* protein was observed as indicated by the lack of an integrated peak at the retention time detected for undigested cytochrome *C* in the control chromatogram (Fig. 1C).

The immobilized pepsinogen column appeared to be as efficient as the pepsin column in its ability to digest the small protein substrate cytochrome *C* at room temperature. The performance of the activated pepsinogen column was evaluated using a larger protein substrate, MnSOD, which is an 88 kDa homotetramer and similar results were obtained (Fig. 2).

3.2. Comparing the specific activity of the immobilized pepsin and activated pepsinogen columns using cytochrome C and MnSOD under slow exchange conditions

Amide H/D exchange experiments require that enzymatic digestion proceed at low pH and low temperature to minimize back exchange or loss of deuterium, therefore the columns were evaluated under these slow exchange conditions. As a control, 500 pmol of cytochrome *C* was loaded onto the analytical C_{18} column and its retention time was determined and the area under the curve at 280 nm was calculated after gradient elution by RP-HPLC (Fig. 3A). The immobilized pepsin column was then connected in-line with the analytical C_{18} column and submerged in an ice bath to lower the temperature of the column to 0 °C. Five hundred picomoles of cytochrome *C* was injected and passed through the column for 3 min and the resultant peptides were collected onto the analytical C_{18} column and gradient eluted by RP-HPLC. The specific activity of the pepsin column at 0 °C was measured by comparing the area calculated under the undigested cytochrome *C* protein peak in the pepsin digested sample and dividing it by the area measured under the cytochrome *C* peak in our undigested control sample. The results indicated that after digestion (200 μL/min for 3 min at 0 °C) with the immobilized pepsin column, 36% of the cytochrome *C* protein remained undigested (Fig. 3B). The experiment was repeated using an immobilized activated pepsinogen column cooled to 0 °C. This resulted in digestion of 84% of the cytochrome *C* protein with the immobilized activated pepsinogen column versus only 64% of cytochrome *C* digested with the immobilized pepsin column (Fig. 3C).

The activated pepsinogen column appeared to be more efficient at cleaving the small cytochrome *C* substrate at 0 °C. The performance of the activated pepsinogen column was evaluated at slow exchange conditions using a larger protein substrate, MnSOD and similar results were obtained as with cytochrome *C* (Fig. 4).

3.3. Evaluating the amount of protein coupled onto pepsinogen resin and the reproducibility of the increased specific of activated pepsinogen columns

The activated pepsinogen column demonstrated greater efficiency in cleaving both cytochrome *C* and MnSOD under slow

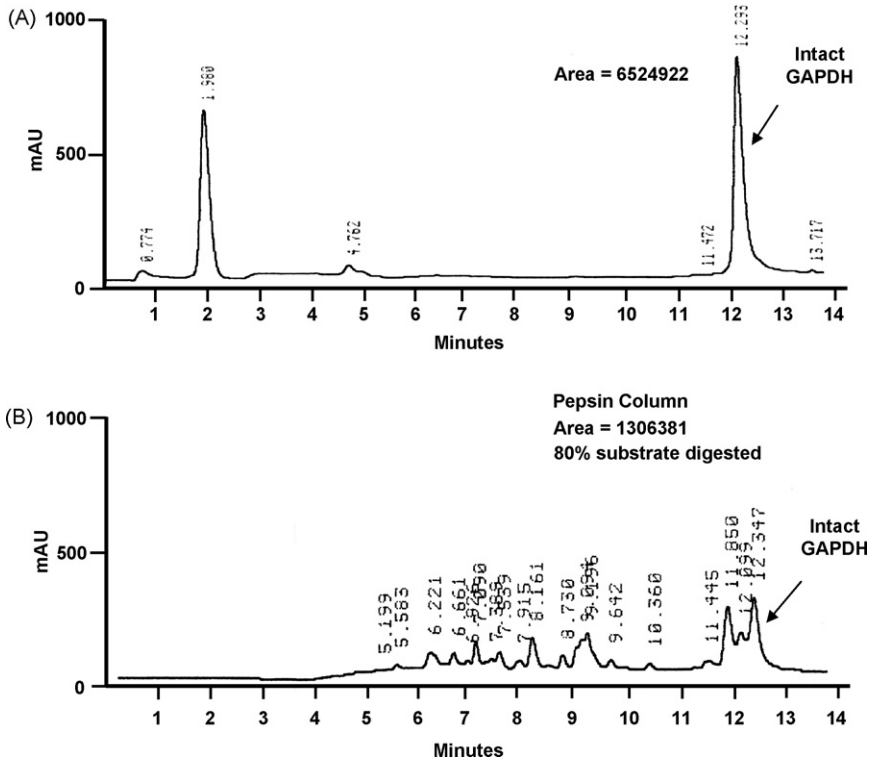

Fig. 5. Evaluating specific activity of pepsin column using GAPDH at 0 °C: (A) 200 pmol of GAPDH was injected onto C_{18} analytical column and eluted over a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated and (B) 200 pmol of GAPDH was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at 0 °C prior to reverse-phase elution and specific activity calculation. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows mark the retention time of intact GAPDH.

exchange conditions. To evaluate the reproducibility of this result, we coupled and activated three pepsinogen columns using the conditions described earlier. Using a BCA protein assay, we calculated the amount of protein bound to each of the independent pepsinogen resins and compared it to the amount of protein bound to same amount of pepsin resin. In all three cases we found that the pepsinogen resins had more protein bound to them than the same amount of pepsin resin (data not shown). The pepsinogen columns averaged 50 μg of pepsinogen per mg of resin where the pepsin column had only 20 μg of pepsin per mg of resin. Having determined more enzyme was reproducibly bound to the pepsinogen resins, we activated the material as described earlier and packed them into columns. We then compared each column's ability to digest 200 pmol of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to that of an identically coupled pepsin column as described earlier. The results indicated that after digestion (200 μL/min for 3 min at 0 °C) with the immobilized pepsin column, 20% of the GAPDH

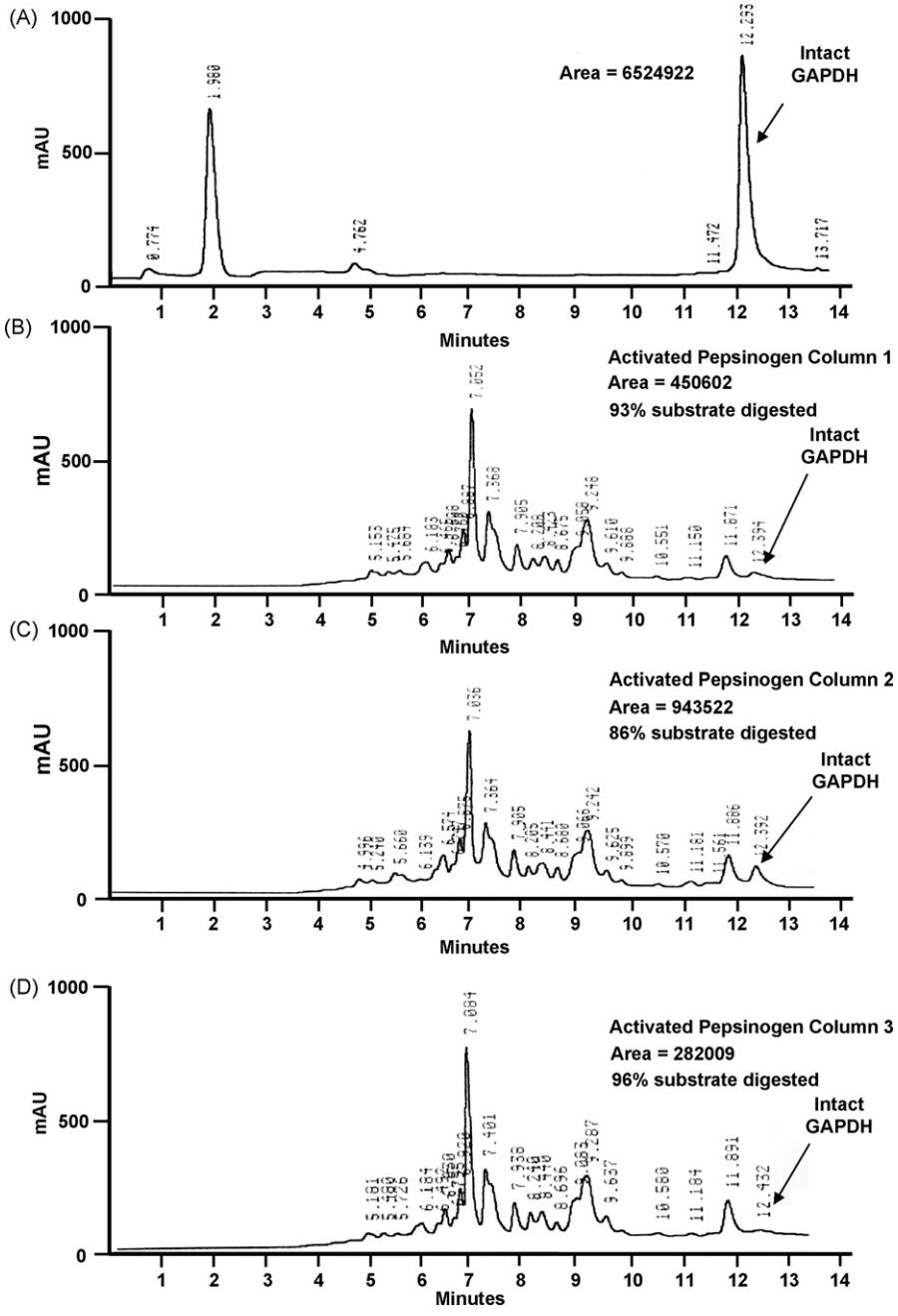

Fig. 6. Evaluating specific activity of three identically coupled and activated pepsinogen columns using GAPDH at 0 °C: (A) 200 pmol of GAPDH was injected onto C_{18} analytical column and eluted over a 20 min reverse-phase gradient. Protein absorbance was monitored at 280 nm and the area under the curve was calculated; (B) 200 pmol of GAPDH was injected onto an on-line activated pepsinogen column connected to a C_{18} analytical column and allowed to digest for 3 min at 0 °C prior to reverse-phase elution and specific activity calculation; (C) 200 pmol of GAPDH was injected onto a second on-line activated pepsinogen column and analyzed as above; (D) 200 pmol of GAPDH was injected onto a third on-line activated pepsinogen column and analyzed as above. The retention times in minutes are listed above each peptide peak in all chromatograms. Arrows mark the retention time of intact GAPDH.

protein remained undigested (Fig. 5B). The experiment was repeated using three identically coupled and activated pepsinogen columns. The amount of GAPDH digested with the three activated pepsinogen columns was 93%, 86% and 96%, respectively (Fig. 6B–D).

3.4. Evaluating peptide digestion coverage of cytochrome C and MnSOD by LC-ESI-MS/MS under slow exchange conditions

Having determined that the activated pepsinogen columns reproducibly digest three different protein substrates more efficiently than pepsin under slow exchange or quench conditions, the columns were evaluated with respect to peptide coverage. Using our automated H/D exchange platform 40 pmol of cytochrome *C* was injected onto the immobilized pepsin column for 3 min at 0 °C and trapped on a C_{18} trap column. The peptides were gradient eluted into a linear ion trap mass spectrometer and subjected to data-dependent product ion analysis as described [1]. Cytochrome *C* peptides were identified with the SEQUEST algorithm and searching the mass spectral data against a single protein database of cytochrome *C* and each product ion spectrum was manually inspected to confirm the peptide assignment. The experiment was repeated using the activated pepsinogen column. Peptide maps containing all the cytochrome *C* peptides identified using either the immobilized pepsin or immobilized activated pepsinogen columns are shown in Fig. 5. The percent coverage was calculated by dividing the number of amino acids contained in the identified peptides in the MS/MS experiment by the total number of amino acids in the protein. It was determined that the peptides generated using the activated pepsinogen column corresponded to 79% of the total protein covered (Fig. 7B) while the peptides generated by the pepsin column corresponded to only 56% coverage (Fig. 7A). The above experiments were repeated with MnSOD as a substrate. A similar trend in protein coverage between the activated pepsinogen and pepsin columns was observed. The MnSOD peptides generated from the activated pepsinogen corresponded to 100% of the total protein (Fig. 8B) while the MnSOD peptides generated by the pepsin column only covered 61% of the total protein (Fig. 8A).

Taken together, the approach described here of immobilizing pepsinogen affords an increase in the specific activity of the acid protease column. This in turn leads directly to an improvement in sensitivity of analysis and an improvement in protein sequence coverage obtained under slow exchange or quench conditions typically employed in amide H/D exchange experiments.

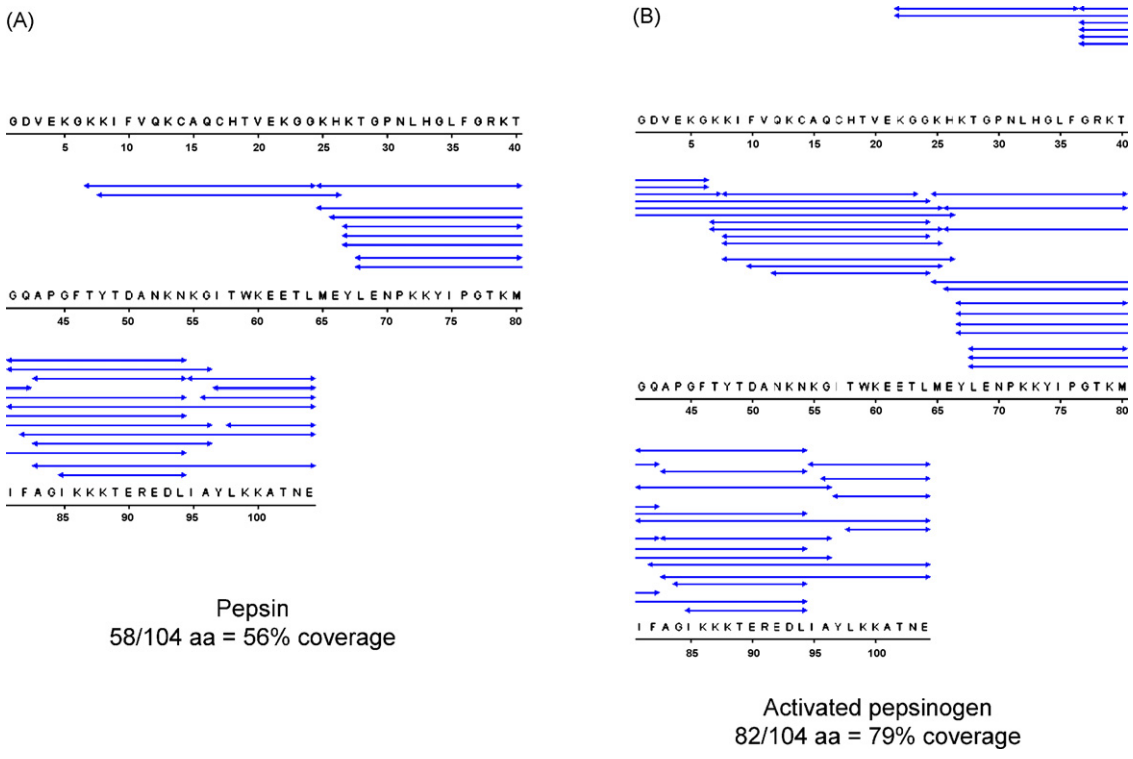

Fig. 7. Evaluating peptide digestion coverage of cytochrome *C* between pepsin and activated pepsinogen columns using LC-ESI-MS/MS under slow exchange conditions: (A) 40 pmol of cytochrome *C* was injected onto an on-line pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 0 °C. The resulting peptides were eluted with a 20 min reverse-phase gradient into a linear ion trap mass spectrometer and subjected to MS/MS product analysis. Peptides were identified by the SEQUEST searching algorithm. Peptide digestion coverage was calculated by dividing the number of amino acids in the identified peptides by the total number of amino acids in the protein; (B) 40 pmol of cytochrome *C* was injected onto an on-line activated pepsinogen column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 0 °C. The resulting peptides were eluted with a 20 min reverse-phase gradient into a linear ion trap mass spectrometer and subjected to MS/MS product analysis. Peptides were identified by the SEQUEST searching algorithm. Peptide digestion coverage was calculated by dividing the number of amino acids in the identified peptides by the total number of amino acids in the protein.

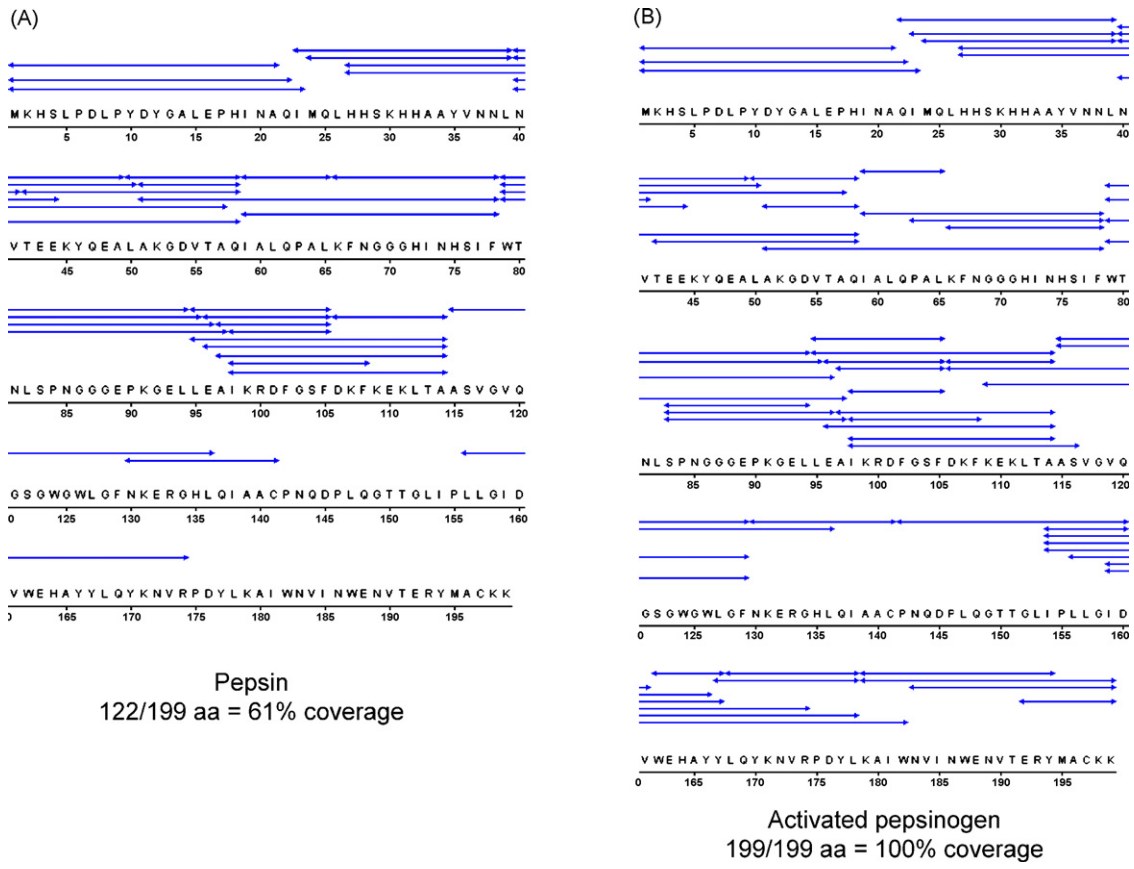

Fig. 8. Evaluating peptide digestion coverage of MnSOD between pepsin and activated pepsinogen columns using LC-ESI-MS/MS under slow exchange conditions: (A) 40 pmol of MnSOD was injected onto an on-line activated pepsin column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 0 °C. The resulting peptides were eluted with a 20 min reverse-phase gradient into a linear ion trap mass spectrometer and subjected to MS/MS product analysis. Peptides were identified by the SEQUEST searching algorithm. Peptide digestion coverage was calculated by dividing the number of amino acids in the identified peptides by the total number of amino acids in the protein; (B) 40 pmol of MnSOD was injected onto an on-line activated pepsinogen column connected to a C_{18} analytical column and allowed to digest for 3 min at a flow rate of 200 μL/min at 0 °C. The resulting peptides were eluted with a 20 min reverse-phase gradient into a linear ion trap mass spectrometer and subjected to MS/MS product analysis. Peptides were identified by the SEQUEST searching algorithm. Peptide digestion coverage was calculated by dividing the number of amino acids in the identified peptides by the total number of amino acids in the protein.

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