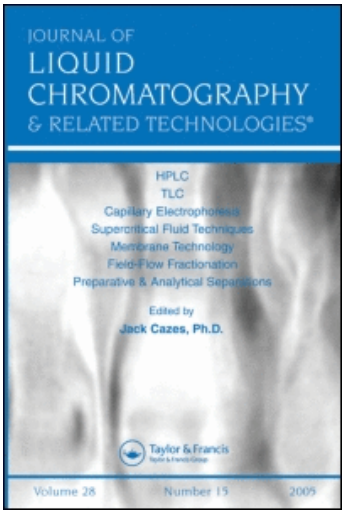

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Two Glutaraldehyde-Immobilized Trypsin Preparations for Peptide Mapping by Capillary Zone Electrophoresis, Liquid Chromatography, and Mass Spectrometry

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Abstract: Trypsin was immobilized using glutaraldehyde either by covalent attachment to aminopropyl controlled pore glass (CPG) or by direct crosslinking without a carrier. As peptide mapping is a comparative method, reproducibility of the analytical separation techniques (liquid chromatography, HPLC, and capillary zone electrophoresis, CZE) and the proteolyses resulting from both enzyme preparations were evaluated. Elution time reproducibilities of 0.3 and 0.6% were found for HPLC and CZE maps, respectively. Proteolysis reproducibility was tested for each trypsin preparation and compared with solution phase proteolysis. Sequence coverages of *ca* 65% were obtained from matrix-assisted laser desorption/ionization−time-of-flight (MALDI-TOF) mass spectral mapping for the two solid phase preparations.

Keywords: Trypsin immobilization, Glutaraldehyde, Crosslinking, Controlled pore glass, Peptide mass mapping

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INTRODUCTION

Peptide mapping is a comparative technique, primarily used for identifying protein modifications, in which enzymatic methods or chemical agents are used to break down proteins into a number of smaller, discrete peptide fragments. Trypsin, which cleaves peptide bonds selectively on the C-terminal side of lysine and arginine residues in a protein, is the most widely used proteolytic enzyme. It is a well characterized serine protease with high specific activity.[1] Liquid chromatography (HPLC) has been the most popular separation technique for generating peptide maps because of its robustness and reproducibility,[2] even though columns are expensive and solvent consumption is high. Capillary zone electrophoresis (CZE), a complementary technique to HPLC, is increasingly attractive because of its simplicity, its aqueous, thus friendly, environment for biomolecules, high separation efficiency, and low consumption of solvent and sample,[3] even though concentration detection sensitivity is not as good as with HPLC. Mass spectrometry (MS) has become the method of choice for rapid peptide mass mapping, primarily for protein identification,[4,5] due to its high resolution and good detection sensitivity. Soft ionization methods, like matrix assisted laser desorption/ionization−time-of-flight (MALDI-TOF) MS, are often employed for the investigation of biomolecules, like the tryptic peptides of isolated proteins. However, not all peptide fragments are detected 100% of the time, unlike with CZE and HPLC, thus MALDI-TOF MS is a used as a complimentary mapping technique.

Immobilized enzymes, defined as enzymes with restricted mobility, include enzymes converted to a water insoluble form by a suitable technique.[6] Their advantages over soluble enzymes or alternative technologies include reusability and easy handling, because they can be separated from the medium by filtration or centrifugation. They also present limited enzyme autolysis and the ability of continuous operation in bioreactors.[7] With the growing need of high throughput and better sensitivity for protein characterization, immobilized proteolytic enzymes are attractive because miniaturized enzyme reactors are easy to fabricate and their flow through design permits automation, reduced sample handling, and rapid proteolysis.[8–12]

Immobilization can be achieved by physical or chemical methods.[13] Physical means of enzyme immobilization include sol-gel entrapment,[14,15] adsorption to modified silica gel via immobilized metal ligands,[16] adsorption to mesoporous silicates,[17] and microencapsulation,[18] to name a few. Chemical methods imply the formation of at least one covalent bond between an amino acid residue of the enzyme and a functionalized support or carrier, or between two or more enzyme molecules using a crosslinking agent. These methods lead to immobilized enzymes with high operational stability because of their resilience to disruption by substrate, by high salt solutions, by organic modifier, etc.[7] Immobilized enzymes often exhibit improved

stability, presumably because of their restricted mobility; however, their activity may not be as high as the free, soluble form of the enzyme. Several forms of carrier, or scaffold, are used for immobilization of proteolytic enzymes. Commercial preparations of trypsin bound to particles of porous silica, agarose, or polymeric materials have been available for many years. Examples of some other scaffolds include silica or polymeric monoliths,[19–21] polyadimethylsiloxane) microfluidic devices,[22] polyethylene plates,[23] magnetic particles,[24] and fused silica capillary surfaces.[25,26] Carrier free covalent methods include the formation of crosslinked enzyme crystals and crosslinked enzyme aggregates (CLEAs).[27] Among the considerable number of agents available for covalent immobilization of enzymes described in the literature,[18,28,29] glutaraldehyde (GA) has found widespread use and is the most commonly employed linking and crosslinking reagent. It reacts rapidly with -NH_2 groups at neutral pH; however, its chemical nature in aqueous solution and the reaction mechanisms of its different possible forms have been the subject of much debate.[30] While effective, many of the immobilization techniques listed above are either time consuming, require specialized equipment, rely on expensive materials, or involve synthesis of the carrier materials. Our goal has been to simplify the immobilization process and provide methods that are accessible to most laboratories. The basis for the present study is, thus, a better comparison between two simple GA based immobilization techniques.

In this work, we decided to use chromatographic and electrophoretic methods to qualitatively compare the peptide mapping results of trypsin immobilization by carrier-free GA crosslinking to that by GA mediated covalent attachment to aminopropyl controlled pore glass (CPG) particles. It was, therefore, necessary to assess the reproducibility of HPLC and CZE as analytical separation tools in peptide mapping, in order to make a direct comparison of the digestions obtained with the immobilized enzyme preparations. Chemically denaturated lysozyme was randomly chosen as a substrate for digestion by the two GA trypsin preparations. The corresponding MALDI-TOF mass spectra were examined and used to compare the lysates obtained with solution phase (soluble) trypsin, CPG-GA trypsin, and GA-crosslinked trypsin. Comparison of the two enzyme preparations by evaluation of their apparent kinetic parameters for hydrolysis of a small, synthetic substrate (*N*-α *p*-tosyl L-arginine methyl ester; TAME) has been previously reported by our group.[31]

EXPERIMENTAL

Reagents

Chicken lysozyme and bovine TPCK-treated trypsin (12,700 U BAEE/mg, Lot: 07K7251), from Sigma-Aldrich (St-Louis, MO), were used without

further purification. Aminopropyl Controlled Pore Glass (CPG, 80–120 mesh, 700 Å average pore size, 35 m^2/g specific surface area), iodoacetamide, *N*-α *p*-tosyl L-arginine methyl ester (TAME), and sodium cyanoborohydride ($NaBH_3CN$) were also from Sigma-Aldrich (St-Louis, MO). α-Cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma (St-Quentin-Fallavier, France) and was used after purification by recrystallization from ethanol. Glutaraldehyde (25% w/v, aqueous solution) and dithiothreitol (DTT) were from Aldrich (Milwaukee, WI). ZipTip® C18 microextraction pipette tips were obtained from Millipore (St-Quentin-en-Yvelines, France).

CZE Separation

CZE peptide maps were acquired on a P/ACE™ MDQ System (Beckman Coulter, Fullerton, CA) equipped with a UV detector monitored at 200 nm. All separations were performed at 25°C in bare fused silica capillary (Polymicro Technologies, Phoenix, AZ) having 50 μm ID × 360 μm OD, 60 cm total length, and 50 cm effective length. The applied voltage was 15 kV and the background electrolyte (BGE) separation buffer was 50 mM sodium phosphate, pH 2.5. The capillary was rinsed with 0.1 M NaOH, water, 0.1 M HCl, and finally with BGE between each separation. Injection of *ca*. 5 nL was accomplished by application of a differential pressure of 0.5 psi for 5 s at the inlet end of the capillary.

HPLC Separation

Peptides maps were obtained on a Hitachi D-7000 chromatographic system (Hitachi, San Jose, CA) using a 250 mm × 4.6 mm Jupiter C18 column (Phenomenex, Torrance, CA). Gradient elution was performed using 0.1% (v/v) trifluoroacetic acid (TFA) in water (eluent A) and 0.08% (v/v) TFA in acetonitrile (ACN) (eluent B). The gradient was from 5 to 35% B over 35 min and the mobile phase flow rate was set at 1 mL/min. The injection volume was 20 μL and the detection wavelength was 200 nm.

MALDI-TOF MS Analysis

Alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix was prepared fresh as a saturated solution in 50:50 ACN:0.1% TFA. Dried lysozyme digests were reconstituted in *ca*. 30 μL 1% formic acid (FA) and desalted using ZipTips®. The loaded ZipTip® was subjected to washing steps with 1% FA followed by a step type elution of the peptides from the tip with 50:50 (v/v) ACN:1% FA, then with 80:20 (v/v) ACN:1% FA. Prior to spotting

on the MALDI target by the dried droplet method, the dried, desalted samples were re-dissolved in 1% FA. The MALDI spectra were collected on a time lag focusing MALDI-TOF (reflectron positive mode) Voyager DE-STR Biospectrometry workstation (Applied Biosystems, Foster City, CA) equipped with a 337 nm N_2 laser. Sequence coverages were obtained using the ProFound database search engine, version 4.10.5.[32]

Trypsin Immobilization Procedures

Carrier-Bound Immobilization Technique: Covalent Binding to CPG Particles

The covalent coupling of TCPK treated trypsin to aminopropyl CPG particles and characterization of the product are described in Migneault et al.[31] Briefly, aminopropyl CPG particles were shaken for 2 h at room temperature with a solution of 1% GA in neutral sodium phosphate buffer. An aliquot of TPCK treated trypsin solution was added to the activated support in slightly alkaline sodium phosphate buffer containing $NaCNBH_3$ and allowed to react for 3 h at room temperature with gentle shaking. Then, unreacted aldehyde groups of the CPG-GA support were deactivated with glycine for 1 h. The CPG-GA trypsin particles were washed and stored at 4°C in neutral phosphate buffer. The protein content of the particles and the specific activity of the immobilized trypsin were determined by UV/Vis absorbance spectrophotometry (protein content 4.9 mg/g support; specific activity of CPG-GA trypsin: 25.8 U TAME/mg protein,[33] or 1483.8 U BAEE/mg protein, or 126.5 U TAME/g support corresponding to 7270.8 U BAEE/g support).

Carrier-Free Immobilization Technique: Crosslinking

Immobilization of trypsin by crosslinking with GA and characterization of the product are described in Migneault et al.[1] Briefly, a 2.5% aqueous GA solution was added dropwise, while stirring, to a TPCK treated trypsin solution in neutral sodium phosphate buffer. After a few minutes, the reaction was allowed to proceed without stirring at room temperature for 2 h. After centrifugation and washing to remove excess GA and non-covalently bound trypsin, the reaction was stopped by addition of a glycine solution in neutral sodium phosphate buffer and allowed to react for 3 h at room temperature. The product was washed and stored in water at 4°C. The protein content and the specific activity of the dried preparation were determined by UV-Spectrophotometry (protein content: 25 mg/g solid; specific activity of GA-crosslinked trypsin: 0.054 U TAME/mg protein,[33] or 3.1 U BAEE/mg protein, or 1.3 U TAME/g solid corresponding to 77.5 U BAEE/g solid).

Enzymatic Proteolysis

Homogeneous Tryptic Proteolysis

Solution phase (homogeneous) tryptic proteolysis was performed batch wise following the procedure described by Stone and Williams,[34] with slight modifications. Prior to proteolysis, lysozyme was chemically denaturated as follows: the protein substrate (8 mg/mL in 400 mM ammonium bicarbonate buffer, pH 8.0, containing urea) was reduced with DTT at 50°C for 15 min, and then alkylated with iodoacetamide at 25°C for 15 min. The protein solution was diluted to 2 mg/mL, then 40 μL trypsin was added to initiate proteolysis. The final enzyme to protein ratio was 1:25 (w/w) and hydrolysis proceeded at 37ºC for 24 h with gentle stirring. The reaction was stopped by acidification with concentrated HCl and the digest was analyzed immediately by CZE. For HPLC and MALDI-TOF MS analyses, the samples were vacuum dried and redissolved in water/TFA buffer (eluent A) and 1% FA, respectively. The concentration of tryptic peptides was the same for CZE and HPLC separations.

Solid Phase Tryptic Proteolysis

Lysozyme (2 mg/mL) was denatured as described above and proteolyses were performed batch wise in 50 mM buffer, pH 8.0 (ammonium bicarbonate for GA-crosslinked trypsin and Tris-HCl for CPG-GA-trypsin) for 2 h at 37°C with gentle stirring. The proteolysis reactions were stopped by centrifugation at 3000 rpm for 2 min, at which point the supernatants (digests containing the peptide fragments) were collected and analyzed immediately by CZE. The immobilized trypsin preparations were rinsed several times with buffer before reuse. Blank proteolyses were performed by incubation of the enzyme preparations with buffer only, under identical conditions. For HPLC and MALDI-TOF MS analyses, samples were vacuum dried and redissolved in water/TFA buffer (eluent A) and 1% FA, respectively.

RESULTS AND DISCUSSION

Routinely, proteolysis by soluble trypsin is performed either “in-gel” on a protein isolated by gel electrophoresis or in solution on protein mixtures (e.g., cell extracts). Both of these methods suffer from long incubation times, loss of catalytic activity, single use of the enzyme, and trypsin autolysis that leads to interfering background peptides during chromatographic separations and ion suppression during mass mapping. Immobilized enzymes can alleviate the drawbacks of soluble enzymes, providing economical and technical advantages. We have investigated two procedures for trypsin immobilization using the same homobifunctional agent, GA: by covalent

coupling to a solid support (covalent attachment to CPG particles) and by crosslinking (carrier-free method), which formed a solid product.

Reproducibility of HPLC and CZE Separations

Lysozyme was used in the peptide mapping studies to compare the proteolyses from the two GA-immobilized trypsin preparations. Lysozyme is a small, well characterized, 14.3 kDa protein containing 4 disulfide bridges, easily cleaved by denaturation and reduction/alkylation to give fourteen peptides and a few individual amino acids, the latter being undetected at 200 nm either by HPLC or CZE and having too low a mass for accurate MALDI-TOF MS detection.

Reproducibility is paramount to the successful application of peptide mapping when used as a comparative technique, so a high degree of elution time reproducibility is required. In HPLC, many factors can affect reproducibility, such as column and instrument performance, especially gradient reproducibility.[35] To test gradient reproducibility, three aliquots of the same solution phase tryptic digest of lysozyme were analyzed consecutively. A mean relative standard deviation (RSD) of less than 0.3% for all peaks was observed. For CZE, adsorption of proteins and peptides on the capillary wall is a serious problem, which can lead to variable migration times, band broadening, and tailing, especially for separations carried out in the intermediate-pH range. Therefore, a low pH separation buffer was employed to ensure minimum interaction between the cationic peptides and neutral wall of the bare fused silica capillary, as well as to profit from slight pK_a differences in the terminal carboxylate groups that help separate peptides having similar structures. To measure the stability of the CZE separation conditions, three analyses of the same digest were performed over a period of 24 h. Migration time reproducibility was $\leq$0.6% RSD for all peaks, a respectable value that was the result of careful consideration to temperature, washing protocols, and capillary wall equilibration with buffer before each separation. Thus, the HPLC and CZE methods both showed excellent performance for peptide mapping applications, as demonstrated by the high elution time reproducibility.

Proteolysis Reproducibility of Each Immobilized Trypsin Preparation by HPLC and CZE

Knowing that these separation techniques can attain at best 0.3% and 0.6% mean elution time RSD, respectively, the proteolysis reproducibility of the two immobilized trypsin preparations was evaluated by comparing peptide maps from three different proteolysis reactions carried out using the same batch of immobilized trypsin preparation. Three aliquots of lysozyme were digested sequentially within the same day, each for 2 h, under the same

conditions (pH and temperature) using a single batch of GA-crosslinked trypsin. Three additional aliquots were digested in the same manner using a single batch of CPG-GA trypsin. The six digests were stored at $-20^{\circ}C$ until their analyses by HPLC (Figures 1A and 1B) and CZE (Figures 1C and 1D) could be made consecutively on the same day.

Repeated use of the two immobilized trypsin preparations for lysozyme proteolysis gave very similar chromatographic (Figures 1A and B) and electrophoretic (Figures 1C and D) peptide maps. Migration time reproducibilities were of the same order as for soluble (solution phase) trypsin. Immobilized trypsin preparations have slower rates of denaturation or inactivation compared to the soluble enzyme and could be reused at least three times without loss of reproducibility. We are currently evaluating the longevity, effective shelf life, and synthesis repeatability of the two immobilized trypsin preparations.

Comparison of HPLC Peptide Maps of Lysozyme with Free and Immobilized Trypsin Preparations

HPLC peptide mapping is generally carried out by ion pairing reversed phase chromatography. The selectivity of reversed phase HPLC results from the relative polarity of the peptide side chain. Figure 2 shows the chromatographic peptide maps of denatured lysozyme digested by soluble trypsin (Figure 2A), GA-crosslinked trypsin (Figure 2B) and CPG-GA trypsin (Figure 2C). The soluble trypsin proteolysis (Figure 2A) was considered as the reference map for our study. In all three cases, more peaks than the expected 14 peptides were seen, presumably due to missed cleavages.

The results on proteolysis reproducibility, combined with the excellent mean RSD values for elution time, suggest that any observed differences in peptide maps would arise from differences in enzyme behavior due to the immobilization processes, such as substrate accessibility to bound trypsin. Moreover, the complete resolution of all peptides derived from a given enzymatic proteolysis is difficult to achieve using a single analytical technique. In general, only hyphenated methods (e.g., LC-MS) have the resolving power sufficient to separate almost all components in a complex mixture. Baseline separations and good peak shapes were achieved in Figure 2 for the most intense peaks, indicating a good choice of separation conditions. Figure 2 shows that almost the same peak pattern was obtained for the three proteolyses, although some extra peaks were observed for solution phase proteolysis. In fact, in Figure 2A, at least two more peaks, at 12.2 and 22.2 min, are seen compared to maps generated by the immobilized enzymes. For the two solid phase proteolyses, the peak profiles are quite similar except the peak at 14.2 min, which is missing for CPG-GA trypsin (Figure 2C) compared to GA-crosslinked trypsin (Figure 2B).

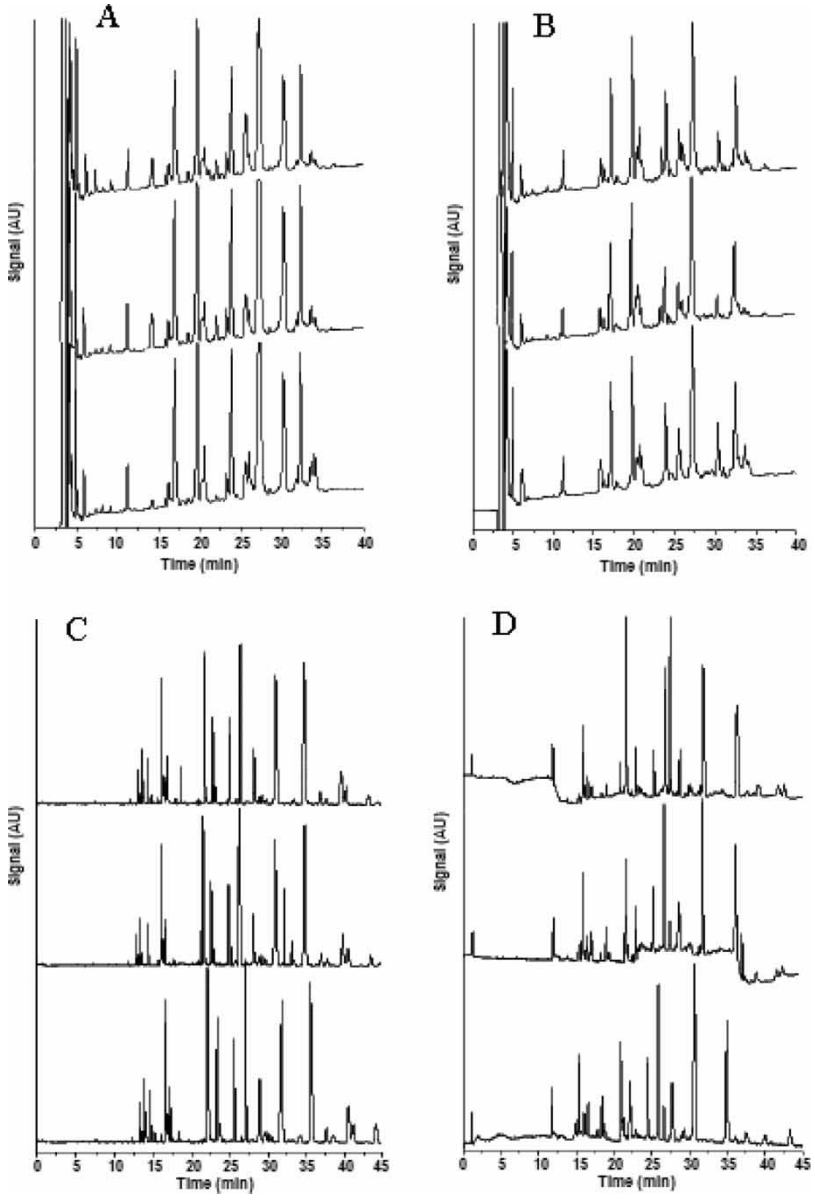

Figure 1. Peptide maps showing the proteolysis reproducibility associated with the two immobilization techniques for six aliquots of lysozyme: 3 aliquots digested sequentially by a single batch of GA-crosslinked-trypsin and 3 aliquots digested sequentially by a single batch of CPG-GA-trypsin, followed by a CZE and HPLC analysis of each aliquot. (A) HPLC maps for GA-crosslinked-trypsin; (B) HPLC maps for CPG-GA-trypsin; (C) CZE maps for GA-crosslinked-trypsin; (D) CZE maps for CPG-GA-trypsin.

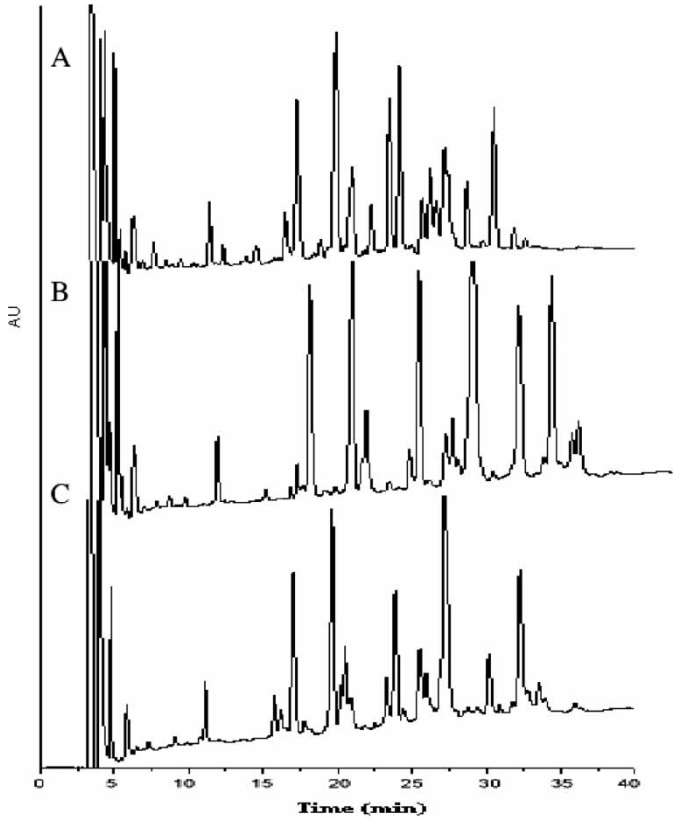

Figure 2. Chromatograms showing the peptide maps of chemically denaturated lysozyme (2 mg/mL) obtained by (A) a 24 h proteolysis with soluble trypsin (enzyme:substrate ratio = 1:25); (B) a 2 h solid-phase proteolysis with GA-crosslinked-trypsin; (C) a 2 h solid-phase proteolysis with CPG-GA-trypsin. The three separations were carried out on the same day.

Comparison of CZE Peptide Maps of Lysozyme with Free and Immobilized Trypsin Preparations

Separation of peptides by CZE is based on the net charge to Stokes's radius ratio, thus leading to different, but complementary, peptide maps compared to HPLC. Figure 3 shows the peptide maps obtained with soluble and immobilized trypsin preparations. The soluble trypsin proteolysis (Figure 3A) was considered as a reference for our study, even though the CZE map showed more peaks than expected, like that seen with the HPLC maps.

The three maps show baseline separations and sharp peak shapes, indicating good resolution and efficiency. The possibility of trypsin autolysis was monitored for the two immobilized trypsin preparations by doing a blank

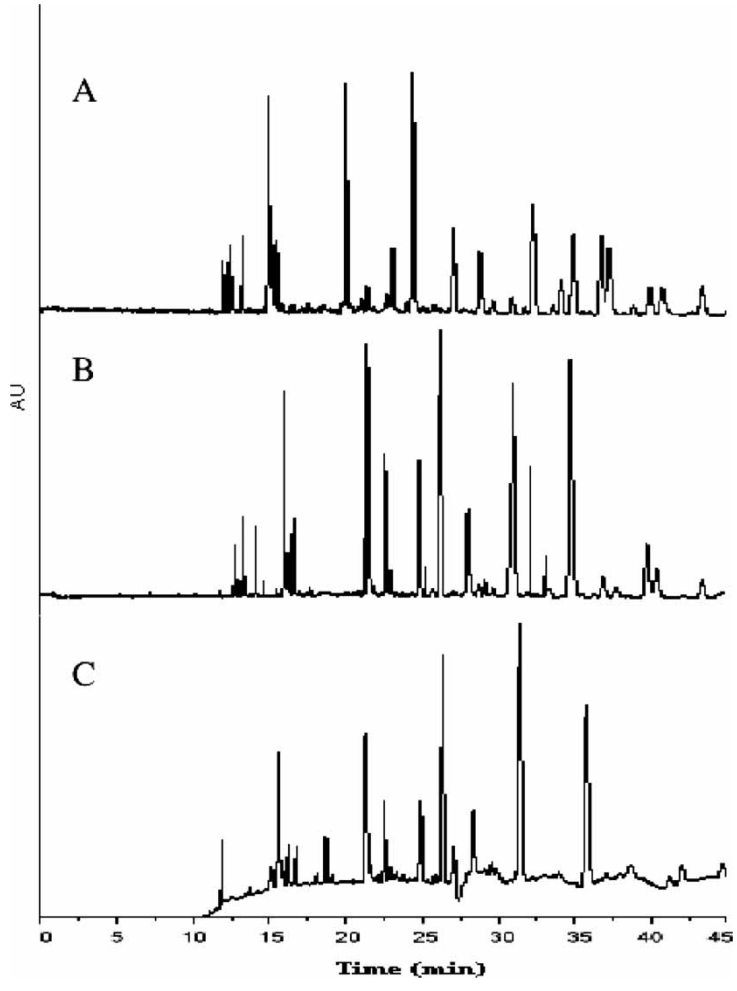

Figure 3. Electropherograms showing the peptide maps of the same three samples described in Fig. 2: (A) a 24 h proteolysis with soluble trypsin (enzyme:substrate ratio = 1:25); (B) a 2 h solid-phase proteolysis with GA-crosslinked-trypsin; (C) a 2 h solid-phase proteolysis with CPG-GA-trypsin.

reaction (i.e., no lysozyme), and autolysis peaks for the two immobilized trypsin preparations were indistinguishable from background noise. Overall, the two immobilized trypsin preparations were able to produce in 2 h a digest similar to that carried out in the solution phase in 24 h, based on major peak patterns present in the three maps in Figure 3, even though a fairly high substrate concentration was used. This concentration (2 mg/mL) was chosen to test the immobilized trypsin preparations, so there would be no detection issues for HPLC and CZE. Studies using reduced substrate

concentrations are currently underway to address the sensitivity needed for many proteomics applications.

A comparison of the electropherograms from the two solid phase digests (Figures 3B and C) reveals close resemblances, although some differences are noteworthy; several peaks between 10 and 13 minutes are missing in the CPG-GA trypsin map (Figure 3C), whereas two additional peaks are found at 18.7 and 27.1 min. These differences are not due to reproducibility of the CZE separations, but are likely a result of differences in trypsin activity, substrate accessibility, and the presence and nature of the CPG carrier in the immobilized preparations. For example, based on the calculated activities and protein loading for the two enzymatic preparations, Figure 3B results from the proteolysis of lysozyme by *ca*. 20 U BAEE (GA-crosslinked trypsin), whereas Figure 3C represents proteolysis by *ca*. 350 U BAEE (CPG-GA trypsin). During batchwise proteolysis, stirring was gentle enough to avoid crushing the CPG particles, yet vigorous enough to disperse them. However, progressively over the 2-h proteolysis period, the relatively heavy CPG particles tended to clump together on one side of the reaction tube, whereas the lighter, crosslinked product was almost uniformly suspended in the reaction solution at all times. This behavior affects the accessibility of the substrate to the trypsin and renders the surface of contact quite different for the two types of immobilized enzyme.

Comparison of MALDI-TOF MS Data of Lysozyme Digested with Soluble and Immobilized Trypsin Preparations

Photometric detection in peptide mapping provides a fingerprint of the parent protein, which can only be compared to a second protein sample; little additional information about the protein is revealed. Conversely, MS allows more detailed characterization due to the mass information obtained. Peptide mass fingerprinting is an important tool for protein identification by matching the measured tryptic peptide accurate masses to theoretical values calculated from genomic or proteic databases.

MALDI-TOF mass spectra suffer from baseline noise namely due to matrix-related background peaks in the lower mass range ($m/z < 850$ Da). As a result, only peptides greater than *ca.* 850 Da can be observed and, thus, 5 of the 14 expected peptides for lysozyme were not considered in the evaluation of the performance of the immobilized trypsin preparations. Instruments using the delayed extraction principle and a reflectron allow high mass accuracy of 5 to 100 ppm for identification of proteins.[36] Therefore, we decided to use the criteria for an unambiguous protein match suggested by Jensen et al.[37] These criteria consider that a minimum of five peptides matched with a maximum allowed mass deviation of 50 ppm and sequence coverage of at least 15% provide a good protein identification. Table 1

summarizes the results obtained by MALDI-TOF MS for the proteolysis of lysozyme by soluble trypsin and the two immobilized trypsin preparations.

For the nine peptide fragments that fall within the scanned mass range, Table 1 shows the observed masses and the accuracy of these results with respect to the theoretically computed masses for soluble and immobilized trypsin preparations. Only the peptides matching the theoretical masses with a mass deviation of ≤ 50 ppm were considered. Table 1 shows that six of the nine theoretical peptides expected were found for the two immobilized trypsin preparations, plus two masses corresponding to peptides having a single missed cleavage site at a lysine residue for CPG-GA trypsin proteolysis. Two of the three undetected peptides are lysine terminated (m/z 893 and 1325), which may be due to the fact that MALDI peptide mass fingerprints of tryptic digests are known to be dominated by peptides containing C-terminal arginine residues.[38] Absence of the peptide at m/z 874, which is small and polar, may be explained by the ZipTip® sample preparation, which is used to eliminate interfering compounds like denaturing agents and salts, and can thus lead to the loss of small or polar peptides that do not effectively adsorb to the C18 phase.

Amongst the two immobilized trypsin preparations, the GA-crosslinked trypsin seems to digest lysozyme more completely compared to CPG-GA trypsin, for which two peptides containing missed cleavage sites were observed. This difference would not be due to the unequal number of units of trypsin (e.g., specific activity) added to the two reaction media because this was *ca.* 20 times higher for the CPG-GA trypsin. Moreover, the concentration of substrate was identical, as well as the reaction time, temperature, and buffer pH for the two immobilized trypsin preparations. Similarly, the difference in detected peptides is unlikely an effect of the composition of the buffer used because we observed very poor proteolysis using CPG-GA trypsin in ammonium bicarbonate versus in Tris-HCl. The different extents of proteolysis might be explained by the physical properties of the two enzymatic preparations (the lighter GA-crosslinked trypsin compared to the heavier CPG-GA trypsin, or differences in their porosity, and thus, surface area), which affect their ability to interact with the substrate, as noted previously for the chromatograms in Figure 2.

By comparing mass data for the solution phase versus solid phase proteolyses, similar results were found, except for the peptides at m/z 1675 and 2508, which were not detected in the soluble trypsin digest. This discrepancy was surprising, but probably not due to the proteolysis itself because peak profiles either by CZE or HPLC were almost the same for soluble versus immobilized trypsin preparations. Instead, there was possibly crystal inhomogeneity during the co-crystallization of sample and matrix during sample preparation.

Determination of primary sequence coverage (Table 2), expressed as the percentage of total amino acid residues of lysozyme accounted for, and as the number of peptides detected in the mass spectrum, was performed using

Table 1. MALDI mass data for denaturated lysozyme peptides from soluble, GA-crosslinked- and CPG-GA-trypsin digests

				Soluble trypsin			GA-crosslinked-trypsin			CPG-GA-trypsin		
Theoretical $[M+H]^+$ (Da)	Position	MC[b]	Sequence	Observed $[M+H]^+$ (Da)	Dev.[c] (Da)	Mass acc.[d] (ppm)	Observed $[M+H]^+$ (Da)	Dev. (Da)	Mass acc. (ppm)	Observed $[M+H]^+$ (Da)	Dev. (Da)	Mass acc. (ppm)
874.42	15–21	0	HGLDNYR									
893.42[a]	6–13	0	CELAAAMK									
993.40[a]	62–68	0	WWCNDGR	993.41	0.01	10	993.41	0.01	10	993.37	−0.03	30
1045.54	117–125	0	GTDVQAWIR	1045.56	0.02	19	1045.56	0.02	19	1045.51	−0.03	29
1325.63	22–33	0	GYSLGNWVCAAK									
1333.67[a]	115–125	1	CKGTDVQAWIR							1333.65	−0.02	15
1428.65	34–45	0	FESNFNTQATNR	1428.70	0.05	35	1428.68	0.03	21	1428.64	−0.01	7
1675.80	98–112	0	IVSDGNGM NAWVA WR				1675.85	0.05	30	1675.83	0.03	18
1753.84	46–61	0	NTDGSTDYGILQIN SR	1753.89	0.05	29	1753.89	0.05	29	1753.87	0.03	17
1803.90	97–112	1	KIVSDGNGM NAWV AWR							1803.93	0.03	17
2508.19	74–96	0	NLCNIPC SALLSSDI TASVNCAK				2508.32	0.13	52	2508.31	0.12	48

[a]Carbamidomethylated peptides; [b]MC: number of missed cleavages; [c]Deviation (Da) compared to theoretical masses; [d]Mass accuracy (ppm) evaluated as the ratio of the deviation (absolute value) from the theoretical mass $\times 10^6$.

Table 2. Comparison of lysozyme sequence coverages identified by profound v. 4.10.5

	Trypsin		
Sequence coverage	Soluble	GA-crosslinked	CPG-GA-
% of Amino acids	34	64	66
Number of peptides	4	6	8

ProFound, one of the many available sequence database search engines. As seen in Table 2, the two immobilized enzymes give similar results in terms of sequence coverage, the difference in number of peptides being due to missed cleavages in the case of CPG-GA trypsin. On the other hand, the lower results for soluble trypsin are probably related to an inhomogeneous co-crystallization during sample preparation. It is noteworthy that 16% of the sequence arises from very small peptides or amino acids, which are not seen by MALDI-TOF analysis. Furthermore, the CHCA background noise can potentially bury one or more low-mass peptides, like the peptide at m/z 874.

The results from Tables 1 and 2, showing a mean mass accuracy of less than 25 ppm and mean sequence coverage of 65% for the immobilized trypsin preparations, demonstrate that peptide mass fingerprinting with both solid phase trypsin preparations allowed reliable identification of lysozyme and is a good method to compare the performance of soluble trypsin to that of immobilized trypsin preparations.

CONCLUSIONS

Trypsin immobilization with GA, either by covalent linking to an insoluble carrier (CPG) or by crosslinking is a powerful strategy for peptide mapping studies. It enables the preparation of reliable and catalytically active enzyme preparations that can be used, and reused, for faster proteolysis of dissolved substrates than solution-phase and in-gel proteolysis. The combination of HPLC and CZE for the analysis of complex peptide mixtures is worthwhile because tryptic proteolysis results in a mixture of peptides of varying physicochemical characteristics. MALDI-TOF MS provides an extra dimension of information that is invaluable for identifying the peptides and confirming protein identity. HPLC and CZE conditions and instruments were highly reproducible (RSD of less than 1%), which validated the choice of peptide mapping as a means for comparison of the proteolyses obtained with the two immobilized trypsin preparations and with the soluble enzyme. Repeated use of the immobilized enzymes produced similar peak patterns, thus indicating good proteolysis reproducibility and a promising alternative to the use of soluble trypsin. Our current

research is now focused on evaluating the long term efficiency of immobilized trypsin preparations, the proteolysis of other substrates and the transfer to the microreactor format, for their application in proteomics.

ABBREVIATIONS

CAM: carbamidomethylation; CHCA: α-cyano-4-hydrocynnamic acid; CPG: controlled pore glass; FA: formic acid; GA: glutaraldehyde; TAME: *N*-α *p*-tosyl-L-arginine methyl ester.

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