Comparison of Two Glutaraldehyde Immobilization Techniques for Solid-Phase Peptide Mapping by CE, HPLC and MALDI-TOF/MS

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INTRODUCTION

The first stage in peptide mapping consists of chemical or enzymatic cleavage of a protein into specific peptides in order to obtain its fingerprint. To address the need for higher throughput in proteomics, fast enzymatic digestions and efficient analysis techniques like capillary electrophoresis (CE), liquid chromatography (HPLC) and mass spectrometry (MS) are essential. Immobilized enzymes, defined as enzymes with restricted mobility, offer technical and economical advantages over soluble enzymes for protein digestion. Enzymes can be immobilized by a variety of techniques. Glutaraldehyde is the reagent used in the following two methods of interest to us for protein digestion applications: 1) covalent attachment onto a support like controlled-pore glass (CPG) particles, and 2) cross-linking of enzyme molecules. In this study, we first measured the kinetic properties of the two solid-phase trypsin preparations and those of the free (soluble) trypsin. Second, we compared the CE, HPLC and MALDI-TOF/MS peptide maps of the two glutaraldehyde-linked preparations (i.e., CPG-glutaraldehyde-trypsin and cross-linked trypsin) with free trypsin for the chemically denaturated standard proteins lysozyme and hemoglobin.

MATERIALS

Enzyme : bovine trypsin (EC 3.4.21.4)

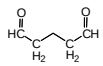
- Endoprotease of 23 800 Da (223 residues with 5 disulfide bridges)
- TPCK-treated to avoid chymotryptic activity (from Sigma Co.)
- High cleavage specificity (hydrolyses peptide bonds only on the C-terminal side of arginine and lysine residues) and high specific activity
- Creates medium-size peptide fragments
- Limited stability in solution as the result of autolysis

Carrier : Controlled Pore Glass (CPG)

- Inorganic support with good mechanical strength and immune to biological degradation
- Particle size: 125-177 µm (80-120 mesh) with 700 Å ave. pore size
- Thermostable and inert to changing conditions except at very alkaline pH
- High surface area (specific surface area = 35 m^2/g) thus high ligand coupling yield
- Surface must be derivatized with reactive functional groups (eg. amino propyl) for covalent binding

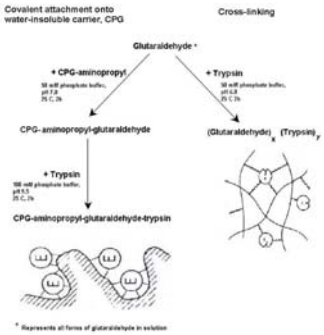
The linker/cross-linker : Glutaraldehyde

- Low molecular weight (100 g/mol)
- Homobifunctionnal reagent
- Inexpensive, easy to use
- Mild reagent
- High reactivity toward NH_2 groups at near neutral pH
- Commercial aqueous solutions (25%) from Sigma Co.
- Reaction mechanism with protein is still a subject of debate



METHODS

1) Trypsin immobilization



2) Trypsin characterization and utilisation

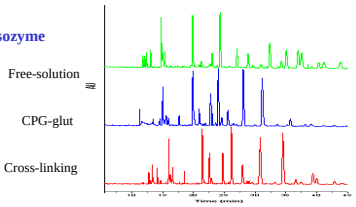
- Quantification of bound trypsin by UV-Vis absorption at 280nm for CPG-trypsin and the 4th derivative of the UV-Vis spectra for cross-linked trypsin
- Kinetic parameters (esterase activity) tested with the artificial peptide-like substrate Tosyl L-Arginine Methyl Ester (TAME, Sigma) at 247 nm
- Batch digestion of 2 µg/µL protein standards (lysozyme and hemoglobin) denaturated by urea, dithiothreitol and iodoacetamide
- Analysis of digests by CE, HPLC and MALDI-TOF/MS to obtain tryptic peptide maps and comparison with free trypsin digests

4) Peptide maps by capillary electrophoresis (CE)

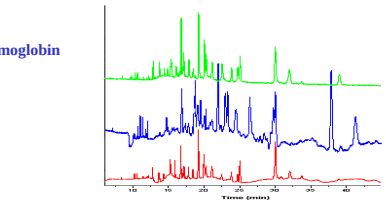
Digestions conditions : pH 8, 50mM ammonium bicarbonate (cross-linked trypsin) or 50mM TRIS buffer (CPG-trypsin) for 2h at 37°C. Free-solution digestion was performed in the same conditions, with a ratio of enzyme to protein of 1:25 for 24h.

Separation conditions : 50 µm i.d. bare f.s. capillary (L_d =50 cm) with 50mM phosphate buffer, pH 2.5, 15kV, detection at 200nm, injection by pressure (0.5 psi x 5s), no sample preconcentration.

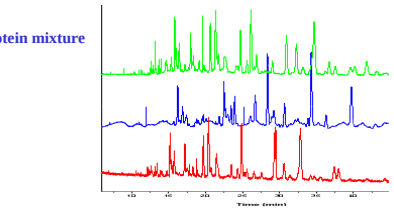
Lysozyme



Hemoglobin



Protein mixture

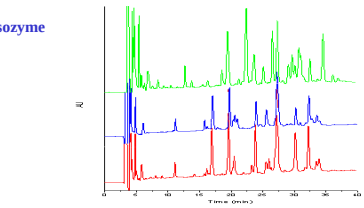


5) Peptide maps by high-performance liquid chromatography (HPLC)

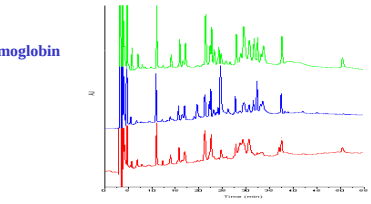
Digestions were performed as above.

Separation conditions : Phenomenex Jupiter 300 Å C18 column, 5µm, 4.6 x 250 mm. (A) 0.1% TFA/H_2O; (B) : 0.08% TFA/ACN. Gradient elution from 5-35% B at 35 min. Flow rate : 1.0 mL/min. Monitoring wavelength : 200 nm. Injection volume: 20 µL.

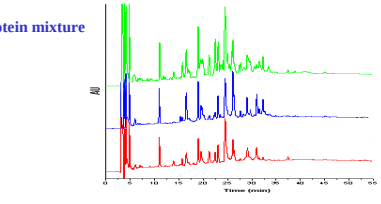
Lysozyme



Hemoglobin



Protein mixture

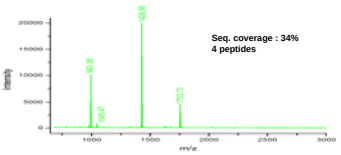


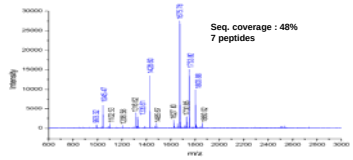
6) Peptide maps by MALDI-TOF/MS

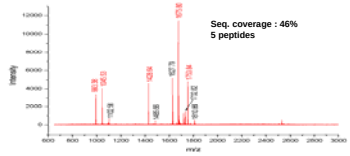
Digestions were performed as before. Approx. 35 µL of digest was desalted/preconcentrated using a C18 ZipTip (Millipore) and reconstituted in 5 µL of 1% formic acid.

Analysis conditions: dried droplet sample preparation was used: 0.5 µl sample in 1% FA + 0.5 µl CHCA matrix (saturated solution in 1:1 ACN:0.1%TFA). Spectra were acquired on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) in reflector mode. Acquisition mass range was 500 or 650 to 4000 m/z.
Sequence coverage was obtained with ProFound 4.10.5 (NCBInr for lysozyme and Swiss-Prot for hemoglobin).

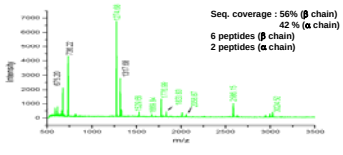
Lysozyme

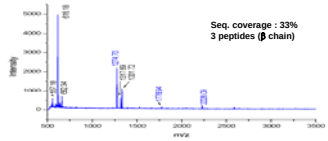




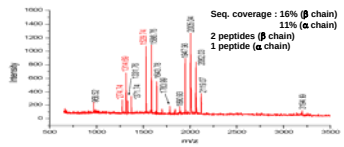


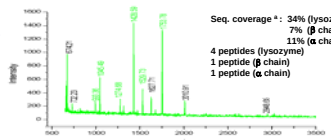
Hemoglobin

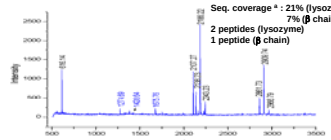


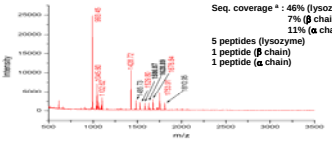


Protein mixture









[a] Sequence coverage was determined by hand because Profound, Mascot and EMBL search engines could not identify the proteins in the mixture (0% sequence coverage) using either NCBInr or Swiss-Prot nr databases.

RESULTS

1) Comparison of the immobilization method conditions

Immobilization Technique	No. of Steps	Time required for preparation		Temp. (C)	Buffer
		Enzyme immob.	To utilization		
CPG-Glutaraldehyde	2	120	405	25	phosphate pH 7.0
Cross-linking	1	120	420		phosphate pH 7.0

2) Enzyme content

Immobilization technique	Immobilization efficiency (%)	Trypsin content (mg/g preparation)
CPG-Glutaraldehyde	68	4.9
Cross-linking	> 95	25

3) Kinetic characterization• of immobilized trypsin preparations

	Trypsin		
	Free	On CPG (solid support)	Cross-linked
		Glutaraldehyde	Glutaraldehyde
Optimum pH	7 to 9	8	8
$K_{M,app}$ (10^{-6} M)	71	190	160
$V_{M,app}$ (10^{-3} M/min)	30	0.79	46
Specific activity (%)	100	11	52

•Apparent kinetic parameters (because of the solid-phase nature of the enzyme after modification) determined with TAME (Tosyl L-Arginine-Methyl Ester) as artificial peptide-like substrate

CONCLUSIONS

- Time (< 4h digestion) and cost savings (enzyme reusability)
- Limited enzyme autolysis, leading to simplification of peptide maps
- Easy separation from the digestion medium by filtration, centrifugation or flow-through format
- Possibility of on-line operation with a separation technique like CE, CE-MS, LC or LC-MS for peptide mapping or peptide mass mapping

ACKNOWLEDGEMENTS

I. Migneault thanks FCAR (Fonds pour la Formation de Chercheurs et Aide à la Recherche du Québec) for a graduate student scholarship. Financial support and equipment (CE, HPLC) were provided by NSERC (Natural Sciences and Engineering Research Council of Canada).