Comparison of Trypsin Immobilization Techniques With or Without a Solid Support for Peptide Mapping

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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 two methods of interest to us for protein digestion applications, which are covalent attachment onto a support like controlled-pore glass (CPG) particles and cross-linking of enzyme molecules. In this presentation, we first measure the kinetic properties of the two solid-phase trypsin preparations and those of the free (soluble) trypsin. Second, we compare 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 and METHODS

- Bovine trypsin (Sigma) was immobilized onto aminopropylated CPG particles (Fig. 1a) or cross-linked with glutaraldehyde (Fig. 1b).
- The inorganic support (CPG, Sigma) has good mechanical strength and resists biological degradation. These particles of 125-177 µm with 700Å average pore size have a high surface area (35 m^2/g), are thermostable and inert except at very alkaline pH.
- Glutaraldehyde (25% in aqueous solution, Sigma) is a small, homo-bifunctionnal reagent with high reactivity toward NH_2 groups at near neutral pH. While it is used extensively for tissue fixation, its reaction mechanism with proteins is still a subject of debate.
- Quantification of bound trypsin was made by measuring the UV-Vis absorption at 280nm for the solid support techniques, and by using the 4th derivative of the UV-Vis spectra to quantify trypsin cross-linked with glutaraldehyde. Immobilized enzymes were tested for esterase activity with the artificial peptide-like substrate Tosylarginine methyl ester (TAME, Sigma) using an absorbance assay at 247 nm.
- The protein standards (140 pmol/µl each) were prepared in solution and chemically denatured (urea, dithiothreitol, iodoacetamide) before batch digestion with either the solid-phase or cross-linked trypsin preparations employing glutaraldehyde.
- Tryptic peptide maps were obtained by CE, HPLC and MALDI-TOF/MS and compared to free trypsin digestions for the two different glutaraldehyde immobilization procedures.

Fig. 1a

- DITC (Diisothiocyanate)-trypsin

- EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)-trypsin

- Glutaraldehyde-

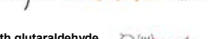


Fig. 1b

- Trypsin cross-linked with glutaraldehyde

RESULTS

1) Comparison of the immobilization method conditions

Immobilization Technique	No. of Steps	Time required for preparation: Enzyme immob.	Time required for preparation: To utilization	Temp. (C)	Buffer	Immobilization Efficiency (%)
CPG-DITC	1	75	240	25	carbonate pH 9.5	60
CPG-EDC	2	180	375		phosphate pH 7.0	60
CPG-Glutaraldehyde	2	120	405		phosphate pH7.0	53
Cross-linking	1	120	420		phosphate pH7.0	≥ 95

2) Kinetic characterization of immobilized trypsin preparations

	Trypsin: Free	On CPG (solid support): DITC	On CPG (solid support): EDC	On CPG (solid support): Glutaraldehyde	Cross-linked: Glutaraldehyde
Optimum pH	7 to 9	8	8	8	8
K_M (µM)	21.4	44,4	190	40	n/a
V_M (µM/min)	11	10,2	20	4.2	n/a
Specific activity (%)	100	39,3	64,8	14.8	n/a

n/a : These results are not available for the moment

3) Expected theoretical tryptic fragments

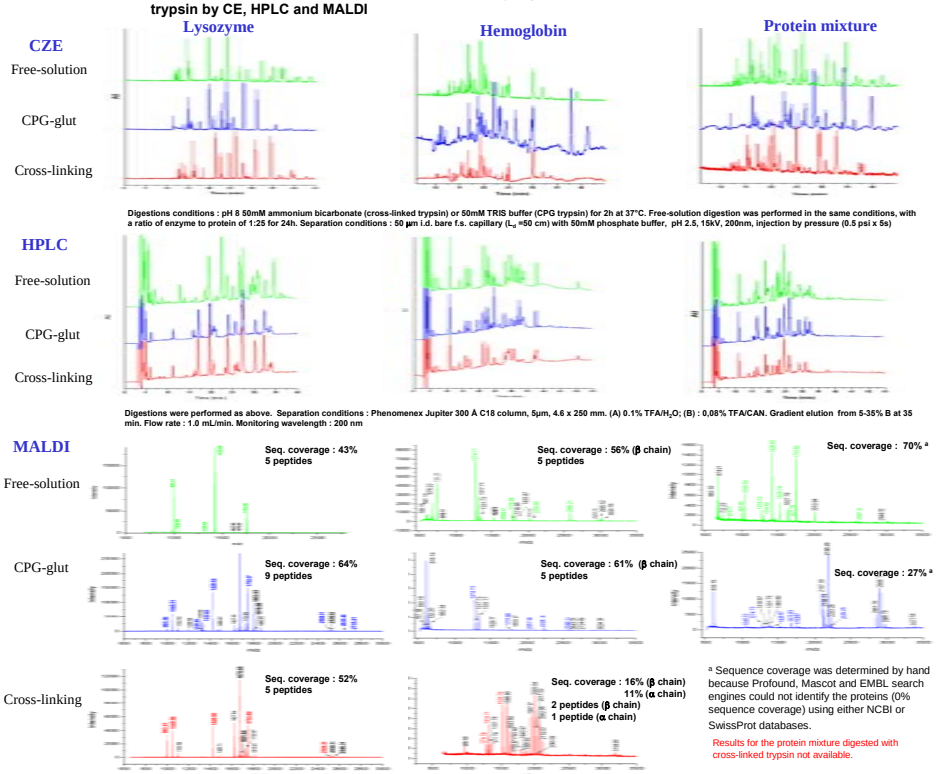
Lysozyme (Chick)

Mass (Da)	Position	Peptide Sequence
2508.1891	74-96	NLCNIPCSALLSSDITASVNCAK
1753.8351	46-61	NTDGSTDYGILQINSR
1675.8009	98-112	IVSDGNGMNAWVAWR
1428.6502	34-45	FESNFNTQATNR
1325.6306	22-33	GYSLGNWVCAAK
1045.5425	117-125	GTDVQAWIR
993.3995	62-68	WWCNDGR
874.4166	15-21	HGLDNYR
893.4219	6-13	CELAAAMK
517.2729	69-73	TPGSR
478.2772	2-5	VFGR
392.1710	126-128	GCR
289.1619	113-114	NR
307.1434	115-116	CK
175.1189	14-14	R
147.1128	1-1	K
147.1128	97-97	K
132.1019	129-129	L

Hemoglobin (Human)

Chain α: Mass (Da)	Position	Peptide Sequence	Chain β: Mass (Da)	Position	Peptide Sequence
3024.6338	100-127	LLSHCLLVTLAAH LAVHASLDK	2058.9477	41-59	FFESFGDLSTPDA VMK
2996.4894	62-90	VADALTNAVAHV DALSDLHAHK	1776.9941	105-120	LLGMVLVCVLAHH FGK
1833.8918	41-56	TYFPHFDLSHGSA QVK	1669.8907	67-82	VLGAFSDGLAHLD NLK
1529.7342	17-31	VGAHAGEYGAEA LER	1478.6944	83-95	GTFATLSELHCDK
1252.7147	128-139	FLASVSTVLTSK	1378.7001	121-132	EFTPPVQAAYQK
1071.5543	32-40	MFLSFPTTK	1314.6648	18-30	VNVDEVGGEALG R
818.4406	93-99	VDPVNFK	1274.7255	31-40	LLVVYPWTQR
729.4141	1-7	VLSPADK	1149.6738	133-144	VVAGVANALAHK
532.2878	12-16	AAWGK	1126.5639	96-104	LHVDPENFR
461.2718	8-11	TNVK	952.5098	1-8	VHLTPEEK
398.2146	57-60	GHGK	932.5200	9-17	SAVTALWGK
338.1823	140-141	YR	412.2303	62-65	AHGK
288.2030	91-92	LR	319.1401	145-146	YH
147.1128	61-61	K	246.1812	60-61	VK
			147.1128	66-66	K

4) Peptide maps of denaturated proteins obtained by digestion with free and immobilized (CPG and cross-linked) trypsin by CE, HPLC and MALDI



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, 200nm, injection by pressure (0.5 psi x 5s)

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/CAN. Gradient elution from 5-35% B at 35 min. Flow rate : 1.0 mL/min. Monitoring wavelength : 200 nm

Digestions were performed as above. Analysis conditions: dried droplet sample preparation was used: 0.5 µl sample in 1% formic acid + 0.5 µl CHCA matrix (saturated solution in 1:1 acetonitrile: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).

[a] Sequence coverage was determined by hand because Profound, Mascot and EMBL search engines could not identify the proteins (0% sequence coverage) using either NCBI or SwissProt databases.

Results for the protein mixture digested with cross-linked trypsin not available.

CONCLUSIONS

- Time saving for digestion : 24 h in solution (free enzyme) versus <4 h with an immobilized enzyme
- Limited enzyme autolysis, leading to simplification of peptide maps
- Easy separation from the digestion medium by filtration, centrifugation or flow-through format
- Cost saving because of possible reusability of immobilized enzyme
- Possibility of on-line operation with a separation technique like CE, CE-MS, LC or LC-MS for peptide mapping or peptide mass mapping
- Cross-linking technique shows a higher immobilization yield but a lower specific activity than covalent binding technique
- Cross-linked enzyme is less sensitive to pH than covalent binding technique

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