IMMOBILIZED TRYPSIN REACTORS FOR PEPTIDE MAPPING : TOWARD THEIR INTEGRATION WITH POLYACRYLAMIDE GEL PROTEIN SEPARATIONS

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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. Since small amounts of proteins of interest are typically isolated and purified from biological samples by polyacrylamide gel separation, a rapid, reliable technique should be established to get peptide maps from nanomole and lower quantities of proteins. To address the need for higher throughput in proteomics, fast enzymatic digestions and efficient analysis techniques like capillary electrophoresis, liquid chromatography and mass spectrometry 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 [1,2]. The two methods of interest to us for protein digestion applications are: covalent attachment onto a water-insoluble support like controlled-pore glass beads and cross-linking with a bifunctional reagent like glutaraldehyde [3]. Among the techniques requiring a solid support, three different linking chemistries have been studied : 1,4-diisothiocyanate (DITC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and glutaraldehyde. Quantification of bound trypsin was made by directly measuring the UV-Vis absorption at 280 nm for the solid support techniques, and by using the fourth derivative of the UV-Vis spectra to quantify trypsin immobilized by cross-linking with glutaraldehyde. Immobilized enzymes were tested for esterase activity with an artificial peptide-like substrate using an absorbance assay at 247 nm [4]. Tryptic maps were obtained by CE and MALDI-TOF/MS for protein standards prepared in solution.

MATERIALS

The enzyme : bovine trypsin (EC 3.4.21.4)

- Serine protease with 223 amino acids (bovine)
- Optimal pH of free enzyme between 7 and 9
- Creates medium-sized peptide fragments by hydrolysis at C-terminal side of arginine and lysine residues
- Limited stability in solution as a result of autolysis
- Creates positively charged peptide fragments which facilitates MS detection
- Numerous databases available for protein identification

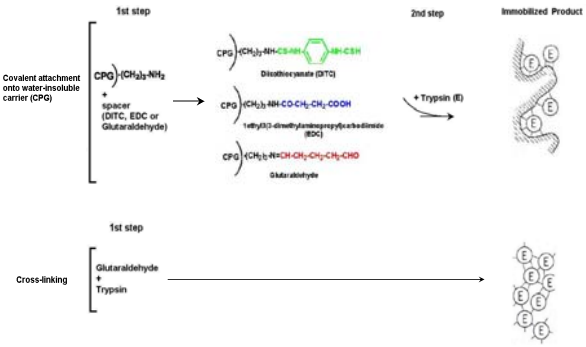
Carrier: Controlled Pore Glass (CPG)

- Inorganic support produced from borosilicate-based material with good mechanical strength; immune to biological degradation
- Particle size: 125-177 mm (80-120 mesh) with 700 Å ave. pore size
- Thermostable and autoclavable
- Inert to changing conditions except at very alkaline pH
- High surface area (specific surface area = 35 m2/g) thus high ligand coupling yield
- Surface must be derivatized with reactive functional groups for covalent binding

Linkers or cross-linking agent :

- DITC (Diisothiocyanate) with aminopropylated CPG
- EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) with aminopropylated CPG
- Glutaraldehyde with aminopropylated CPG
- Glutaraldehyde for direct cross-linking with trypsin

IMMOBILIZATION TECHNIQUES



RESULTS

1) Comparison of the immobilization method conditions

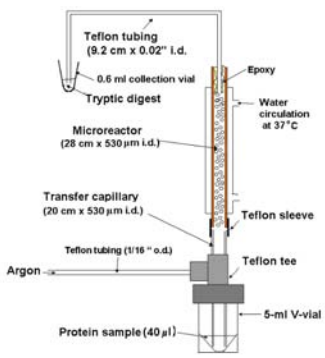
Immobilization Technique	No. of Steps	Time required for preparation		Temp. (°C)	Buffer	Immobilization Efficiency (%)
		Enzyme immob.	To utilization			
CPG-DITC	1	75	240	25	carbonate pH 9.5	60
CPG-EDC	2	180	375		phosphate pH 7.0	60
CPG-Glutaraldehyde	2	120	405		phosphate pH 7.0	53
Cross-linking	1	120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

2) Kinetic characterization of immobilized trypsin preparations

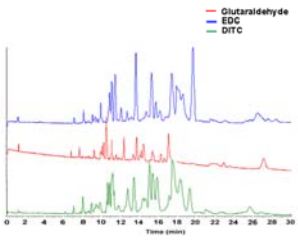
	Trypsin				
	Free	On CPG (solid support)			Cross-linked
		DITC	EDC	Glutaraldehyde	Glutaraldehyde
Optimum pH	7 to 9	8	8	8	8
K_M (µM)	21.4	44,4	190	40	n/a
V_M (µM/min)	11	10,2	20	4.2	n/a
Specific activity (%)	100	39,3	64,8	14.8	n/a

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

3) Microreactor design [5]



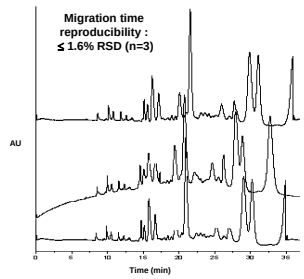
4) Resulting β-casein digestions in microreactor format



Sample: 40 µl β-casein (5 mg/ml) + 80 µl wash with digestion buffer
Separation: PACE/MDQ Beckman Coulter; 15kV; 50 mM sodium phosphate, pH 2.5
Capillary: 75 µm I.D., 360 µm O.D., L_t= 60 cm; L_d = 50 cm
Injection: 0.5 psi x 5s; Detection: λ = 200 nm.

5) Analysis of β-casein digests from the CPG-DITC-Trypsin microreactor

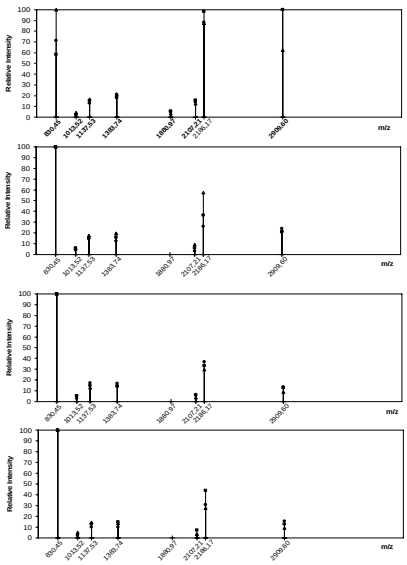
5.1 Reproducibility of CE peptide maps



The same sample (β-casein digest plus microreactor wash) was run 3 times within 24 hr. Separation conditions: 75 µm ID, 60 cm total L, 50 cm effective length, bare f.s. capillary. Run: 50 mM phosphate, pH 2.5, injection 0.5 s by pressure, 15 kV, detection at 200nm.

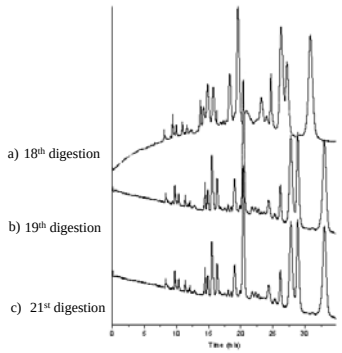
5.2 Reproducibility of MALDI peptide maps for CPG-DITC-trypsin

- Digestion of β-casein (50 pmol/µL)
- CPG-DITC-trypsin
- Digestion buffer: 50 mM ammonium carbonate, pH 8.0
- Digestion time: 6 h
- 4 aliquots of the same digest



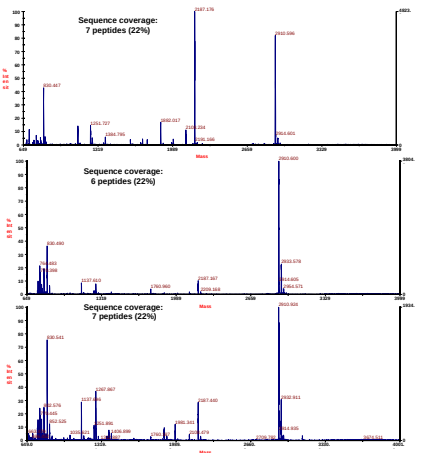
- Same 4 aliquots dried, reconstituted in 1% formic acid to give 130 pmol/µL final concentration for MALDI-TOF analysis (0.5 µL sample + 0.5 µL CHCA matrix);
- 3 spectra acquired per aliquot
- No trypsin peaks observed; no keratin observed; β-casein peaks at 830.4 Da, 1013.5 Da, 2186.1 Da, 2909.6 Da to give 18% sequence coverage. (1137.5 Da for B-variant of β-casein)

5.3 Reusability: CE peptide maps for sequential digestions carried out in the same CPG-DITC-trypsin microreactor



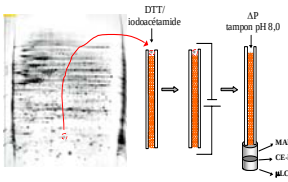
Three different β-casein samples (2.9x10^{-4}M, from same lot) were digested in the same microreactor and stored. As example, 18th, 19th and 21st digestions are presented. Separation conditions: same as above.

5.4 Reusability: MALDI peptide maps for sequential digestions carried out in the same CPG-DITC-trypsin microreactor



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.

6.0 Integration with gel electrophoresis



- Gel-separated proteins are chemically denatured the electromigrated into an immobilized enzyme reactor (IMER) for digestion.
- Experiments are still underway.

CONCLUSIONS

- Immobilization methods with or without a solid support are convenient
- Time saving for digestion (<4h depending on protein)
- Cost saving because of possible reusability of immobilized enzymes
- Possibility of on-line operation with a separation technique like CE, CE-MS, LC or LC-MS for peptide mapping or peptide mass mapping
- Variability in relative peak intensity for MALDI-TOF/MS peptide mapping is observed ⇒ automated MALDI analysis may help to reduce this problem

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