Optimization of Enzyme Immobilization Techniques and Miniaturized Peptide Mapping Devices for Structural Proteomics Applications

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INTRODUCTION

The application of capillary electrophoresis for peptide mapping is illustrated in the analysis of the peptide fragments produced by tryptic digestion of β-casein. Complete enzymatic digestion is usually desired for peptide mapping. The use of enzymes (such as trypsin) with high specificity for a particular type of peptide bond results in a reduction in the number of fragments generated during digestion. Another way to simplify peptide maps and reduce the number of peaks is to use immobilized enzymes. Immobilized enzymes are currently the object of considerable interest due to the expected benefits over soluble enzymes: separation from the product, reuse of the enzyme, facile automation.

The kinetic behavior of a bound enzyme can differ significantly from that of the same enzyme in free solution. These changes may be due to conformational alterations within the enzyme resulting from the immobilization protocol or to the presence and nature of the carrier (support) and linker. Therefore, we studied three immobilization techniques in the following manner:

1) immobilized trypsin was characterized in batch form by determination of its kinetic properties using the substrate N-α-*p*-tosyl-L-arginine methyl ester (TAME);

2) each immobilized enzyme preparation was packed in a capillary-based microreactor and the substrate β-casein was digested;

3) digests were separated by capillary electrophoresis (CE) for comparison of the peptide maps with respect to each immobilization technique;

4) for one of the immobilization techniques, the reproducibility of peptide maps was evaluated and the maps compared with those obtained in the same type of microreactor that had been packed with commercially available trypsin immobilized using the same linker and support;

5) rapid digestion in this particular microreactor is demonstrated showing CE-based peptide maps as well as MALDI-TOF peptide mass maps.

MATERIALS and IMMOBILIZATION TECHNIQUES

Enzyme: bovine trypsin (EC 3.4.21.4)

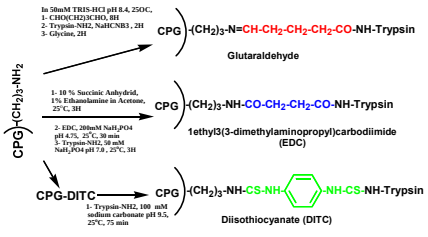
- **Endoprotease of 23800 Da (223 residues with 5 disulfide bridges)**
- **TPCK-treated to avoid chymotryptic activity**
- **High cleavage specificity (hydrolyses peptide bonds only on the C' side of arginine and lysine residues) and high specific activity**
- **Limited stability in solution as the result of autolysis**

Carrier: Controlled Pore Glass (CPG)

- **Inorganic support produced from borosilicate-based material with good mechanical strength; immune to biological degradation**
- **Thermostable and autoclavable;**
- **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 for covalent binding**
- **Particle size: 125-177 μm (80-120 mesh); narrow pore size distribution; 700 Å ave. pore size; specific pore volume of 1.0 ml/g; packed density of 0.41 g/ml**

Linkers:

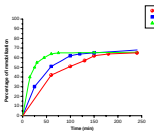
- **Glutaraldehyde with aminopropylated CPG**
- **EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) with aminopropylated CPG**
- **DITC (Diisothiocyanate) with aminopropylated CPG**



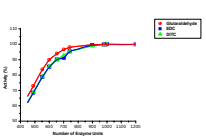
1) BATCH CHARACTERIZATION OF IMMOBILIZED TRYPSIN PREPARATIONS

1.1 Optimization of the immobilization

1- Length and percentage of immobilization



2- Number of enzyme units



The yields of trypsin immobilization are 65% but immobilization times are 240 min, 150 min and 75 min for Glutaraldehyde, EDC and DITC, respectively.

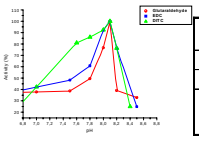
Percentage of immobilization was determined at λ = 280 nm.

For 10 mg of CPG , the best activity is obtained at 1000 enzyme units.

At 60 % immobilization yield, the amount of trypsinimmobilized is 4.4 mg/g of carrier

1.2 Effect of immobilization on the kinetic properties of trypsin

1- Optimal pH



optimal pH of free trypsin: 7.0 - 9.0

Determined with TAME as substrate in 50 mM TRIS-HCl containing 11.5 mM $CaCl_2$ at 25° C

2- K_m, V_{max} and specific activity

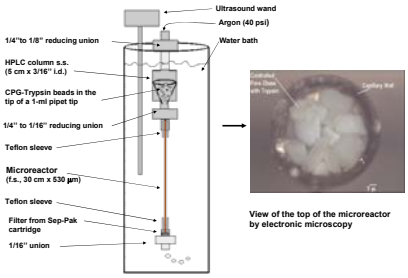
Trypsin	Free	Immobilized		
		Glutaraldehyde	EDC	DITC
K_m (10^{-4} M)	21.4	40	190	44.4
V_{max} (10^{-4} M/min)	11	4.2	20	10.2
Specific* Activity	13600	2012.5	8808	5344.9
	100%	14.8%	64.8%	39.3%

* BAEE units per mg of enzyme and in percentage

⇔ effect of carrier

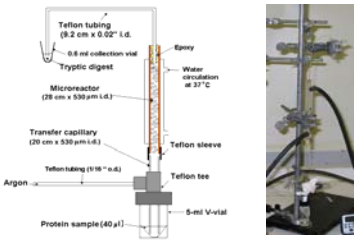
⇔ effect of spacer

2) PACKING AND OPERATION OF THE IMMOBILIZED TRYPSIN MICROREACTOR



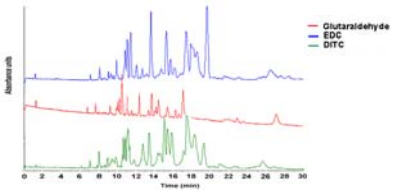
View of the top of the microreactor by electronic microscopy

The microreactor was dry-packed with trypsin-CPG beads (~50 mg) under argon pressure and simultaneous sonication in 30 s. The packing system could be constructed in about 2-5 min.



Protein substrate (β-casein was infused through the microreactor by application of approx. 1-2 psi Ar, over a period of 2 hr (or less). The digest was collected in a microtube for mapping by CE.

3) PEPTIDE MAPPING BY CAPILLARY ELECTROPHORESIS

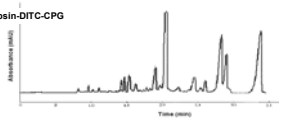


Comparison of peptide maps for each immobilization technique. Sample: 40 μl β-casein (5 mg/ml) + 80 μl wash with digestion buffer. Separation: PACE/MDQ CE (Beckman Coulter);15kV in 50 mM sodium phosphate, pH 2.5 Capillary: 75 μm I.D., 360 μm O.D., Lt= 60 cm; Ld = 50 cm Injection: 0.5 psi x 5s; Detection: 200 nm.

4) EVALUATION OF A TRYPSIN-DITC-CPG MICROREACTOR FOR PEPTIDE MAPPING

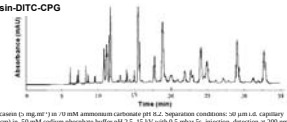
4.1 Comparison of two sources of immobilized trypsin

Home-made trypsin-DITC-CPG



digestion of 40 μl β-casein (5 mg.ml^{-1}) in 50 mM sodium phosphate pH 8.0. Separation conditions: 75 μm i.d. capillary (Lt 74 cm, Ld 43.5 cm) in 50 mM sodium phosphate buffer pH 2.5. 15 kV with 0.5 s injection by vacuum. detection at 200 nm.

Commercial trypsin-DITC-CPG

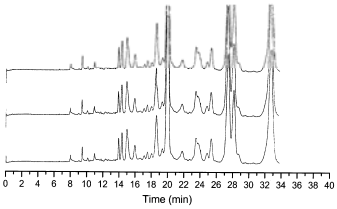


digestion of 40 μl β-casein (5 mg.ml^{-1}) in 70 mM ammonium carbonate pH 8.2. Separation conditions: 50 μm i.d. capillary (Lt 48.5 cm, Ld 40.0 cm) in 50 mM sodium phosphate buffer pH 2.5, 15 kV with 0.5 mbar 5s injection, detection at 200 nm.

4.2 Reproducibility of CE separations

The same 120 μL sample (β-casein digest plus microreactor wash) was run 3 times within 24 hr. Migration time reproducibility was ≤ 1.02% RSD (n=3) over the elution window.

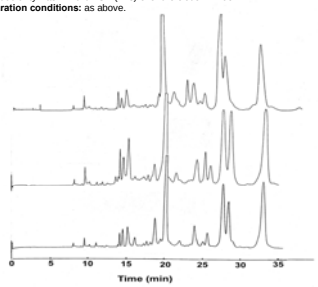
Separation conditions: Capillary: 75 μm ID, 363 μm OD, 74 cm total L, 43.5 cm effective length. Run: 50 mM phosphate, pH 2.5, injection 0.5 s by vacuum, 15 kV, detection at 200nm.



4.3 Reproducibility of digestions in the same microreactor

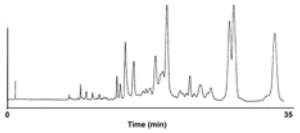
Three different β-casein samples (from same lot) were digested in the same microreactor and stored. Electropherograms were run consecutively on the same day. Migration time reproducibility was ≤ 1.6% RSD (n=3) over the elution window.

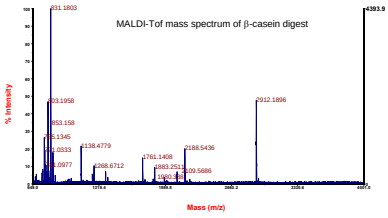
Separation conditions: as above.



5) RAPID DIGESTION IN THE TRYPSIN-DITC-CPG MICROREACTOR

Digestion of 40 μ L β-casein (290 μM) in 50 mM phosphate buffer, pH 8.0, followed by 80 μ L wash through reactor and injection of pooled 120 μ L into CE (separation conditions as above). **Total digestion time = 82 s**





Tryptic peptide masses matched (M-H$^+$) using database search (ProFound v. 4.10.5) and corresponding sequence:

742.081 GPFPIIV
748.015 EMPFPK
780.093 VLPVPQK
830.173 AVPYPQR
1137.469 VKEAMAPKHK
1980.338 FQSEEQQQTEDELQDK

Tryptic peptide masses corresponding to possible sodium adducts (M-Na$^+$) :

765.133 GPFPIIV
771.033 EMPFPK
803.195 VLPVPQK
853.158 AVPYPQR

6 peptides: 23% sequence converage

CONCLUSIONS AND FUTURE STUDIES

- **Enzyme immobilization process required less than 2 h and the microreactor could be constructed in about 30 min including its packing with the trypsin beads**
- **Peptide maps of β-casein (5 mg/ml) were obtained with good reproducibility in less than 3 h from sample introduction to map completion.**
- **The CE-based analysis of the digest resulted in electropherograms (maps) comparable to those obtained with commercially-immobilized trypsin**
- **Digestion in phosphate buffer impedes MS analysis (low sequence coverage)**
- **We need to evaluate the effective microreactor life-time, influence of substrate concentration and substrate composition on microreactor performance and make quantitative peptide recovery studies**

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