Electronic Supplementary Material (ESI) for Analytical Methods.
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Title: Development of Novel Enzyme Immobilization Methods Employing Formaldehyde or Triethoxysilylbutyraldehyde To Fabricate Immobilized Enzyme Microreactors For Peptide Mapping

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Supplementary Data

Table S1 – Effects of using phosphate buffer vs phosphate buffered saline (PBS) on formation of FA-CT enzyme particle. Reactions were carried out for two hours at 21°C using 40 µL of 1.3 mM chymotrypsin and 78 µL of formaldehyde 37%. "Large" indicates an enzyme particle which is greater in size than 2 mm × 2 mm in terms of the width and height for the total size of the enzyme particle. "Small" refers to any enzyme particle which is less than 2mm × 2mm mm in terms of the width and height for the total size of the enzyme particle. "Robust" indicates the FA-CT enzyme particle formed without breaking apart during the washing procedure. "Poor" robustness refers to breakage of the enzyme particle during washing.

	Buffer Conditions	Enzyme Particle Size	Enzyme Particle Robustness
Phosphate Buffer	50 mM, pH 6.4	Large	Robust
Phosphate Buffer Saline (PBS)	10 mM, pH 7.4	Small	Robust

Table S2 – Effects of various FA:CT ratios (mass:mass) on immobilization of 1.3 mg CT. Crosslinking reaction was performed for two hours at 21°C using 50 mM phosphate buffer (pH 6.4)."Large/Small" and "Robust/Poor" were defined same as Table 1.

FA:CT ratio (mass:mass)	Enzyme Particle Size	Enzyme Particle Robustness
38.5:1.0	Small	Robust
46.1:1.0	Small	Robust
69.2:1.0	Large	Robust
123.1:1.0	Large	Poor

Table S3 – Effects of reaction time on FA-CT immobilization. The reaction was carried out at 30 °C using a FA:CT ratio of 69.2:1.0 (mass:mass). CT was dissolved in 50 mM phosphate buffer (pH 6.4). Immobilized amount of CT was calculated to be 1.3 mg. "Large" and "Robust" were defined same as Table 1.

Immobilization Time (Hours)	Enzyme Particle Size	Enzyme Particle Robustness
Two	Large	Robust
Four	Large	Robust
Seven	Large	Poor

Table S4 – Effects of temperature on FA-CT immobilization performed for two hours using a ratio of FA:CT of 69.2:1.0 (mass:mass) with 1.3 mg CT in 50 mM phosphate buffer (pH 6.4). "Large" and "Robust" were defined same as Table 1.

Immobilization Temperature (°C)	Enzyme Particle Size	Enzyme Particle Robustness
0	Small	Poor
21	Small	Poor
30	Large	Robust
37	Large	Robust

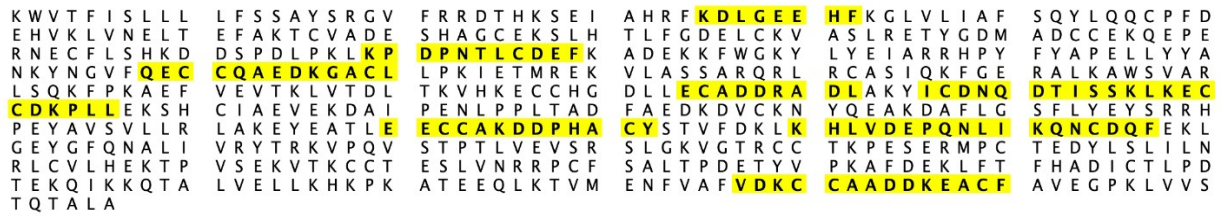

Figure S1. Primary sequence of BSA highlighted indicating the MS identified peptides. BSA was denatured and digested in-solution by free chymotrypsin (33.1 mg/mL) using an E:S ratio of 10:1 and incubated at 37 °C for 4 hours. MS data shows 11 unique peptides with a coverage of 18 % (107/606 amino acids).

Figure S2. Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.30mg CT:0.13mg BSA) using chymotrypsin dissolved in phosphate buffer (50mM, pH 6.4) indicates 42% coverage (256/607 amino acids), generating 54 chymotryptic peptides.

Figure S3. Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.30mg CT:0.13mg BSA) using chymotrypsin dissolved in PBS (10mM, pH 7.4) indicates 35% coverage (214/607 amino acids), generating 54 chymotryptic peptides

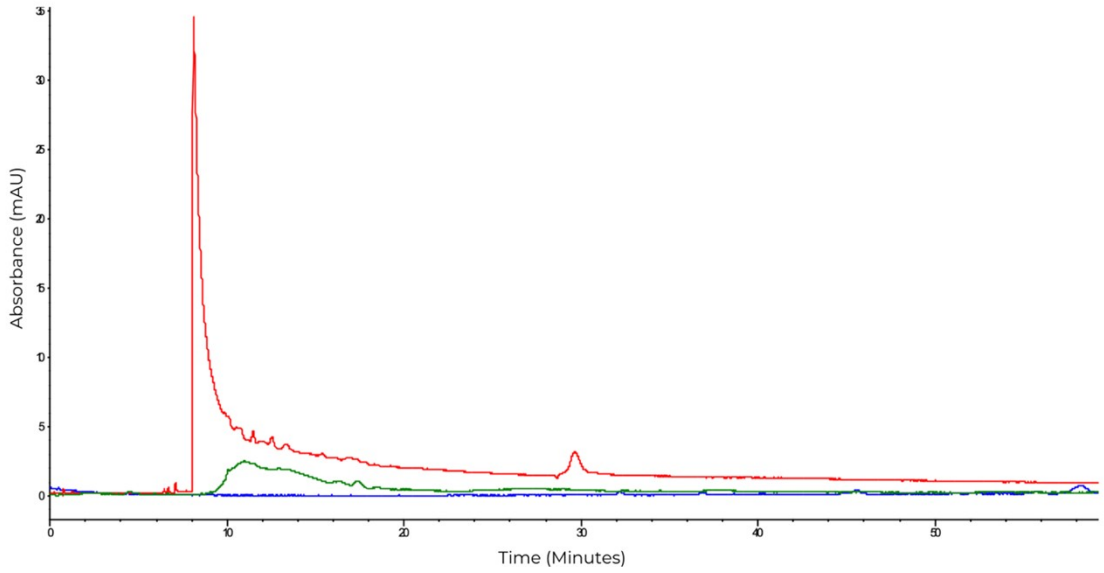

Figure S4 – Electropherograms from the chymotrypsin 0.13 mM standard (red) and the supernatants from the FA-CT (green) and TESB-CT (blue) enzyme particles. This shows the supernatant for the TESB-CT below the limit of detection for the G7100A Capillary Electrophoresis.

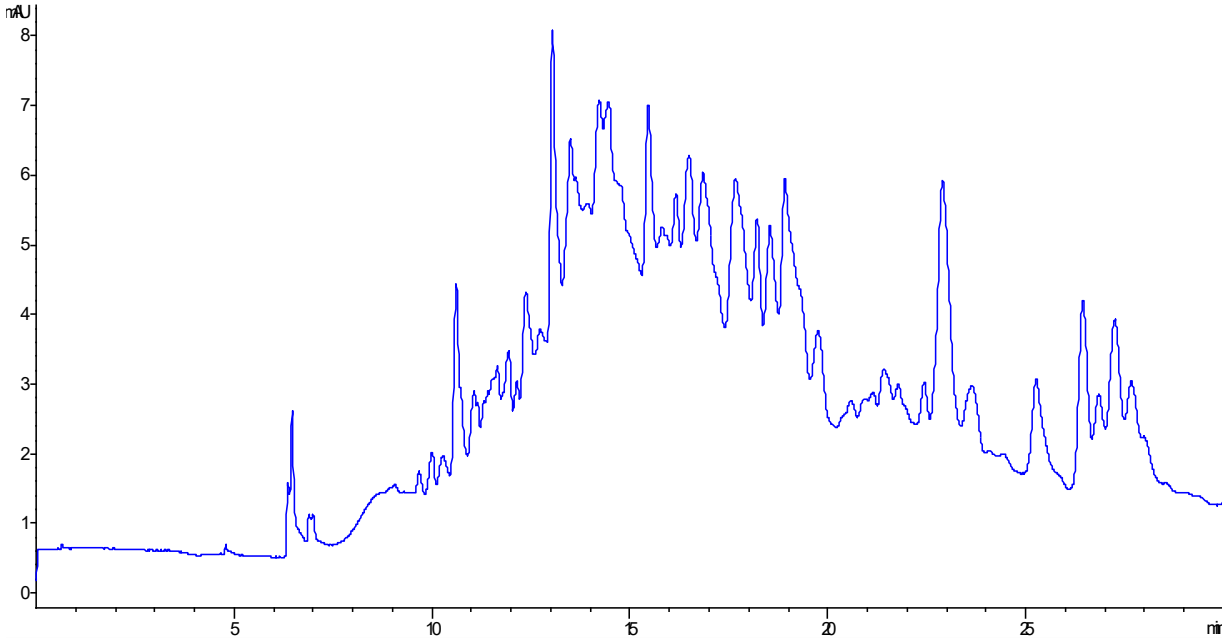

Figure S5. Electropherogram of FA-CT enzyme particle digestion of denatured BSA at 1:1 enzyme-to-substrate ratio (1.30mg CT:1.30mg BSA). Separations were performed at +15 kV using 50 mM phosphate buffer, pH 2.5 with detection at 200 nm.

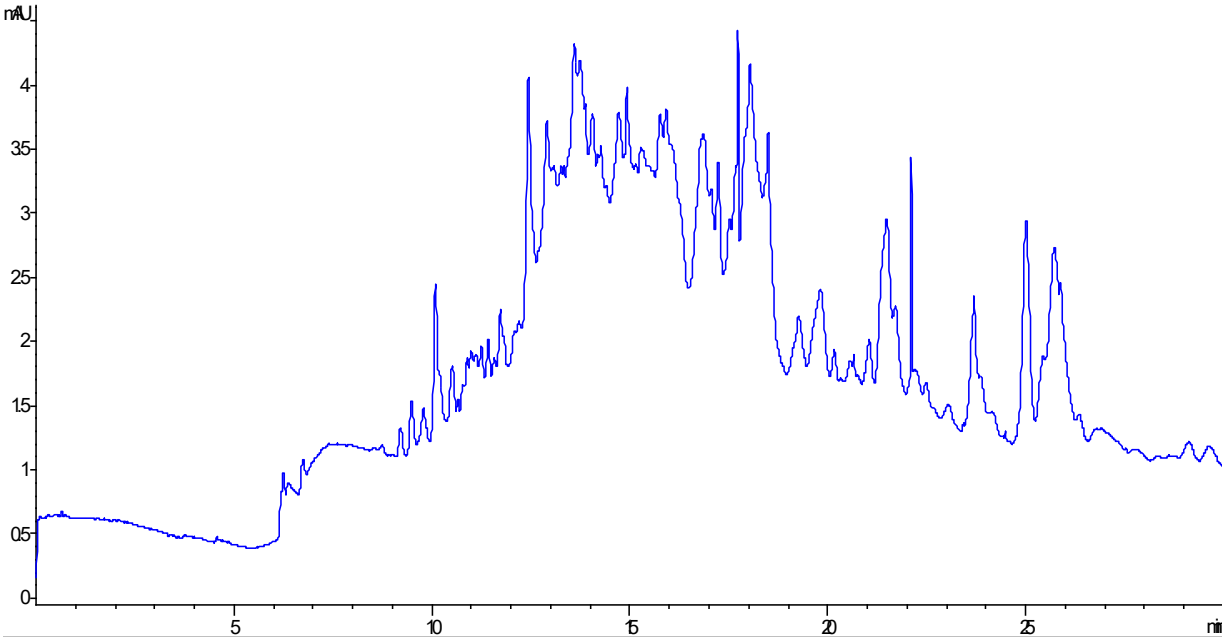

Figure S6 – Electropherogram of FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.30mg CT:0.13mg BSA). Separation condition was the same as Fig. S1.

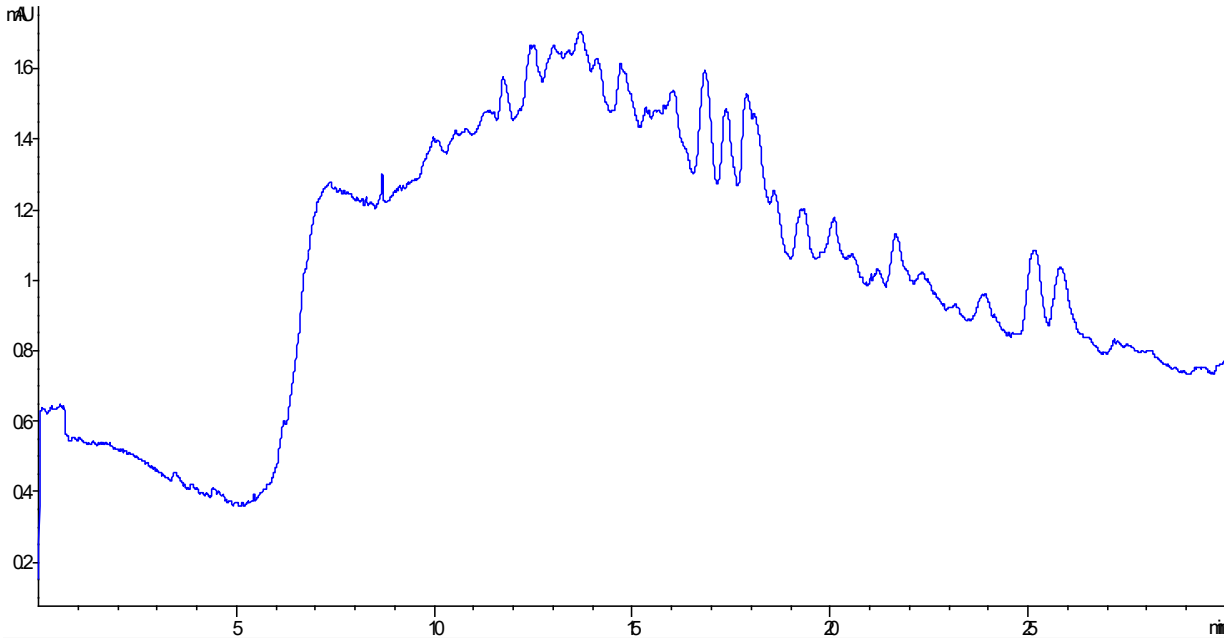

Figure S7 – Electropherogram of FA-CT enzyme particle digestion of denatured BSA at 100:1 enzyme-to-substrate ratio (1.30mg CT:0.013mg BSA). Separation condition was the same as Fig. S1.

Figure S8 – Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 1:1 enzyme-to-substrate ratio (1.30mg CT:1.30mg BSA) indicates 68% coverage (415/606 amino acids), generating 54 unique peptides.

Figure S9. Primary sequence of BSA with highlighted indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.3mg CT:0.13mg BSA) shows a 67% coverage (407/606 amino acids), generating 44 unique peptides.

Figure S10 – Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 100:1 enzyme-to-substrate ratio (1.30mg CT:0.013mg BSA) shows a 66% coverage (400/607 amino acids), generating 29 unique peptides.

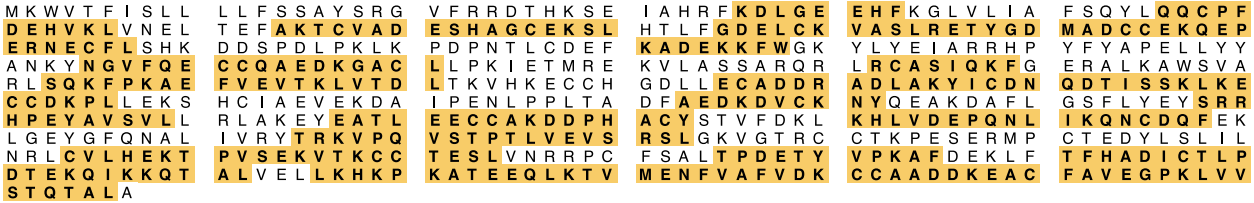

Figure S11 - Primary sequence of BSA with highlights indicating the MS identified peptides. TESB-CT enzyme particle digestion of denatured BSA at 1:1 enzyme-to-substrate ratio (1.30mg CT:1.30mg BSA) shows a 55% coverage (334/607 amino acids) generating 26 chymotryptic peptides.

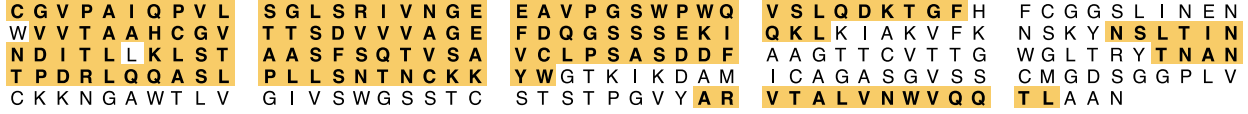

Figure S12 - Primary sequence of chymotrypsin with highlights indicating the MS identified peptides. TESB-CT autolysis shows a 67% coverage (164/245 amino acids) generating 14 chymotryptic peptides.