

**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  $\mu$ L of 1.3 mM chymotrypsin and 78  $\mu$ L of formaldehyde 37%. “Large” indicates an enzyme particle which is greater in size than 2 mm  $\times$  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  $\times$  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

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KWVTFISLL  L FSSAYS RGV  FRRDTHKSEI  AHRFKDLGEE  HFKGLVLI AF  SQYLQQCPFD
EHVKLVNELT  EFAKTCVADE  SHAGCEKSLH  TLFGDDELCKV  ASLRETYGDM  ADCCEKQEP E
RNECFLSHKD  DSPDLPKLP  DPNTLCDEF  ADEK KFWGKY  LYEIARRHPY  FYAPEL LYYA
NKYNGVFQEC  CQAEDKGACL  LPKIETMREK  VLASSARQRL  RCASIQKFGE  RALKAWSVA R
LSQKFPKAEF  VEVTKLVTDL  TKVHKECCHG  DLL ECADDRA  DLAKY ICDNQ  DTISSK LKEC
CDKPLL EKSH  CIAEVEKDAI  PENLPPLTAD  FAEDKDVCKN  YQEA KDAFLG  SFLY EYSRRH
PEYAVSVLLR  LAKEYEATLE  ECCAKDDPHA  CYS TVFDK LK  HLVDEPQNL I  KQNC DQFEKL
GEYGFQNALI  VRYTRKVPQV  STPTLVEVSR  SLGKVGTRCC  TKPESERMPC  TEDYLSL I LN
RLCVLHEKTP  VSEKVTKCC  ESLVNR R PC  SALT PDETYV  PKAFDEKLF  T  FHADICTLP D
TEKQIKKQT  ALVELLKHK  P  KATEEQ LKT V  MENFVA FVDK  CCAADDKEAC  F  AVEGPKLVV S
TQTALA

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**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).

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MKWVTFISLL  L L FSSAYS R G  V FRRDTHKSE  I AHRFKDLGE  E HFKGLVLI A
FSQYLQQCPF  DEHVKLVNEL  T EFAKTCVAD  E SHAGCEKSL  HTLFGDELCK
VASLRETYGD  MADCCCEKQEP  ERNECFLSHK  D DSPDLPKLP  PDPNTLCDEF
KADEKKFWGK  YLYE IARRHP  YFYAPEL LYY  ANKYNGVFQE  CCQAEDKGAC
LLPKIETMRE  KVLASSARQR  LRCASIQKF  G ERALKAWSVA  RLSQKFPKAE
FVEVTKLVT D  LTKVHKECCH  GD LLECADDR  ADLAKY ICDN  QDTISSK LKE
CCDKP LLEKS  HCIAEVEKDA  I PENLPPLTA  DFAEDKDVCK  NYQEA KDAFL
GSFLY EYSRR  HPEYAVSVLL  R LAKEYEATL  E ECCAKDDPH  ACYSTVFDKL
KHLVDEPQNL  IKQNC DQFEK  LGEYGFQNAL  I VRYTRKVPQ  VSTPTLVEVS
RSLGKVGTRC  CTKPESERMPC  CTEDYLSLIL  NRLCVLHEKT  PVSEKVTKCC
TESLVNRRPC  F SALT PDETY  VPKAFDEKLF  T FHADICTLP  DTEKQIKKQT
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S TQTALA

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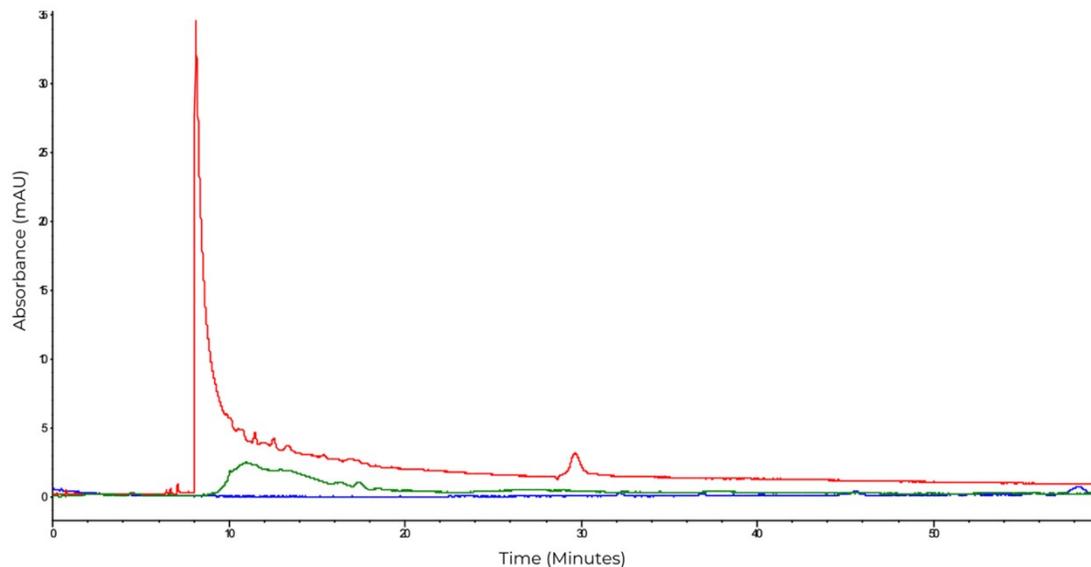
**Figure S2.** Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.30mg CT:0.13mg BSA) using chymotrypsin dissolved in phosphate buffer (50mM, pH 6.4) indicates 42% coverage (256/607 amino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

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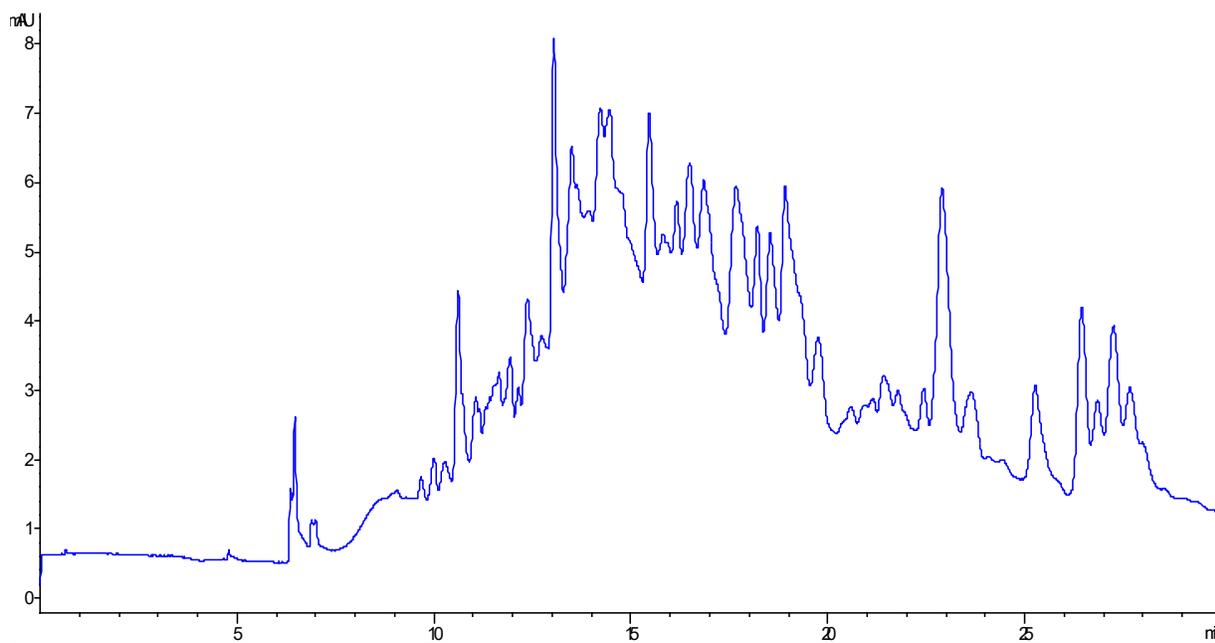
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VASLRETYGD  MADCCCEKQEP  ERNECFLSHK  D DSPDLPKLP  PDPNTLCDEF
KADEKKFWGK  YLYE IARRHP  YFYAPEL LYY  ANKYNGVFQE  CCQAEDKGAC
LLPKIETMRE  KVLASSARQR  LRCASIQKF  G ERALKAWSVA  RLSQKFPKAE
FVEVTKLVT D  LTKVHKECCH  GD LLECADDR  ADLAKY ICDN  QDTISSK LKE
CCDKP LLEKS  HCIAEVEKDA  I PENLPPLTA  DFAEDKDVCK  NYQEA KDAFL
GSFLY EYSRR  HPEYAVSVLL  R LAKEYEATL  E ECCAKDDPH  ACYSTVFDKL
KHLVDEPQNL  IKQNC DQFEK  LGEYGFQNAL  I VRYTRKVPQ  VSTPTLVEVS
RSLGKVGTRC  CTKPESERMPC  CTEDYLSLIL  NRLCVLHEKT  PVSEKVTKCC
TESLVNRRPC  F SALT PDETY  VPKAFDEKLF  T FHADICTLP  DTEKQIKKQT
ALVELLKHK  P  KATEEQ LKT V  MENFVA FVDK  CCAADDKEAC  F AVEGPKLVV
S TQTALA

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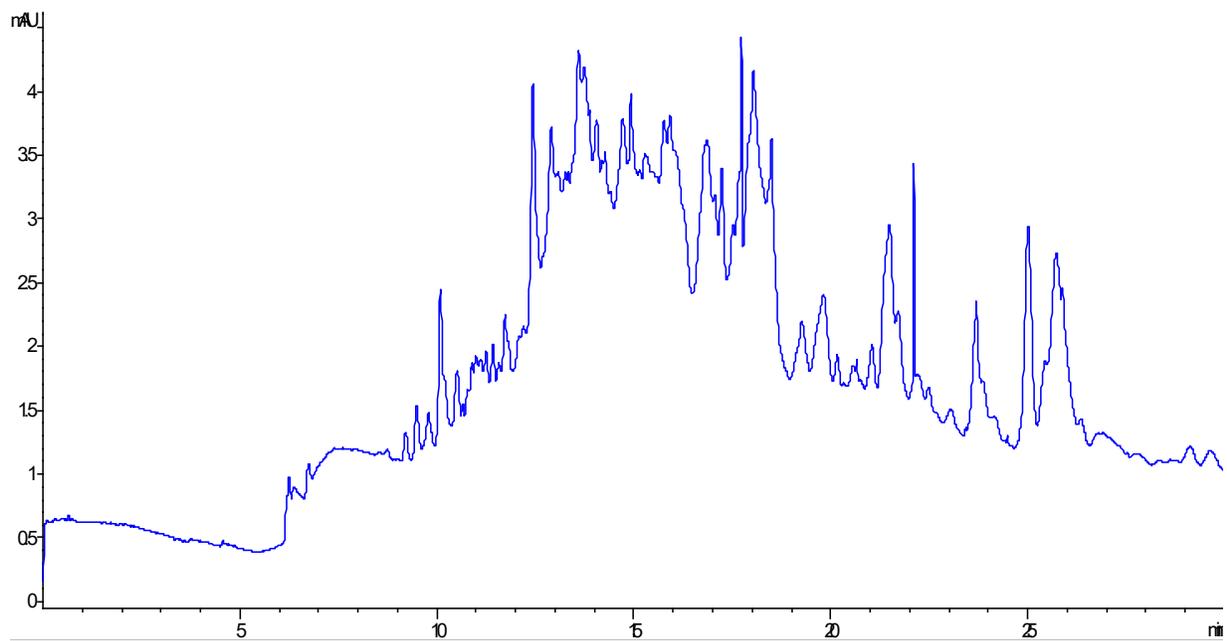
**Figure S3.** Primary sequence of BSA with highlights indicating the MS identified peptides. FA-CT enzyme particle digestion of denatured BSA at 10:1 enzyme-to-substrate ratio (1.30mg CT:0.13mg BSA) using chymotrypsin dissolved in PBS (10mM, pH 7.4) indicates 35% coverage (214/607 amino acids), generating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



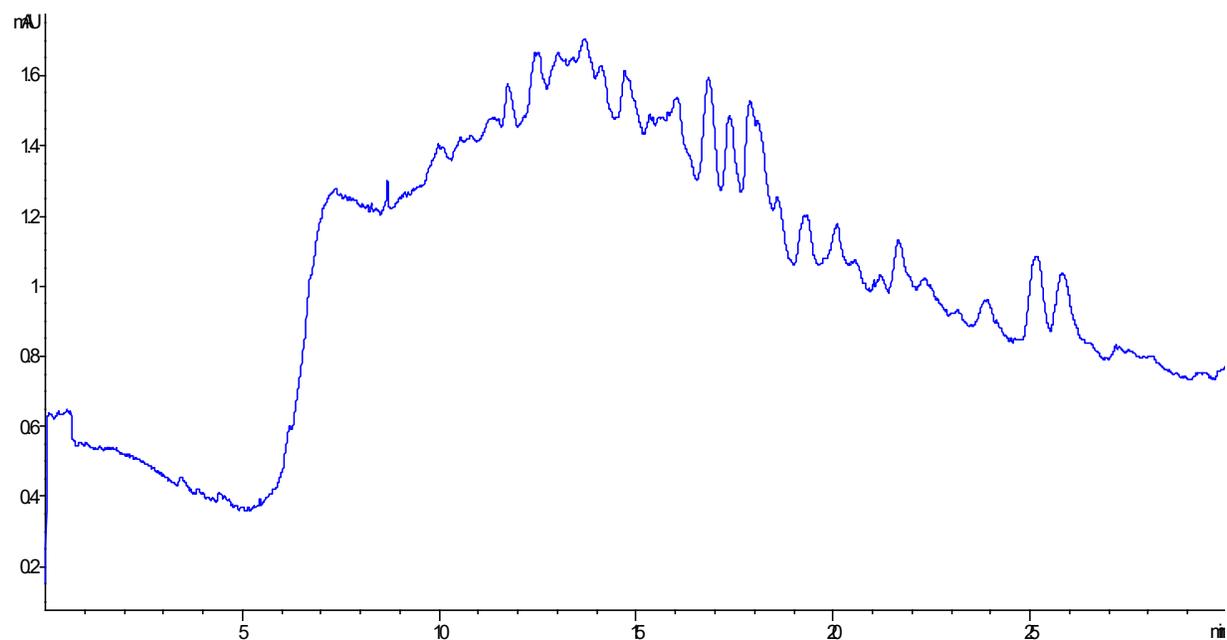
**Figure S4** – Electropherograms from the chymotrypsin 0.13 mM standard (red) and the supernatants from the FA-CT (green) and TESB-CT (blue) enzyme particles. This shows the supernatant for the TESB-CT below the limit of detection for the G7100A Capillary Electrophoresis.



**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.

KWVTFISLLL	LFSSAYS SRGV	FRRDTHKSEI	AHRFKDLGEE	HFKGLVLI AF	SQYLQQCPFD
EHVKLVNELT	EFAKTCVADE	SHAGCEKSLH	TLFGDELCKV	ASLRETYGDM	ADCCEKQEP E
RNECFLSHKD	DSPDLPKLLK	DPNTLDCDEFK	ADEKKFWGKY	LYE IARRHPY	FYAPPELLYYA
NKYNGVFQEC	CQAEDKKGACL	LPKIETMREK	VLASSARQRL	RCASIQKFGE	RALKAWSVAR
LSQKFPKAEF	VEVTKLVTDL	TKVHKECCHG	DLLECADDDRA	DLAKYICDNQ	DTISSKLEK E
CDKPLLEKSH	CIAEVEKDAI	PENLPPPLTAD	FAEDKDVCKN	YQEAKDAFLG	SFLYEYSRRH
PEYAVSVLLR	LAKEYEATLE	ECCAADDPHA	CYSTVFDKLLK	HLVDEPQNL I	KQNCDDQFEK L
GEYGFQNALI	VRYTRKVPQV	STPTLVVEVSR	SLGKVGTRCC	TKPESERMPC	TEDYLSLILN
RLCVLHKEKT	VSEKVTKCC	ESLVNRRPCF	SALTPDETYV	PKAFDEKLF	FHADICTLPD
TEKQIKKQTA	LVELLKHKPK	ATEEQLKTVM	ENFVAFVDKC	CAADDKEACF	AVEGPKLVVS
TQTALA					

**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.

KWVTFISLLL	LFSSAYS SRGV	FRRDTHKSEI	AHRFKDLGEE	HFKGLVLI AF	SQYLQQCPFD
EHVKLVNELT	EFAKTCVADE	SHAGCEKSLH	TLFGDELCKV	ASLRETYGDM	ADCCEKQEP E
RNECFLSHKD	DSPDLPKLLK	DPNTLDCDEFK	ADEKKFWGKY	LYE IARRHPY	FYAPPELLYYA
NKYNGVFQEC	CQAEDKKGACL	LPKIETMREK	VLASSARQRL	RCASIQKFGE	RALKAWSVAR
L SQKFPKAEF	VEVTKLVTDL	TKVHKECCHG	DLLECADDDRA	DLAKYICDNQ	DTISSKLEK E
CDKPLLEKSH	CIAEVEKDAI	PENLPPPLTAD	FAEDKDVCKN	YQEAKDAFLG	SFLYEYSRRH
PEYAVSVLLR	LAKEYEATLE	ECCAADDPHA	CYSTVFDKLLK	HLVDEPQNL I	KQNCDDQFEK L
GEYGFQNALI	VRYTRKVPQV	STPTLVVEVSR	SLGKVGTRCC	TKPESERMPC	TEDYLSLILN
RLCVLHKEKT	VSEKVTKCC	ESLVNRRPCF	SALTPDETYV	PKAFDEKLF	FHADICTLPD
TEKQIKKQTA	LVELLKHKPK	ATEEQLKTVM	ENFVAFVDKC	CAADDKEACF	AVEGPKLVVS
TQTALA					

**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.

MKWVTFISLL	LLFSSAYS SRG	VFRRDTHKSE	I AHRFKDLGE	EHFKGLVLI A	FSQYLQQCPF
DEHVKLVNEL	TEFAKTCVAD	ESHAGCEKSL	HTLFGDELCK	VASLRETYGD	MADCCEKQEP
ERNECFLSHK	DDSPDLPKLLK	PDPNTLDCDEF	KADEKKFWGK	YLYE IARRHP	YFYAPPELLYY
ANKYNGVFQEC	CQAEDKKGAC	LLPKIETMRE	KVLASSARQRL	LRCASIQKFGE	ERALKAWSVA
RLSQKFPKAE	FVEVTKLVTD	LTKVHKECCH	GDLLECADDD	ADLAKYICDN	QDTISSKLEK E
CCDKPLLEKS	HCIAEVEKDA	I PENLPPPLTA	DFAEDKDVCK	NYQEAKDAFL	GSFLYEYSRR
HPEYAVSVLL	RLAKEYEATL	EECCAADDPH	ACYSTVFDKL	KHLVDEPQNL	IKQNCDDQFEK
LGEYGFQNAL	IVRYTRKVPQ	VSTPTLVVEV	RSLGKVGTRC	CTKPESERMPC	CTEDYLSLIL
NRLCVLHKEK	PVSEKVTKCC	TESLVNRRPC	FSALTPDETY	VPKAFDEKLF	TFHADICTLP
DTEKQIKKQT	ALVELLKHKPK	KATEEQLKT V	MENFVAFVDK	CAADDKEAC	FAVEGPKLVV
STQTALA					

**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.

MKWVTFISLL	LLFSSAYS SRG	VFRRDTHKSE	I AHRFKDLGE	EHFKGLVLI A	FSQYLQQCPF
DEHVKLVNEL	TEFAKTCVAD	ESHAGCEKSL	HTLFGDELCK	VASLRETYGD	MADCCEKQEP
ERNECFLSHK	DDSPDLPKLLK	PDPNTLDCDEF	KADEKKFWGK	YLYE IARRHP	YFYAPPELLYY
ANKYNGVFQEC	CQAEDKKGAC	LLPKIETMRE	KVLASSARQRL	LRCASIQKFGE	ERALKAWSVA
RLSQKFPKAE	FVEVTKLVTD	LTKVHKECCH	GDLLECADDD	ADLAKYICDN	QDTISSKLEK E
CCDKPLLEKS	HCIAEVEKDA	I PENLPPPLTA	DFAEDKDVCK	NYQEAKDAFL	GSFLYEYSRR
HPEYAVSVLL	RLAKEYEATL	EECCAADDPH	ACYSTVFDKL	KHLVDEPQNL	IKQNCDDQFEK
LGEYGFQNAL	IVRYTRKVPQ	VSTPTLVVEV	RSLGKVGTRC	CTKPESERMPC	CTEDYLSLIL
NRLCVLHKEK	PVSEKVTKCC	TESLVNRRPC	FSALTPDETY	VPKAFDEKLF	TFHADICTLP
DTEKQIKKQT	ALVELLKHKPK	KATEEQLKT V	MENFVAFVDK	CAADDKEAC	FAVEGPKLVV
STQTALA					

**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.

CGVPAIQPVL	SGLSRIVNGE	EAVPGSWPWQ	VSLQDKTGFH	FCGGSLINEN
WVVTAAHCGV	TTSDVIVVAGE	FDQGSSEK I	QKLIKIAKVFK	NSKYNSLTIN
NDITLLKLLST	AASFVQTVSA	VCLPSASDDF	AAGTTCVTTG	WGLTRYTNAN
TPDRLLQQASL	PLLSNTNCKK	YWGTKIKDAM	ICAGASGVSS	CMGDSGGPLV
CKKNGAWTLV	GIVSWGSSCT	STSTPGVYAR	VTALVNWVQQ	TLAN

**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.