MT-Trypsin mediated rapid in-gel digestion with trypsin removal

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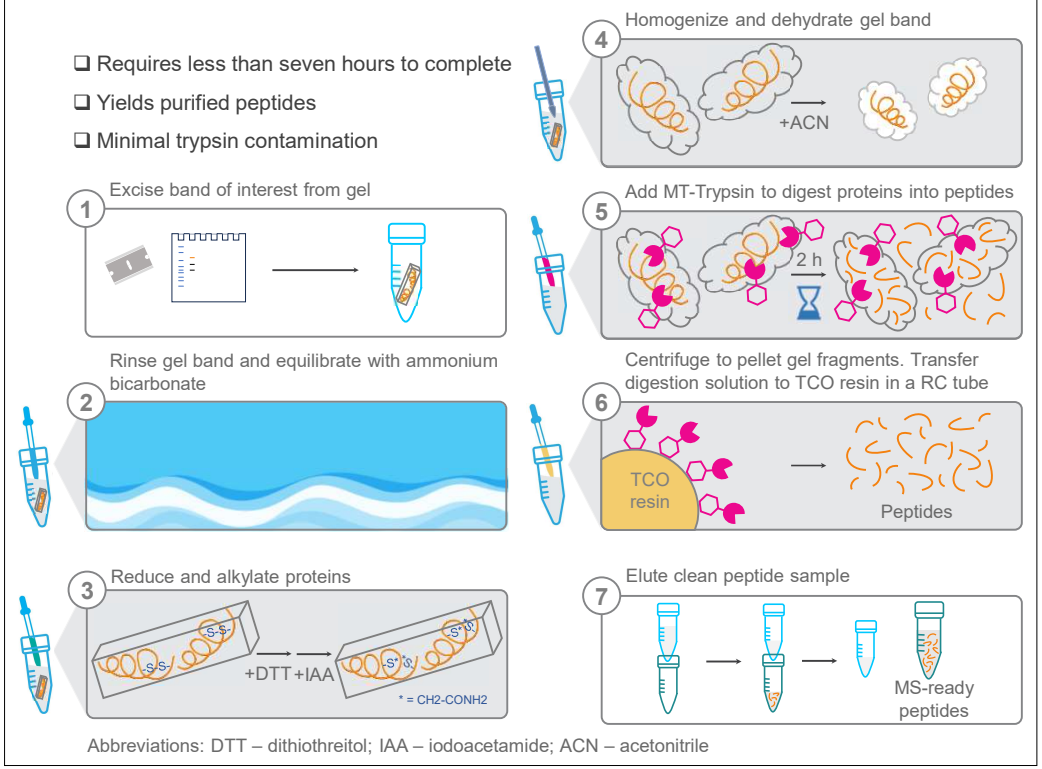
Abstract

In-gel digestion, the enzymatic digestion of proteins in bands or plugs excised from one- or two-dimensional polyacrylamide gels, is a commonly used method of preparing peptides for mass spectrometry (MS) analysis in bottom-up proteomics workflows.

Utilizing click chemistry technology, we developed an in-gel digestion protocol that enables rapid, two-hour enzymatic digestion of proteins. This workflow includes aspects of a canonical method of in-gel digestion (Shevchenko et al, 2007) combined with the utilization of methyltetrazine (MT) modified trypsin (MT-Trypsin) that covalently binds to trans-cyclooctene (TCO) resin (Biedka et al., 2021), allowing its removal from the digestion reaction. We also introduced homogenization steps using a pestle to crush the gel band into small fragments that facilitate MT-Trypsin migration into the gel matrix.

Using Cy3-labeled proteins of various molecular weights, we demonstrate the efficacy of this in-gel digestion procedure and show that this method enables detection of proteins present in gel samples at femtomolar quantities.

Schematic of rapid in-gel protein digestion workflow



Abbreviations: DTT – dithiothreitol; IAA – iodoacetamide; ACN – acetonitrile

References

Shevchenko, A., Tomas, H., Havliš, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1, 2856–2860 (2007).

Biedka, S. et al. Reversible Click Chemistry Tag for Universal Proteome Sample Preparation for Top-Down and Bottom-Up Analysis. J Proteome Res 20, 4787–4800 (2021).

Efficacy of in-gel digestion

To demonstrate the efficacy of the workflow, we performed MT-Trypsin mediated rapid in-gel digestion to purify for MS analysis peptides on three representative proteins, apo-myoglobin (17 kDa), alcohol dehydrogenase (ADH, 37 kDa), and fibrinogen alpha chain (95 kDa), labeled with Cy3-NHS dye.

The results show high sequence coverage for all three proteins: 95.5% for apomyoglobin, 73.9% for ADH, and 64.1% for fibrinogen alpha chain (**Table 1**). Trypsin was detected by a single unique peptide in the ADH and fibrinogen alpha chain digest samples, and two peptides in the apo-myoglobin sample.

For the fibrinogen alpha chain sample, both the beta and gamma chains of fibrinogen were identified in addition to the alpha chain. This is likely due to imperfect excision of the fibrinogen alpha chain band, as these bands were closely clustered on the SDS-PAGE gel. This is supported by the higher counts of unique peptides of the beta subunit, which runs closer to alpha, versus gamma.

Table 1. Mass spectrometry data of peptide samples after in-gel digestion of 160 pmol gel bands of apomyoglobin, ADH, and fibrinogen alpha chain.

Protein IDs	Major protein names	Unique peptides	Sequence coverage [%]	Score	Intensity	MS/MS count
Apomyoglobin gel band						
P68082	Myoglobin	21	95.5	323.3	7.3E+07	77
P07477	*Trypsin-1*	*2*	*7.3*	*38.3*	*6.9E+06*	*6*
Q2KIG3	*Carboxypeptidase B2*	*1*	*2.4*	*5.5*	*4.4E+04*	*3*
P05619	Leukocyte elastase inhibitor	2	4.7	11.1	1.1E+06	2
P00443	Superoxide dismutase [Cu-Zn]	2	32.5	13.3	2.5E+04	2
ADH gel band						
P00330	Alcohol dehydrogenase 1	31	73.9	323.3	1.3E+08	83
P00331	Alcohol dehydrogenase 2	1	26.4	84.3	1287500	2
P07477	*Trypsin-1*	*1*	*4*	*25.8*	*6409200*	*2*
P07246	Alcohol dehydrogenase 3, mitochondria	1	2.1	13.1	954080	2
Fibrinogen alpha chain gel band						
P02671	Fibrinogen alpha chain	83	64.1	323.3	3.6E+08	286
P02675	Fibrinogen beta chain	37	74.9	323.3	3.2E+07	80
P02768	Serum albumin	31	53	323.3	8.4E+06	44
P02679	Fibrinogen gamma chain	15	44.6	285.5	1.5E+06	16
P01024	Complement C3	13	12.5	145.9	8.4E+05	15
P0C0L5	Complement C4-B	10	6.9	107.1	5.4E+05	12
P01876	Ig alpha-1 chain C region	7	26.9	78.4	8.6E+05	10
P01860	Ig gamma-3 chain C region	4	22	48.0	3.3E+05	6
P02751	Fibronectin	6	4	114.2	2.5E+05	6
P00488	Coagulation factor XIII A chain	6	9.2	38.9	2.1E+05	6
P05160	Coagulation factor XIII B chain	6	10.7	37.6	1.5E+05	6
P04003	C4b-binding protein alpha chain	3	7.2	22.3	2.1E+05	4
P04004	Vitronectin	2	6.3	15.0	1.3E+05	3
P07477	*Trypsin-1*	*1*	*4*	*6.6*	*5.3E+06*	*2*
Q9NQZ2	Something about silencing protein 10	1	3.8	5.7	3.1E+06	2
Q9UIL4	Kinesin-like protein KIF25	1	2.9	6.3	9.5E+05	2
Q13243	Serine/arginine-rich splicing factor 5	1	2.9	5.9	2.3E+05	2
P23142	Fibulin-1	2	3.1	11.7	8.6E+04	2

The table includes protein IDs with MS/MS count 2 and above. Keratin contaminants filtered out. Trypsin and foreign species contaminants are in italics.

Detection limit of rapid in-gel digestion

To test the protein detection limit of our method, we prepared gel bands with pico- and femto-molar quantities of ADH. Gel bands with 16 pmol, 48 fmol and 16 fmol of ADH were processed following the rapid MT-Trypsin mediated in-gel digestion workflow, and the final peptide samples were analyzed by MS. To account for potential carryover of protein, we used a fresh razor blade for the excision of each band, and samples were injected into the MS starting with the 16 fmol sample, followed by the 48 fmol sample, and then the 16 pmol sample.

ADH was detected in all three samples (**Table 2**). In the 16 fmol sample, five unique peptides were detected, with coverage of 24.1%. In samples prepared with higher molar quantities of ADH, the peptide coverage increased to 35.6% (48 fmol) and 70.7% (16 pmol).

Table 2. Mass spectrometry results of peptide samples after in-gel digestion of ADH gel bands loaded with serial fmolar and pmolar quantities of protein.

Molar quantity per gel band	Protein IDs	Major protein names	Unique peptides	Sequence coverage	Intensity	MS/MS count
16 pmol	P00330;I	Alcohol dehydrogenase 1	27	70.7	124640000	69
48 fmol	P00330;I	Alcohol dehydrogenase 1	8	35.6	582890	11
16 fmol	P00330;I	Alcohol dehydrogenase 1	5	24.1	249210	6

Conclusion

We developed an in-gel digestion protocol that enables rapid 2-hour enzymatic in-gel digestion followed by trypsin removal from peptide sample. The workflow provides high-quality protein identification and sensitivity.