

Trypsin in-gel digestion of proteins

(adapted from Shevchenko et al. *Anal. Chem.* 68, pp. 850-858, 1996)

Always wear gloves to avoid keratin contamination.

This protocol is compatible with mass spectrometric protein analysis. Generally two days are required for completion of the analysis: on the first day samples are prepared for overnight digestion. the following day samples are lyophilised and then reconstituted in a solution for ms analysis.

We have two methods for protein analysis. One method is *short* and is practically always used for protein identification purposes. The second method is longer and involves reduction and alkylation of cysteine-containing peptides, this is useful when a higher coverage of the protein is required, *e.g.* for protein sequencing and for protein obtained from gel of unknown quality. Both the [short](#) and the [long](#) methods are described below.

Trypsin digestion - short method (ID purposes only)

1. Excise protein spot/band, cut into smaller pieces (<1mm) and dehydrate in CH₃CN for approx. 10 min. Remove CH₃CN and SpeedVac untill dry. (use gel loading tip not to loose gel pieces)
2. Reswell gel pieces at 4 °C for 45 min in buffer containing trypsin and 50 mM NH₄HCO₃. (Approx. 5 µL/mm² gel). The gel pieces should *just* be covered.

Suggested amount of trypsin is 12.5 ng/µL of buffer for proteins that have been silver stained.

Digest overnight at 37 °C (or at least for 3 h).

3. Centrifuge gel pieces and collect supernatant. Further extract peptides by one change of 20mM NH₄HCO₃.

(centrifuge then collect), and 3 changes of 5% formic acid (acetic acid is fine too) in 50% CH₃CN (10-20 min between changes) at room temp.

4. Dry sample down in speedvac untill desired volume has been reached (try not to dry to completion for low level samples).

Note NEVER use more than 1 ug trypsin per sample for MS analysis.

Trypsin digestion - long method (higher coverage or gel of unknown quality)

1. Excise protein spot/band, cut into smaller pieces (<1mm) and dehydrate in CH₃CN for approx. 10 min. Remove CH₃CN and SpeedVac until dry (use gel loading tips).
2. Cover gel pieces with 10 mM DTT in 100 mM NH₄HCO₃. Reduce proteins for 1 h at 56 °C.
3. Cool to room temp, remove DTT solution and add equal volume of 55 mM iodoacetamide in 100 mM NH₄HCO₃. Incubate for 45 min in dark place at room temp with occasional mixing.
4. Wash gel pieces with 50-100 ul aliquots of 100 mM NH₄HCO₃ for 10 min. Dehydrate with plenty of CH₃CN, reswell in 100 mM NH₄HCO₃, and shrink again with CH₃CN.
5. Remove liquid phase and speedvac.
6. Reswell gel pieces at 4 °C for 45 min in buffer containing trypsin and 50 mM NH₄HCO₃. (Approx. 5 μ L/mm² gel). The gel pieces should *just* be covered.

Suggested amount of trypsin is 12.5 ng/ μ L of buffer for proteins that have been silver stained.

Digest overnight at 37 °C (or at least for 3 h).

7. Centrifuge gel pieces and collect supernatant. Further extract peptides by one change of 20mM NH₄HCO₃.

(centrifuge then collect), and 3 changes of 5% formic acid (acetic acid is fine) in 50% CH₃CN (20 min between changes) at room temp.
8. Dry sample down in speedvac until desired volume has been reached.

Note NEVER use more than 1 ug trypsin per sample for MS analysis.

reasons for failure of digestion

-Gel piece is still too acidic.

Solution: rinse the gel piece with buffer. This should not be the cause of the problem in the long digestion method.

-the enzyme solution is too old.

Solution: change to a new trypsin vial every few weeks.

-protein is resistant to trypsin.

Solution: change enzyme (see list below).

Table 1. Proteolytic enzymes and their cleavage specificities useful for protein digestion

Enzyme	Cleavage site	Exception
Arg-C	RX Some KX	Some RX
AspN	DX X-cysteic acid Some XE	-
Chymotrypsin	FX, YX, WX, LX Some MX, IX, SX, TX, VX, HX, GX, AX.	XP
Clostripain	R	-
Cyanogen_Bromide	M	-
Elastase	GX, AX, SX, VX, LX, IX	XP
Glu-C	EX, DX in phosphate buffers EX ammonium bicarbonate buffers	XP
IodosoBenzoate	W	-
Lys-C	KX Some NX	
Pepsin	FX, LX EX	XV, XA, XG
Proline_Endopept	PX	-

Pronase	most peptide bonds	
Staph_Protease (V8)	EX	-
Trypsin	KX, RX	XP
Trypsin_K	KX	XP
Trypsin_R	RX	XP

X represents any amino acid