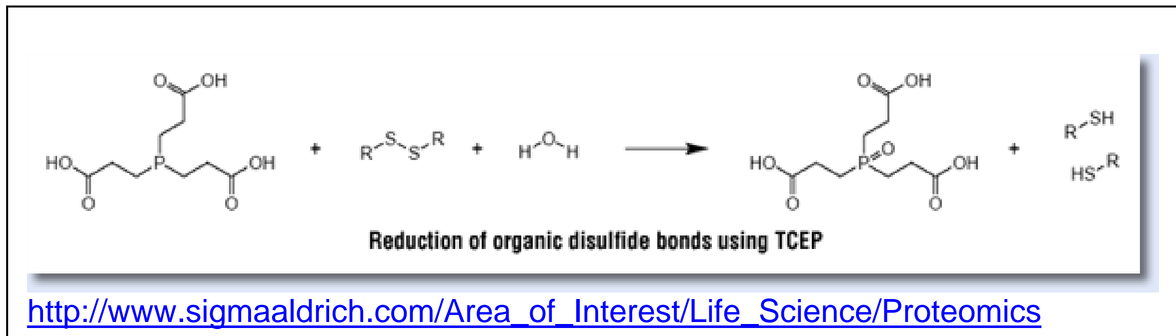
Protein Digestion Handout

For protein identification purposes, mass spectral analysis of proteins typically requires breaking intact proteins down into discrete peptide fragments. Primarily this is accomplished by treating proteins with proteases, most commonly, trypsin. Measuring the mass/charge (m/z) of multiple proteolytic peptide ions, rather than intact proteins allows more precise measurements and therefore higher confidence identifications.

Denaturation, reduction and alkylation. Prior to digestion, proteins must be treated to allow full access by the proteolytic enzyme. Proteins can be digested in solution, or within a gel fragment after one- or two-dimensional gel electrophoresis. In either case the basic steps are the same. With the exception that proteins within gel fragments must first be destained to remove coomassie (or other in-gel stain) prior to digestion.

For in-solution digests, proteins are first reconstituted in 8M urea. This step **denatures** proteins and prevents aggregation. Next they are treated with a **reducing agent**, typically dithiolthreitol (DTT), to disrupt disulfide bonds between cysteine residues. To prevent disulfide bonds from reforming, free sulfhydryl groups are then treated with an **alkylating agent**, iodoacetamide (IAA). Although DTT is commonly used for reduction, tris[2-carboxyethyl] phosphine (TCEP) is an attractive alternative. TCEP offers several advantages over DTT. It is more resistant to oxidation and therefore more stable. It is faster acting and doesn't react with other functional groups. And because it is non-reactive with alkylating agents, reduction and alkylation can be accomplished in the same step.



http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Proteomics

In-gel digestion of proteins is accomplished by applying the same basic steps as for in-solution digests. The entire process is carried out with the proteins still in the gel fragment. Because proteins separated by gel electrophoresis are stained for visualization, stains are removed prior to digestion. Destaining is accomplished by incubating gel slices in a acetonitrile (ACN), ammonium bicarbonate (ABC) solution. Dilute ACN forces water out of the gel fragment, causing the gel to shrink slightly. This helps force free dye out of the gel. After destaining, proteins separated by 1-dimensional electrophoresis are reduced and alkylated. Proteins separated by 2-dimensional electrophoresis only require destaining prior to digestion, as the reduction and alkylation steps are

accomplished between first and second dimension separation (see 2D gel lab for more information).

Digestion. By far the most commonly used enzyme for digestion of proteins is trypsin. Trypsin cleaves after (on the carboxyl side of) arginine and lysine. Other enzymes can be used if different cleavage site specificity is required. Several enzymes and their cleavage sites are listed in the table 1 below. When very broad coverage of a protein is required, for example when post-translational modification (PTM) mapping is being performed, using more than one enzyme (in separate reactions) is necessary. This is because even good mass spectral data won't include m/z information for every peptide in a protein. **This is an important point: for identification, 20-30% coverage is often fine. Whereas for PTM mapping, this level of coverage falls short. Modifications will likely exist in the 70-80% you missed!** By using different proteases you can alter the size of the peptides as well as their relative ionization efficiencies, allowing detection of peptides and PTMs you would otherwise have missed. Table 2 shows the

Table 1. Common enzymes and their specificity sites. The chemical cyanogen bromide is also included

Enzyme	Specificity
Trypsin	Carboxyl side of Arg and Lys
Chymotrypsin (hs)	Trp, Tyr, Phe
Asp-N	Amine side of Asp
Glu-C	Carboxyl side of Glu and Asp
Lys-C	Carboxyl side of Lys
Arg-C	Carboxyl side of Arg
Chemical	**Specificity**
CNBr	Met

Table 2. Cleavage pattern of IL-4 when exposed to various enzymes and CnBR

Enzyme	Cleavage sites	# of sites
Trypsin	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	21
Chymotrypsin	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	12
Asp-N	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSTEQKTLCTETVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	4
Glu-C	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	14
Lys-C	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	12
CnBR	MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGL NSCPVKEANQSTLENFLERLKTIMREKYSKCSS	2

peptide fragments generated when the protein IL-4 is exposed to various enzymes. Note the number and size of peptides produced in each case. The chemical cyanogen bromide (CNBr) is also included. CNBr, which cleaves at methionine residues, is also used to cleave proteins for subsequent proteomic analysis.

Handling and storage of proteases. It is critical that you handle and store proteolytic enzymes properly. Working solutions should be aliquoted and frozen at -80°C for long-term storage. For immediate use solutions can be stored at -20°C. Enzymes should NEVER be left at room temperature, even for very short periods of time. Prolonged placement on ice prior to use should be avoided. Trypsin, particularly in the absence of substrate, will undergo autoproteolysis and there will be insufficient intact enzyme to cleave your sample proteins. Repeated freeze-thaw cycles must be avoided. After an aliquot is thawed, any unused volume should be discarded. Therefore aliquot volumes should be adjusted to minimize waste. Working concentrations. For in-gel digests we use trypsin solutions at concentrations between 25ng and 100ng/μl. For in-solution digests, we use approximately 1:30 trypsin (by weight) to protein sample.

Note: When performing any digest it is important to minimize keratin contamination. Keratin is abundant on skin and hair, and also in room dust on your clothing. It is critical to perform sample preparation steps up to and including digestion, in a keratin-free whenever possible.

Web resources for more information on proteases used for proteomics applications.

http://www.expasy.ch/tools/peptidecutter/
http://prospector.ucsf.edu/prospector/4.0.7/mshome.htm
http://www.soltecventures.com/New%20Product1.htm
http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Proteomics