

Efficient and Specific Trypsin Digestion of Microgram and Nanogram Amounts of Proteins in Organic Solvents

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OVERVIEW

We systematically tested different solvent systems to optimize trypsin digestion of samples containing 10 µg, 2 µg, 1 µg and 200 ng of a protein standard mixture.

- MS data collected from the digested protein standard mixture indicated that a solvent containing 80% acetonitrile consistently resulted in the most complete digestion, producing more peptides than the other solvents tested.
- For example, a one hour digestion in 80% acetonitrile resulted in more than 50% more peptides than the overnight digestion of 1 µg of a protein mixture in aqueous solvent.
- The shorter digestion times possible with the organic solvent improved trypsin specificity, resulting in smaller numbers of semi-tryptic peptides than an overnight digestion in aqueous solvent.
- Analysis ion elution profiles for each LC-MS-MS run indicated that the 80% acetonitrile digestion was the most complete

The goal of this project was to develop methods for improving sample preparation of protein complexes purified from *Rhodospseudomonas palustris*. We compared the two solvents that led to the most efficient digestions for samples of 2 complexes from *R. palustris*: 70S ribosome and GroEL complexes

- The 80% acetonitrile digestion resulted in the most efficient digestion for the 70S ribosome and GroEL

INTRODUCTION

One of the major goals of our group is to develop a pipeline for the high throughput MS analysis of protein complexes from microbes. Currently, we use dual affinity purification to isolate protein complexes, a method that results in low micrograms or less of protein for each purified complex. Because limited sample amounts can complicate enzymatic digestion and protein identifications, we have focused on improving processes in dual affinity purification sample preparation for MS analysis. Current enzymatic protein digestion methods usually require chaotropes or surfactants to denature proteins before digestion. The use of these reagents require procedural steps that often result in reduced digestion efficiency and peptide loss. Because caution should be taken to minimize sample loss when digesting low microgram or nanogram amounts of protein, the drawbacks associated with these reagents make their use detrimental to sensitivity for small amounts of protein. Russell et al. (Anal Chem. 2001, 73, 2682-2685) has used MALDI MS to demonstrate the potential use of organic solvents to efficiently digest proteolytically resistant proteins. We, therefore, present a study testing (See Figure 1) different organic solvent systems for optimizing trypsin digestion of samples containing microgram to nanogram amounts of total protein for subsequent analysis by LC-MS-MS.

EXPERIMENTAL STUDY

Construction of protein standard mixture (PSM)

- PSM was generated using equal weights of 6 proteins
 - Bovine Serum Albumin (BSA) (MW 69 kDa)
 - Yeast alcohol dehydrogenase I (MW 37 kDa)
 - Bovine carbonic anhydrase II (MW 29 kDa)
 - Horse myoglobin (MW 17 kDa)
 - Bovine hemoglobin (MW 15 kDa) Included a and b polypeptide chains
 - Chicken egg lysozyme C (MW 14 kDa)
- Each protein was dissolved in 50 mM Tris-HCl, 10 mM CaCl₂ (pH 7.6) and then combined in equal masses to give samples containing 10 µg, 2µg, 1µg and 200 ng of total protein. The final volume of each mixture was adjusted to 100 µl of one of the digestion solvents listed in Figure 1.

Proteolytic digestion of PSM

- Using the 10 mg samples of the PSM, we compared a digestion protocol including the chaotrope guanidine HCl and a desalting step against protocols that relied upon the solvents alone for denaturation. This protocol is shown in Figure 1a. Because of substantial sample loss we did not perform 1a on smaller sample amounts.
- For all digestions (done in triplicate), the same amount of trypsin (200ng) was added. Figure 1b shows the protocols used to compare different solvent systems.
 - For all PSMs digestions were performed in 100 µl of each solvent shown in Figure 1b.
 - The aqueous solvent was 50 mM Tris-HCl, 10 mM CaCl₂ (pH 7.6). All organic-containing solvents used in this study were mixtures of this aqueous solvent.

Figure 1a



Figure 1b

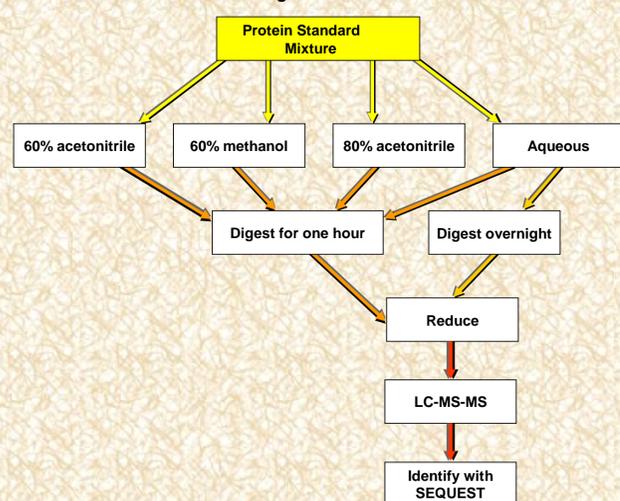
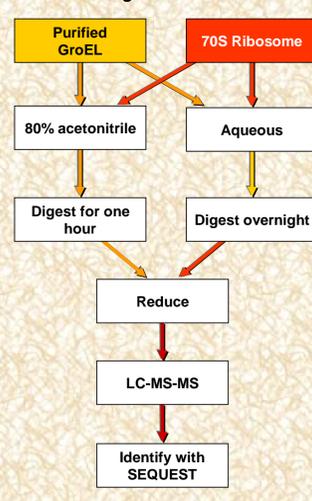


Figure 1c



Digestion of 70S ribosome and GroEL complexes

- Samples of the 70S ribosome and GroEL complexes were digested with the two most efficient digestion solvent conditions as determined by evaluation of the PSM results. These included aqueous overnight and 1 hour 80% acetonitrile digestions (see Figure 1c).

LC-MS-MS analysis

- 1D experiments were performed using an Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA) coupled to an LCQ-DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a nanospray source. Injections were made with a Famos (LC Packings) autosampler onto a 50µl loop. Flow rate was 150 nL/min with a 160 minute gradient for each.
 - An LC Packings nano C18 precolumn (300mM x 5 mm) preconcentrated and desalted the samples on-line with the analytical column.
 - A Vydac (Grace-Vydac, Hesperia, CA) C18 analytical column (300mM id x 25 cm, 300Å with 5mM particles) was directly connected to the nanospray source.
 - For all 1D LC/MS/MS experiments, the LCQ was operated in the data dependent mode with dynamic exclusion enabled, where the top 4 peaks in the 400-2000 m/z range of every full MS scan were subjected to MS/MS analysis.

MS1PeakFinder algorithm to analyze ion elution profiles for MS-MS runs

- We visualized the ions observed during each separation using the "MS1PeakFinder" algorithm developed at ORNL to reveal the retention times and m/z ratios of identifiable and unknown ions for each sample. MatchMap images plot retention time versus m/z and use color to emphasize three classes of ions:
 - Ions observed only during ms (yellow)
 - Ions for which a tandem mass spectrum was collected but not identified (orange)
 - Ions for which a tandem mass spectrum was collected and confidently identified. (red)

Table 1. # of peptides and % tryptic peptides for best LC-MS-MS run out of 3.

Digestion Solvent	Total protein amount							
	0.2 mg		1 mg		2 mg		10 mg	
	Peptides Identified	Tryptic	Peptides Identified	Tryptic	Peptides Identified	Tryptic	Peptides Identified	Tryptic
Overnight Aqueous	105	90%	130	82%	148	68%	138	62%
One hour Aqueous	95	91%	94	93%	125	81%	123	70%
60% Acetonitrile	95	97%	113	93%	165	96%	117	91%
80% Acetonitrile	132	97%	197	97%	197	96%	146	91%
60% Methanol	67	97%	134	96%	137	97%	120	91%

Table 2. Sequence coverage and # of peptides per protein for 1 µg PSM

Proteins	Overnight Aqueous		One-hour Aqueous		60% CH ₃ CN		80% CH ₃ CN		60% CH ₃ OH	
	% seq. cov.	num. pep ^c	% seq. cov.	num. pep ^c	% seq. cov.	num. pep ^c	% seq. cov.	num. pep ^c	% seq. cov.	num. pep ^c
yeast alcohol dehydrogenase I	59	26	51	29	40	16	56	25	36	21
yeast alcohol dehydrogenase II	27	10	32	14	14	5	20	10	10	6
bovine serum albumin	23	16	0	0	19	9	70	61	25	17
horse myoglobin	75	24	50	11	70	25	94	30	98	27
bovine hemoglobin a	79	14	62	13	59	13	71	12	54	11
bovine hemoglobin b	73	22	72	17	77	19	82	20	76	23
chicken lysozyme	38	5	22	2	86	20	66	17	95	16
bovine carbonic anhydrase	59	22	54	14	34	10	72	20	48	17

Figure 2

