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Rapid Detergent Removal From Peptide Samples With Ethyl Acetate For Mass Spectrometry Analysis

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Abstract

Detergents are required for the extraction of hydrophobic proteins and for the maintenance of their solubility in solution. However, the presence of detergents in the peptide samples severely suppresses ionization in mass spectrometry (MS) analysis and decreases chromatographic resolution in LC-MS. Thus detergents must be removed for sensitive detection of peptides by MS. This unit describes a rapid protocol in which ethyl acetate extraction is used to remove octylglycoside from protease digests without loss of peptides. This procedure can also be used to reduce interference by sodium dodecyl sulfate, Nonidet P-40, or Triton X-100 in peptide samples for MS analysis.

Keywords

Detergent removal; mass spectrometry; ethyl acetate extraction; octylglucoside

Introduction

Mass spectrometry (MS) is both the preferred and the dominant analytical tool for proteomic studies, including the identification of proteins and their post-translational modification (Cravatt et al., 2007). Protein sample preparation for MS analysis usually involves purification of proteins followed by site-specific enzymatic cleavage into small peptides. To extract integral membrane proteins and to maintain the solubility of hydrophobic proteins throughout the protease digestion, detergents such as Triton X-100, Nonidet P-40 (NP-40), or octylglucoside (OG) at concentrations of 0.5 − 1% are used in these procedures. The use of detergents during protein purification also reduces non-specific protein-protein association and loss of protein due to adsorption to surfaces. OG has been described as a MS-compatible detergent and is commonly used in protein extraction and isolation for MS analysis (Katayama et al., 2001; Zhang and Li, 2004; Zhang and Neubert, 2006). However, 0.5-1% OG, Triton X-100 or NP-40 severely suppress ionization in MALDI-MS (Katayama et al., 2001; Zhang and Li, 2004) and decrease chromatographic resolution in LC-MS. Thus they must be removed prior to MS analysis. Procedures for the removal of detergents from protein solutions prior to protease digestion have been described (UNITS 4.8 and 23.6). Usually, the protein is precipitated with trichloroacetic acid (TCA), the precipitate washed with acetone to remove TCA, the acetone evaporated by SpeedVac and the dried protein precipitate dissolved in 8M urea. Prior to protease digestion, the urea concentration is reduced by dilution to a level tolerated by the protease. However, the low microgram or sub-microgram quantities of protein often obtained from purification for protein identification

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and structural analysis will not produce visible precipitates with TCA. Furthermore, the acetone-precipitated and dried protein is very difficult to dissolve in 8M urea and the urea solution must be diluted many fold prior to protease digestion. Thus this procedure is unsuitable for the removal of detergent from the small amounts of protein usually available for MS analysis.

Removal of detergent from the peptides after protease digestion of a protein can also be achieved by ion-exchange chromatography. However, this procedure needs optimization for each digest, results in an increase in volume and salt content, and will not remove peptide-bound detergent. Although an acid-labile detergent has been used with the liquid phase separation and digestion method (Nomura et al., 2004; Chen et al., 2007), its high cost and the need for larger volumes severely limits its application. In view of these limitations, we developed a simple method to extract OG from protease-digested samples with the water-immiscible organic solvent, ethyl acetate (Yeung et al., 2008).

Extraction of Detergent from Peptide Samples

Since OG can be extracted completely with ethyl acetate without detectable loss of peptides, it is desirable to exchange other existing detergents in the protein solution with OG before digestion. Methods for detergent exchange have been described in UNIT 4.8. Following exchange, the protein in OG is denatured with 6M GnHCl, then reduced, carboxymethylated and the denaturing reagent removed by ultrafiltration or dialysis against 0.5% OG prior to digestion. As an alternative to the GnHCl denaturation treatment, organic solvents compatible with the protease used (e.g. 5-20% acetonitrile for trypsin) are included in the digestion mixture to increase the efficiency of the digestion (Chen et al., 2006; Masuda et al., 2006).

Materials

Ethyl acetate (HPLC grade or better, in a glass bottle)

Milli-Q (Millipore) or glass double-distilled water

Acetic acid, formic acid, or trifluoroacetic acid (HPLC grade or better)

pH 1-12 pH paper

Equipment

125ml glass bottle with glass stopper

1.5ml microfuge tubes

200μl round gel-loading micropipette tips

Vortex mixer

Microcentrifuge (13,000 to 16,000 × g maximum speed)

Speed vacuum system with a Savant SpeedVac, refrigerated vapor trap, vacuum gauge and vacuum pump, or equivalent system

Extraction of peptide solution by water-saturated ethyl acetate—Extraction can be performed immediately after digestion or on stored samples. Acidification of the protein digest is not necessary. The volume of the peptide solution should be between 50 and 100μl in a 1.5ml microfuge tube. Larger volumes can either be split into two tubes or reduced to 100μl by SpeedVac. However, the salt concentration in the peptide solution should be

maintained at less than 200mM to prevent possible loss of peptide to the organic phase during extraction.

1. Add 1ml of water-saturated ethyl acetate to the peptide solution, close the cap and vortex the contents vigorously for 1min at room temperature.
2. Centrifuge for 15 sec in a microcentrifuge at 13,000 rpm (10,000 – 16,000 × g) at room temperature to separate the ethyl acetate phase from the aqueous phase.
3. Carefully aspirate the upper ethyl acetate layer using a gel loading tip attached to a vacuum line until 1mm of the ethyl acetate layer remains (do not aspirate all the ethyl acetate layer as this will result in loss from the aqueous peptide-containing phase).
4. Repeat the extraction steps 1 – 3, five times.
5. Remove the small amount of ethyl acetate on top of the sample in a SpeedVac for 3min with heating.
6. If ice forms, warm the tube with your palm to thaw the ice before the next step.
7. Mix the solution gently (after thawing) and SpeedVac it for 4min, two or more times, until it is reduced to less than 50% of the original volume. Thaw out any ice that forms before the next round of SpeedVac drying. This volume reduction will remove nearly all of the remaining dissolved ethyl acetate from the sample. The volume of the sample can be reduced to 10 to 20μl. Complete dryness is not recommended.
8. Acidify the sample to pH 2-3 by adding 1μl 50% acetic acid at a time. Check the pH of the sample after each addition by spotting 0.2-0.5μl of the resultant mixture on pH paper. The acidified sample can then be stored either at 4°C for 3 days or less, or alternatively frozen quickly with liquid nitrogen or dry ice and stored at -80°C for weeks.

If the sample is to be processed immediately, it can be acidified with formic acid or TFA as needed.

Support protocol

Preparation of water-saturated ethyl acetate—All glassware is acid-washed, rinsed with Milli-Q water followed by ethyl acetate. Prepare only the volume needed for the next 2 weeks.

1. Add approximately 10ml water to approximately 80ml of ethyl acetate in the 125ml glass bottle. Accurate measurement of the solution is not required here.
2. Cap the glass bottle with its glass stopper and shake the contents vigorously for 1 min at room temperature so that the water breaks into tiny droplets in the ethyl acetate.
3. Rest the bottle on the work bench for 5min and repeat the shaking two more times.
4. Rest the bottle on the bench for about 30min or until a clear layer of ethyl acetate formed on top of water.
5. Leave the water at the bottom with the solvent in the bottle and use only the clear upper layer of ethyl acetate for extraction.
6. Store the water-saturated ethyl acetate at room temperature in the dark and discard after 2 weeks.

Commentary

a. Background information—The most commonly used procedure for protein preparation for MS analysis is in in-gel digestion of purified or partially purified proteins separated by one dimensional or two dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. In this protocol, detergent is extracted by organic solvent from the gel before digestion, but the resulting peptide yield is very low. Peptide recovery from in-gel protein digestion can be increased by using an expensive, acid-labile detergent that is degraded under acidic conditions into products that do not interfere with MS analysis (Nomura et al., 2004). Inclusion of low concentrations of saccharide-based detergents, OG or 5-cyclohexyl-1-pentyl-β-D-maltoside, in the digestion buffer has also been shown to increase peptide recovery from in-gel digestion without severely decreasing the sensitivity of MS analysis (Katayama et al., 2001, 2004). Even with these improvements, peptide yield from in-gel digestion is much less than that from liquid phase protein separation and digestion. Protocols utilizing liquid phase separation result in high peptide yields but they require the use of high concentrations of detergents (Nagele et al., 2004). Indeed, Zhang and Neubert (2006) have demonstrated a dramatic improvement of selectivity in immunopurification of tyrosine phosphorylated peptides by including 1% OG in the immunoprecipitation buffer. Since protocols currently available for removal of detergent from protein sample are not suitable for the small amounts of protein usually available for MS analysis, a simple procedure to remove detergents after protease digestion of protein samples for MS analysis is desirable.

To develop the simple method described above for the extraction of OG after protease digestion, we tested the solubility of OG against a variety of organic solvents, including ethyl acetate, n-butanol, hexane, n-heptan, n-octane and trichloro-triflluoro-ethane. OG was most soluble in ethyl acetate, less soluble in n-butanol and not significantly soluble in the other solvents tested. Ethyl acetate was chosen for the extraction because of its high volatility, low solubility in water and its efficacy as a solvent for OG.

Other than removal of OG, ethyl acetate extraction is also able to eliminate interference by SDS, but with some loss of larger peptides. Ethyl acetate extraction can also greatly reduce the interference in MALDI-MS analysis by NP-40 and Triton X-100 (Yeung et al., 2008). Recently, Masuda, et al. (2008) demonstrated that ethyl acetate effectively extracted sodium deoxycholate from tryptic peptides and that no peptides were found in the ethyl acetate phase. Thus ethyl acetate extraction may also remove detergents other than those mentioned above from peptide samples to reduce detergent interference in MS analysis. Even without detergent, ethyl acetate extraction results in a significant enhancement of peptide detection in the MS analysis (Yeung et al., 2008). Furthermore, this extraction protocol can be performed rapidly for multiple samples without sophisticated equipment.

b. Critical parameters and trouble shooting—It should be emphasized that extraction is a two-way process. Hydrophobic molecules can be partitioned to the organic solvent from the aqueous solution, but at the same time, hydrophilic molecules in the organic solvent can also be partitioned to the aqueous phase. Hence it is very important to use the highest quality extraction solvent and avoid contaminated utensils in the storage and transfer of the solvent. Acid washed glass bottles and pipettes should be used for the storage and transfer of ethyl acetate. Poly-propylene or poly-ethylene tubes and pipette tips can be used for short term extraction and transfer provided they are rinsed with water-saturated ethyl acetate before use. Poly-propylene or poly-ethylene containers should not be used for long-term storage of the solvent. We observed many low m/z contamination peaks in our peptide mass spectra when we extracted our peptide samples with ethyl acetate that had been stored in a polyethylene tube for a few days.

Extraction should be performed at room temperature with all the reagents and equipment equilibrated at this temperature. At 0-4°C, detergents like SDS tend to form insoluble precipitates at the junction of the two phases and a significant amount of the detergent will remain with the peptides after extraction. Common to all extraction procedures, mixing must be vigorous to ensure equilibrium for partition. Furthermore, extraction efficiency improves the higher the organic- to aqueous-phase ratio. We therefore recommend five to ten times the volume of solvent to peptide solution in each extraction.

c. Anticipated results—The ethyl acetate extracted peptide solution will contain only trace amounts of ethyl acetate (or other volatile organic solvents added during digestion or purification) and all the non-volatile ingredients in the original digestion buffer. It should be ready for direct LC/ESI-MS/MS or indirect LC-MALDI-MS/MS analysis. For less complex protein digests or peptide mixtures, the extracted sample can be desalted by C18 Ziptips (Millipore) and analyzed directly by MALDI-MS/MS.

d. Time considerations—The time taken to perform the extraction of one sample is about 35min. Within this period, 1min will be spent on vortexing, 2min for separation of the two phases by centrifugation, 30sec for aspiration, 30sec for addition of the solvent, 16min repeating the above procedure four more times, and 15min for removal of solvent by SpeedVac. For multiple samples, vortexing (with a multi-tube holder), centrifugation and SpeedVac can be carried out at the same time. Hence it takes a shorter time per sample to process multiple samples (about 50 to 60min for 10 samples).

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