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COMPLETE TRYPTIC DIGESTION OF MEMBRANE PROTEIN BACTERIORHODOPSIN

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Overview

Integral membrane proteins play vital roles in many cellular and physiological processes. Due to their hydrophobic nature, characterization of the integral membrane proteins is very difficult. Surfactants are typically used to enhance protein solubility prior to enzymatic digestion. However, introduction of surfactant usually causes reduction in proteolytic enzyme activity and compromises the downstream liquid chromatography and mass spectrometry analysis. We applied a novel acid degradable surfactant, RapiGest™ SF, to improve the solubility of a model membrane protein, Bacteriorhodopsin (BR) upon enzymatic digestion. Observed peptides from LC/MS analysis cover 99% of the protein sequence.

Introduction

Enzymatic digestion of membrane proteins requires the use of surfactants in order to obtain peptides in the transmembrane region. However, most surfactants are disruptive to enzyme activity and interfere with LC and MS analysis. We developed an improved membrane protein digestion method using a novel acid degradable surfactant, Waters RapiGest SF. This surfactant has been shown to be compatible with proteolytic enzymes, while denaturing the protein substrates [1]. When RapiGest SF was applied to denature membrane protein, Bacteriorhodopsin (BR), it facilitated the solubilization of the protein hence complete enzymatic digestion of BR was achieved without using a large amount of protein.

It is well known that surfactants interfere with LC and MS analysis [2]. Dialysis is the typical method for removing surfactants with the risk of significant sample loss. The surfactant described here was designed to be degradable under acidic condition, therefore, minimizing the surfactant caused LC or MS interferences. The degradation greatly simplifies sample treatment for LC or MS analysis. In addition, it has been found that in low quantity (< 2 µg), intact RapiGest SF was tolerated by RP LC/MS (LC column,1.0 x150 mm) (Fig.1).

RapiGest SF rapidly degrades at low pH to two products; one is water soluble and the other one is water immiscible (forms an oily layer). We found that all hydrophobic transmembrane tryptic peptides from the BR were trapped in the oily layer after the surfactant was degraded (Figure 2). These trapped hydrophobic peptides were extracted with isopropanol. This unique property of RapiGest SF was utilized to fractionate hydrophilic and hydrophobic peptides prior to LC/MS or LC/MS/MS analysis.

Methods

Enzamatic Digestion of Bacteriorhodopsin

BR (1 mg, 75% pure, Sigma) was solubilized in 500 µL of 0.1% RapiGest SF solution. The sample was boiled for 5 minutes and then cooled to room temperature. Five hundred µL of 50 mM NH_4HCO_3 buffer (pH 7.9) was added to the sample followed by addition of 20 µg porcine trypsin (Promega). The protein sample was digested overnight at 37 °C.

Sample Preparation for LC/MS and LC/MS/MS

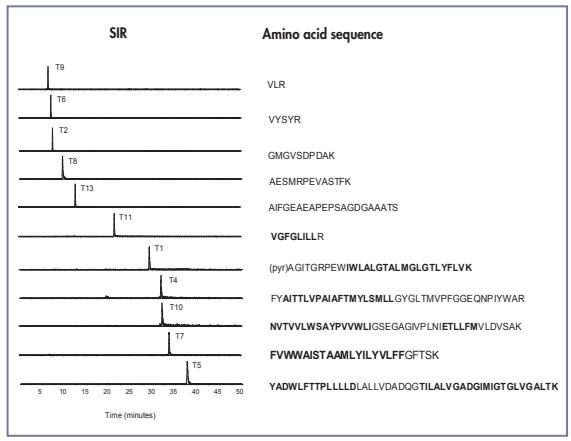
RapiGest SF was degraded with acid prior to LC/MS analysis (TFA, 0.5%, v/v). This sample was incubated at 37 °C for 30 minutes to ensure the complete degradation of RapiGest SF. Formation of a water immiscible pellet was observed. The sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant containing hydrophilic peptides was analyzed by LC/MS (Fig. 2A). The pellet containing the hydrophobic peptides was washed with Milli-Q® water, followed by isopropanol (70%) extraction. Sonication was applied to improve the extraction yield. After the extraction, the BR digest sample was centrifuged once more before LC/MS analysis (Fig. 2B). Experimental results also suggest that low quantities of intact RapiGest SF did not interfere with LC separations (Figure 1).

Liquid Chromatography and Mass Spectrometry Instrumentation

BR peptides were operated by a RP-HPLC system (Waters CapLC®). A C_{18} microbore RP LC column (1.0 x 150 mm, 3.5 µm, Waters) and a capillary RP LC column (0.32 x 100 mm, 3.5 µm, Waters) were used to separate the tryptic peptides (LC conditions are specified in the Figures). The LC separated peptides were interfaced to either a Tof MS (Waters Micromass LCT™) or a Waters Micromass Q-Tof micro™ MS instrument. Automatic Data Directed Analysis (DDA™) controls the switching between MS and MS/MS mode for CID experiments.

Results

LC:	Waters CapLC®
Column:	Waters Symmetry300™ C_{18}, 1.0 x 150 mm, 3.5 µm
Solvent A:	0.1% Formic acid
Solvent B:	0.065% Formic acid in ACN:isopropanal (1:1)
Gradient:	0 - 90% B, 2% per min
Flow rate:	35 µL/min
Injection:	4 µL (~ 3 µg)
MS:	TOF MS (Waters Micromass LCT™)



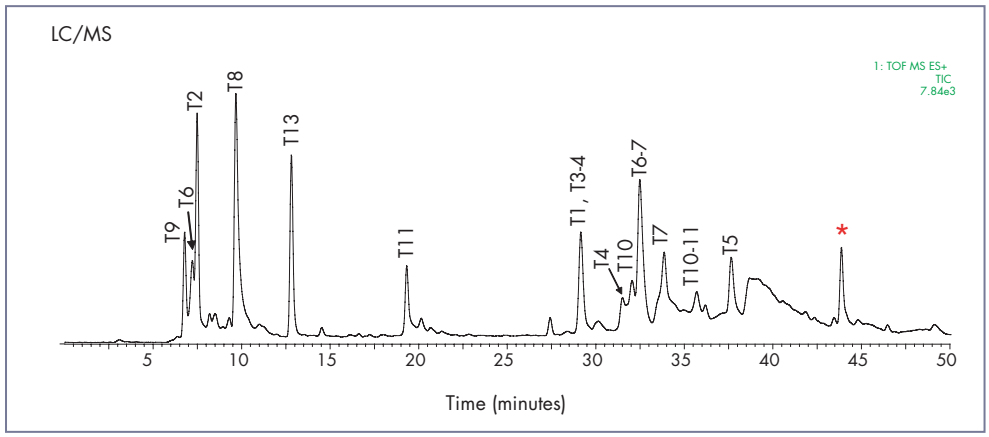


Figure 1. Trypsin digested BR (~ 3.0 µg) was separated and mass analyzed by LC/MS. The sample was loaded onto the RP LC column directly without degrading the RapiGest SF. The mass identified tryptic peptides were labeled in the Figure (indicates an impurity from the sample). The SIR chromatograms for the tryptic peptides were shown on the above right along with their amino acid sequences (transmembrane portions are labeled in bold). The identified peptides cover about 99% of the amino acid sequence of BR. Note: the missing peptides, T3 and T12 were not detected since they contain only one or two amino acids.*

LC:	Waters CapLC®
Column:	Waters Symmetry300™ C_{18}, 0.32mm x 100 mm, 3.5 µm
Solvent A:	0.1% Formic acid in 100% Water
Solvent B:	0.065% Formic acid in ACN:isopropanal (1:1)
Flow rate:	10 µL/min
Gradient:	0 - 90% B, 2% per minute
Injection:	1 µL (~ 0.75 µg)
MS:	TOF MS (Waters Micromass LCT™)

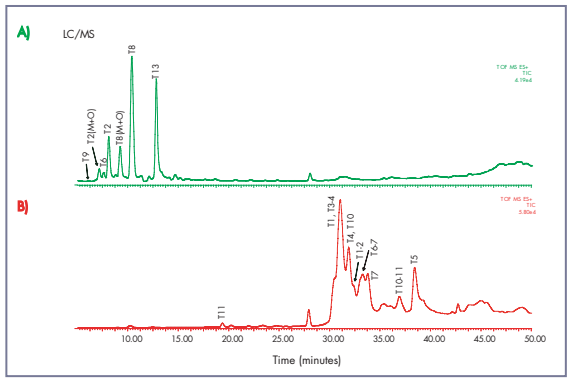


Figure 2. LC/MS (TIC) of BR peptides generated from trypsin enzymatic digestion. RapiGest SF was degraded with acid prior to the LC/MS analysis. The degradation of the surfactant enables the fractionation of hydrophilic/hydrophobic peptides to two phases, one of which is in aqueous and the other is highly hydrophobic. A) LC/MS separation of the hydrophilic peptides. B) LC/MS separation of the hydrophobic peptides that are in transmembrane regions of BR. The identified peptides from the combined LC/MS runs cover about 99% of the amino acid sequence of BR.

LC:	Waters CapLC®
LC conditions:	see Figure 1
MS:	Waters Micromass® Q-Tof micro™

Collision energy: 30-60 eV (depends on the charge states)

Switch threshold: 5 counts/s

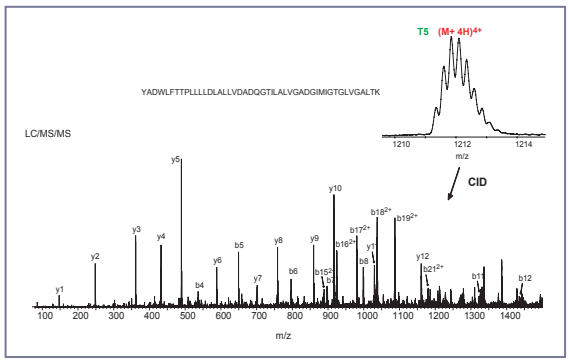


Figure 3. LC/ESI/MS/MS of the BR peptides was performed to further confirm their identity. For example, partial sequence information was obtained for the largest peptide T5. Pepseq program in MassLynx™ 4.0 software was used to assign the fragmented ions (y and b series).

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Conclusions

Results suggest that RapiGest SF:

- Improves the enzymatic digestion of membrane proteins.
- Enables a complete digestion of membrane proteins, all transmembrane peptides from bacteriorhodopsin were generated and identified by LC/MS (99% sequence coverage).
- Simplifies digestion and sample handling.
- Degrades rapidly under acidic condition to two phases (water soluble/water immiscible), hence, allows for the fractionation of hydrophilic and hydrophobic peptides.
- Can be directly analyzed with LC/MS even without sample preparation (low quantities injected on RP-HPLC column).

References

1. Waters Applications Note at http://www2.waters.com/Watprod.nsf/docs/WA20774.html

2. K. M. Swiderek, A. J. Alpert, A. Heckendorf, K. Nugent and S. D. Patterson, "Structure Analysis of Proteins and Peptides in the Presence of Detergents:Tricks of the Trade", *Journal of Biomolecular Techniques,* December **1997**.

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