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EVALUATION OF RAPIGEST EFFICACY FOR THE DIGESTION OF PROTEINS FROM CELL CULTURES AND HEART TISSUE

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Abstract

Introduction. *Rapigest is an acid-labile detergent used in proteomics for the improvement of protein digestion.*

Materials and method. *To test the efficacy of Rapigest for proteomics analysis of different sample types we used protein extracts from S9 cell line and mouse heart tissue and performed protein isolation, digestion and mass spectrometry analysis.*

Results. *For the S9 cell line, there was no significant difference concerning the number of identifications (peptides, proteins) between Rapigest and No Rapigest samples, though slightly more peptides and proteins were identified in the Rapigest samples. For the mouse heart tissue samples, Rapigest use resulted in the identification of a higher number of proteins. Rapigest did not modify the protein profile with respect to the biological compartments covered by the identified proteins in S9 cell line samples, but produced a small increase in the representation of cytoplasm proteins and a small decrease in the representation of membrane proteins in the mouse heart tissue samples.*

Discussions. *Results are comparable to other studies that evaluated the efficacy of Rapigest for the analysis of tissue samples, recommending Rapigest for the improvement of protein digestion and implicitly identification, without the modification of the protein profile in the samples.*

Conclusion. *Rapigest may be successfully used for the improvement of protein identification from heart tissue samples using mass spectrometry.*

Keywords: Proteomics, Rapigest, cell line, mouse heart ventricles.

Introduction

Proteomics is the sequencing and study of the abundance of all proteins in a biological system [1]. Proteomics has recently focused on the discovery of molecules useful for the diagnosis, monitoring and prognosis of diseases, also known as biomarkers [2].

The use of animal models in proteomics research focuses on identifying those biomarkers that could have a translational value - biomarkers with useful applications in clinical settings [2]. However, animal samples, as well as human samples, may present with challenges concerning protein isolation/extraction and identification. Thus, in the search for more accurate and efficient methods of protein identification, improvement of all aspects of protein analysis is necessary, especially protein extraction from tissue and protein digestion for mass spectrometry analysis.

Rapigest, also known as sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxy]-1-propanesulfonate is an acid-cleavable anionic detergent used to enhance the enzymatic digestion of proteins. Rapigest negatively charges the surface of the proteins, exposing the important cleavage sites for enzymes such as trypsin, Lys-C, Asp-N and Glu-C [3], and presumably enhancing the efficacy of the protein digestion resulting in more protein identifications during proteomics analysis.

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To test the efficacy of Rapigest for proteomics analysis of different sample types we designed a simple study, using cells from S9 cell line and mouse heart tissue, and performed protein isolation, digestion (with and without Rapigest) and mass spectrometry analysis.

Materials

Cell culture

The first type of biological sample used was adeno12-SV40-immortalized human airway epithelial cell line S9 (ATCCs number CRL-2778) cultured in minimal essential medium (MEM; PromoCell, Heidelberg, Germany) supplemented with 4% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 1% non-essential amino acids (NEAA), and 4 mM glutamine (both PAA Laboratories GmbH, Pasching, Austria) in a humidified incubator at 37°C with 5% CO2. Cells were divided into six replicates (cell density approximately $5x10^6$ cells for each replicate). Three replicates from every sample type were used for protein digestion with Rapigest (S9R1, S9R2 and S9R3) and in comparison, the other three samples (S9R*1, S9R*2 and S9R*3) were digested without the addition of Rapigest.

Mouse ventricular tissue

The second type of biological samples used was left ventricular tissue sections from C57BL/6 wild type mice. The animals were maintained in the animal facility of the University Medicine of Greifswald in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S.NIH [4]. Experimental protocols were approved by the local authorities.

Samples were divided into six replicates each. Three replicates were used for protein digestion and mass spectrometry analysis using Rapigest (TR1, TR2 and TR3). The other three replicates were digested without Rapigest (TR*1, TR*2 and TR*3).

Method

Protein extraction

S9 cells were harvested by aspirating the medium and washing the cells twice with phosphate buffered saline (PAA), before cells were detached in 300 µl of 8M urea/2M thiourea. Cell lysis was supported by subsequent freezing (liquid N_2) and thawing (15 min, 37°C, 1400 rpm) of cells three times.

Whole left ventricle tissue samples were first snap-frozen in liquid nitrogen and then homogenized into a fine powder using a Mikro dismembrator (Braun, Melsungen, Germany) at 2600 rpm for 2 min. Urea/thiourea 2M/8M (1000 µl) was then added to the samples for cell lyses and protein denaturation.

Further, all samples were sonicated for nucleic acid fragmentation using a Sonoplus (Bandelin, Berlin, Germany) and cell debris pelleted by centrifugation (60 min, 4°C, 16.000 x *g*). The protein containing supernatants were transferred to new tubes and stored at -80°C until further use. Protein quantitation was performed using Bradford assay kit with bovine serum albumin as standard protein [5].

Protein digestion

Rapigest in 50 mM ammonium bicarbonate was added to the samples (TR1, TR2, TR3 and S9R1, S9R2 and S9R3) containing 4 µg protein, final concentration 0.1% Rapigest w/v. The other samples (TR*1, TR*2, TR*3 and S9R*1, S9R*2 and S9R*3) were further processed without the addition of Rapigest. All samples were afterwards subjected to reduction with 2.5 mM DTT for 1h at 60°C and alkylation with 10 mM iodoacetamide for 30 min at 37°C. Further, samples were digested with Lys C (Sigma) in a ratio of 1:100 for 3h and then overnight (~16h) with trypsin (Promega) in a ratio of 1:10. Digestion was stopped with 1% acetic acid and the samples were purified with C-18 resin tips with a binding capacity of 2 µg (Merck Millipore, Darmstadt, Germany).

Mass spectrometry analysis

Mass spectrometry analysis was performed using LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) after pre-fractionation of peptides by reverse phase nano-HPLC. Proteins were identified using Proteome discoverer 1.3 searching against a UniProt Swiss-Prot release 2010_11 database limited to human or mouse entries respectively, with a mass tolerance of 10 ppm for peptide identifications. Methionine oxidation was set as variable, carbamidomethylation as fixed modification and up to two missed tryptic cleavages were considered. Peptide identifications were considered significant if: peptide confidence was high corresponding to a false discovery rate of <1%; charge dependent Xcorrelation score for two-fold charged peptides was 2.2, and for three and four-fold charged peptides 3.75.

Results

The study included two different sample types: at first cells of a eukaryotic cell line were processed, which lyse and are digest readily. As a second sample type, heart tissue was chosen. Heart tissue constitutes of a complex cytoskeletal structure and contains a lot of membrane proteins from the high number of mitochondria, the sarcolemma and the cytoplasmic membrane, which are known to be solubilized and digested more difficult [6].

For the S9 cell line samples, in total more than 4000 peptides and 1000 proteins per replicate were identified. The mass spectrometry analysis revealed that there was no significant difference between the samples digested in the presence of Rapigest or without Rapigest (*Figure 1*) regarding the number of peptides (p=0.15), the number of proteins identified based on at least one unique peptide (p=0.053) or the number of proteins identified based on at least two unique peptides (p=0.103). However, an improvement of protein coverage in the presence of Rapigest

was registered, even if not significant, with a slightly higher identification rate observed in samples digested with Rapigest (combined analysis for proteins identified by at least one unique peptide: Rapigest proteins=1281; No Rapigest proteins=1197).

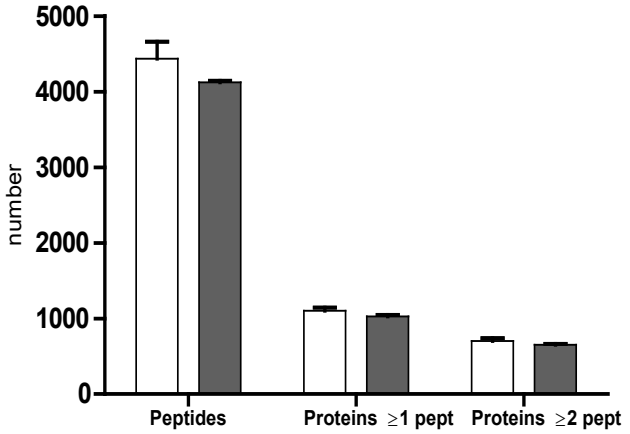

Figure 1. Number of peptides and proteins identified by mass spectrometry analysis in the S9 cell line samples. Samples were digested with (white bars) or without Rapigest (grey bars).

Figure 2 represents the number of peptides and proteins identified by mass spectrometry analysis in the mouse ventricular tissue samples. There was no significant difference between the number of peptides identified in Rapigest and No Rapigest samples (p=0.90). However, proteins identified based on at least one unique peptide were found to be significantly more in the Rapigest samples, compared to the No Rapigest samples (p=0.009), (combined analysis Rapigest proteins=651; No Rapigest proteins=624). Additionally, proteins identified based on at least two unique peptides were identified significantly more in the Rapigest samples than in the No Rapigest samples (p=0.03).

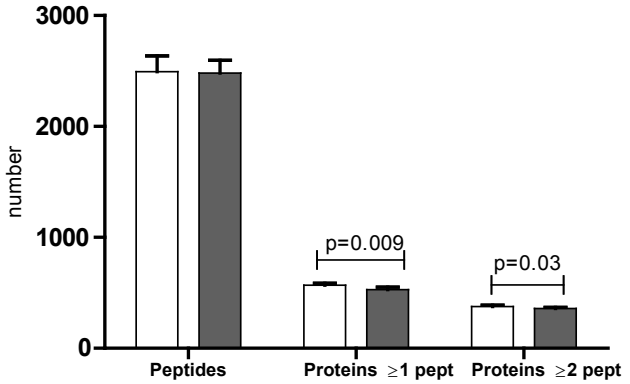

Figure 2. Number of peptides and proteins identified by mass spectrometry analysis in the mouse ventricular tissue samples. Samples were digested either with (white bars) or without Rapigest (grey bars).

For the S9 cell line (*Figure 3A*), cellular components coverage of the identified proteins was similar between the two sample types (Rapigest and No Rapigest), with cytoplasm proteins being the most abundant (23.72%; n=304 - Rapigest, 23.53%; n=282 - No Rapigest), followed by nuclear proteins (14.82%; n=190 - Rapigest, 15.21%; n=182 - No Rapigest). Membrane proteins (13.67%; n=175 proteins - Rapigest, 13.73%; n=164 proteins - No Rapigest) were also well covered by the extracted proteins resulting in a similar protein profile. Cytoskeletal proteins were well identified in both sample types (6.10%; n=78 Rapigest, 6.22%; n=74 No Rapigest).

The proteins identified in the mouse ventricular tissue covered approximately in the same proportion the cellular components, independently of the use of Rapigest (*Figure 3B*). However, there was a small over-representation of the cytoplasm proteins (24.32%; n=158 - Rapigest, 23.58%; n=147 - No Rapigest) and a small under-representation of the membrane proteins (14.95%; n=97 - Rapigest, 15.44%; n=96 - No Rapigest) in the Rapigest samples.

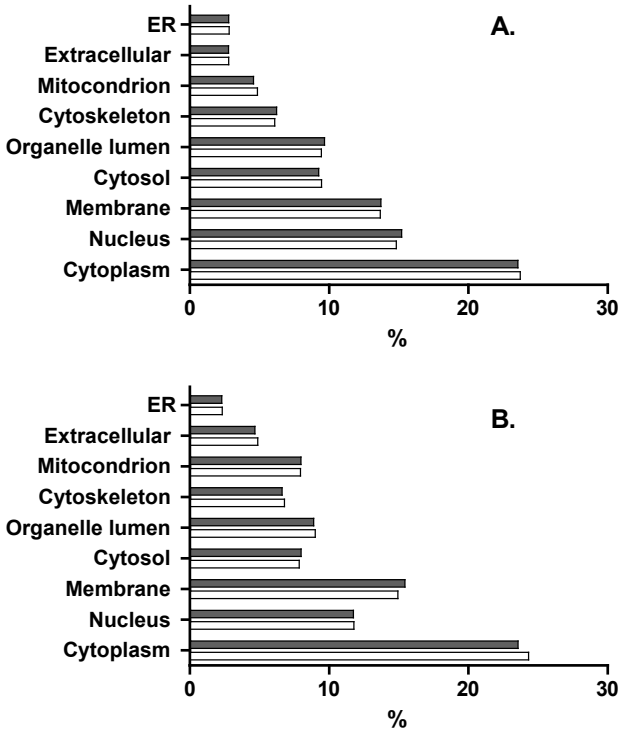

Figure 3. Cellular components (%) covered by the proteins identified in the cell line samples (A.) and in the mouse ventricular tissue samples (B.). Proteins were digested with (white bars) or without (grey bars) Rapigest.

Discussion

Our analysis revealed that augmentation in the number of protein identifications for the mouse heart samples encourages the use of Rapigest in proteomics

analysis for the discovery of translational cardiovascular biomarkers, and makes it more suitable for the enhancement of the digestion of complex samples such as tissue samples, especially cardiac tissue, without any obvious benefit for S9 cell line extracted proteins. In this respect, *Kline et al* have recently used Rapigest successfully for the digestion of proteins from human heart explants in order to enhance protein digestion efficacy [7]. Although a significant effect on the proportion of membrane proteins identified was not observed, Rapigest might support the destruction of the cytoskeletal structure in the cardiomyocytes, thereby supporting the release of cytoplasmic proteins, over-represented in the Rapigest treated samples but not in the samples without Rapigest.

Rapigest has been used successfully by others to help analyze orthopoxviruses that were grown in HeLa cells [8]. In a method optimization study for peptide profiling of micro-dissected breast carcinoma tissue, Rapigest efficacy was compared to that of SDS, resulting in detection of the highest number of sample specific peak signals with the use of 0.1% SDS. However, there was little overlap in detectable peaks using the different buffers, implying that they can be used complementarily to each other [9].

Another report evaluated detergent-based sample preparation workflows for the MS-based analysis of bacterial proteomes, performed on *Escherichia coli*. SDS performed best in terms of total protein yields and overall number of MS identifications, mainly due to a higher efficiency in extracting high molecular weight (MW) and membrane proteins, while Rapigest led to enrichment in periplasmic and fimbrial proteins [10]. However, Rapigest performed better at enabling digestion of membrane proteins from *Escherichia coli* than SDS in another study [11]. Rapigest has also proven to be useful in the identification of proteins with post-translational modification, such as N-linked glycosylation, particularly important for the biopharmaceutical analysis field [12].

In our study, Rapigest did not influence significantly the protein cellular component coverage profile of the samples. Rapigest did not enhance the identification of membrane proteins, whose hydrophobic nature complicates their extraction, proteolysis and identification. For the improvement of membrane protein identification, other detergents such as sodium laurate or sodium deoxycholate have been used successfully [13]. Nevertheless, other reports state similar efficacy in the identification of membrane proteins with the use of Rapigest and sodium cholate [14].

Conclusions

Rapigest helps solubilize proteins, making them more susceptible to enzymatic cleavage without inhibiting enzyme activity. Unlike other commonly used denaturants, such as SDS or urea, Rapigest does not modify peptides or suppress protease activity. It is compatible with enzymes such as trypsin, Lys-C, Asp-N and Glu-C and other enzymes. Rapigest is also compatible with mass spectrometry analysis, resulting in the increase of the number of identified proteins in mouse heart samples, without modifying the protein profile of the sample.

Our study revealed that by using Rapigest for the digestion of proteins from the S9 cell line we obtained a small, but not statistically significant improvement in the number of peptides and proteins identified. However, mouse heart tissue benefited from the addition of Rapigest before digestion of proteins and showed significant increases in the identification of proteins. Rapigest did not modify significantly the profile of the protein cellular components identified in the samples, although for the mouse heart tissue samples, the cytoplasm proteins were slightly over-represented in the Rapigest samples as compared to the No Rapigest samples and membrane proteins were slightly under-represented in the Rapigest samples.

All in all, our study supports the use of Rapigest for the proteomics analysis of heart tissue samples.

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