Analysis of Single Amino Acid Mutations in Intact Proteins

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Abstract
With the increasing availability of high resolution mass spectrometers the analysis of intact proteins becomes a feasible alternative to the more commonly employed shotgun techniques in proteomics. High resolution separations of proteins are hindered by the size of the analyte molecules and the resulting low diffusion rates especially inside the pore system. The stationary phase of the column employed in these studies is a 3 µm C8 silica with 1000 Å pore size. By using a dedicated protein separation column (ProteCol™ C8 HQ1003) we demonstrate that it is possible to separate single amino acid mutants in a 33 kDa protein.

Protein Separation and Diffusion
Diffusion is the time-limiting process in the separation of macro-molecules such as proteins. Due to their size the diffusion rate in free solution is significantly lower than the diffusion rate of small molecules. Diffusion is the time-limiting process in the separation of macro-molecules such as proteins. Due to their size the diffusion rate in free solution is significantly lower than the diffusion rate of small molecules. In the most common reversed phase chromatography, it is important to have unrestricted access to the surfaces inside the pores even when the column is made of a packed capillary, with 1/32” OD, 50 µm ID PEEKsil™-G C8 HQ1003, 300 µm ID x 100 mm. The change in hydrophobicity and the retention time usually correlates well with the shift in retention time of the intact protein. However, with the increasing resolving power of the modern mass spectrometer and the inherent compatibility of the reversed phase solvent systems with MS analysis, we explored the potential of wide pore silicas for the accurate mass analysis of intact proteins. In the examples reported here we were able to separate variations of a 33 kDa protein which differed by a single amino acid mutation.

Experimental
Nine single amino acid mutations of a 33 kDa protein were analysed by RP-µLC-MS to confirm the right structure of the mutant protein. Samples supplied at concentrations ranging from 0.3 to 1 mg/ml. 100 µl of samples were desalted with SE spin column and resultant filtrate directly injected at a volume to equal the injection of 1 µg i.e. 2 µl injected of 0.5 mg/ml solution assuming no losses at filtration step.

Chromatographic conditions:
Instrument: Agilent 1200 Cap-LC with Bruker micrOTOF-Q MS
Column: ProteCol™-G C8 HQ1003, 300 µm ID x 100 mm
Flow rate: 5 µl/min
Mobile phase A: 0.1 % formic acid
Mobile phase B: 95 % acetonitrile, 0.1 % formic acid
Gradient: 0 min 5 % B 30 min 55 % B 33 min 70 % B 38 min 70 % B 40 min 5 % B

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The shift in retention time can be correlated to the change in hydrophobicity. A hydrophobicity scale published by Cowan and Whitaker was used because it is based on HPLC retention data (R.Cowan and R.G. Whitaker, Peptide Research, 3 (1990) 75-80). As shown in figure 6, there is a good correlation between the change in hydrophobicity and the retention time. The open circles indicate mutated proteins where the measured mass differs significantly from the predicted mass. The shift in retention time can be correlated to the change in hydrophobicity. A hydrophobicity scale published by Cowan and Whitaker was used because it is based on HPLC retention data (R.Cowan and R.G. Whitaker, Peptide Research, 3 (1990) 75-80). As shown in figure 6, there is a good correlation between the change in hydrophobicity and the retention time. The open circles indicate mutated proteins where the measured mass differs significantly from the predicted mass.

Discussion/ Conclusion
The separation of proteins by reversed phase chromatography remains an uncommon technique due to the poor recovery of proteins from the commonly used 300 Å pore size stationary phase. However, with the increasing resolving power of the modern mass spectrometer and the inherent compatibility of the reversed phase solvent systems with MS analysis, we explored the potential of wide pore silicas for the accurate mass analysis of intact proteins. In the examples reported here we were able to separate variations of a 33 kDa protein which differed by a single amino acid mutation.

The change of a single amino acid influences the hydrophobicity and this change correlates well with the shift in retention time of the intact protein. In the separation of large molecules, especially in an adsorption mode such as reversed phase chromatography, it is important to have unrestricted access to the surfaces inside the pores even when an analyte molecule is already adsorbed at the pore entrance.