Analysis of Single Amino Acid Mutations in Intact Proteins

H.J. Wirth[1], D. Steer[2], X Yan[3], T. Bannam[3], J. Rood[3] and A.Gooley[1]
1 SGE Analytical Science, 7 Argent Place, Ringwood, Vic. 3134, Australia
2 Monash Biomedical Proteomics Facility, Monash University, Clayton, Vic. 3128, Australia
3 Department of Microbiology, Monash University, Clayton, Vic. 3128, Australia

Abstract

With the increasing availability of high resolution mass spectrometers the analysis of intact proteins becomes a feasible alternative to the more commonly employed shot-gun 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 plus the effect of restriction inside pores is far more pronounced. To minimize the effects of hindered pore diffusion a 1000 Å, 3 µm C8-silica column (ProteCol™-G HQ1003) was employed for this study.

PHYSICO-CHEMICAL BACKGROUND

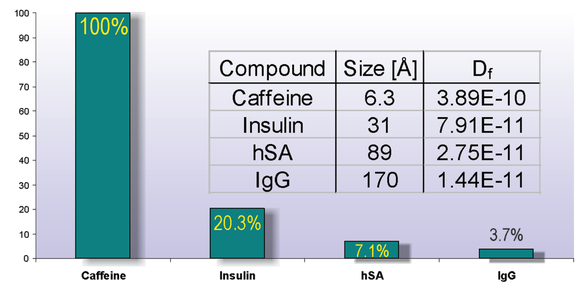
Diffusion in Free Solution

The diffusion rate is described by:

$$\frac{k_B T}{6\pi\mu R}$$

Einstein-Stokes Equation

D_f = Diffusion rate constant in free solution
k_B = Boltzmann constant
T = Temperature
R = Stokes radius
µ = Viscosity



Compound	Size [Å]	D_f
Caffeine	6.3	3.89E-10
Insulin	31	7.91E-11
hSA	89	2.75E-11
IgG	170	1.44E-11

Figure1: Normalised diffusion rates of small, medium and large molecules in free solution

Diffusion Inside Pores

$$D_f\left(1-\frac{r_s}{r_p}\right)^2\left[1-2.104\left(\frac{r_s}{r_p}\right)+2.09\left(\frac{r_s}{r_p}\right)^3-0.95\left(\frac{r_s}{r_p}\right)^5\right]$$

Renkin Equation (E.M. Renkin, J.Gen.Physio., 38 (1954) 225.)

D_p = Diffusion coefficient inside the pores
r_s = Strokes radius of the analyte
r_p = Pore radius

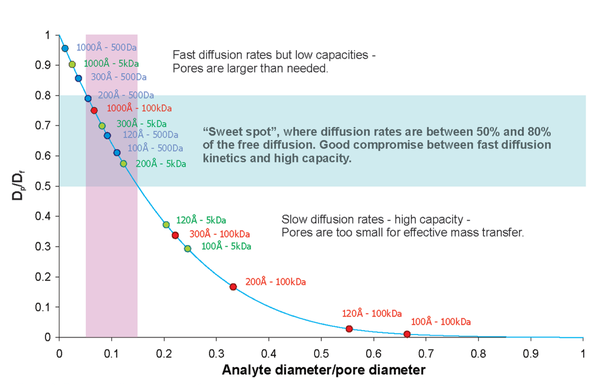


Figure2: Relative diffusion rates in free solution and inside pores as function of molecule size to pore size ratio

The Capillary Column Design

The column is made of a packed 1/16" OD PEEKsil™ (polyether ether ketone coated fused silica) capillary, with 1/32" OD, 50 µm ID PEEKsil™ connection capillaries permanently attached. The frits are made from a 100 µm thick woven mesh with 0.5 µm pore size. The column is protected by a stainless steel sleeve. Using PEEKsil™ rather than fused silica increases the robustness of the column while maintaining flexibility to easily attach the column to the LC system. The C8-silica stationary phase with 3 µm particles with 1000 Å pore size is tailored for the separation of intact proteins.

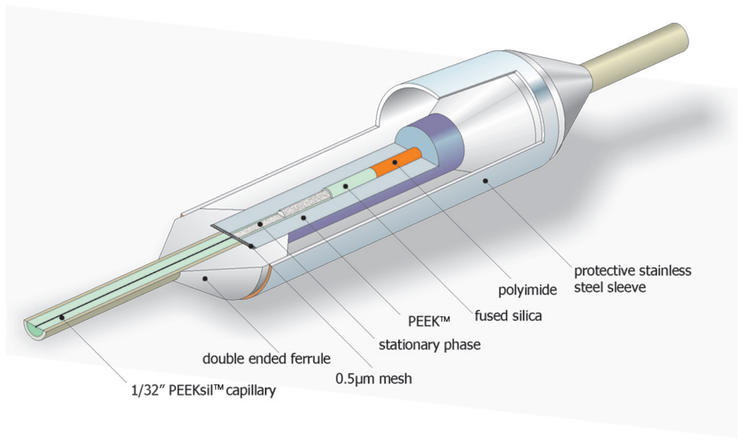


Figure 3. Schematic view of the ProteCol™ capillary column.

Experimental

Nine single-amino acid mutations of a 33 kDa protein were analysed by RP-µLC-MS in order 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

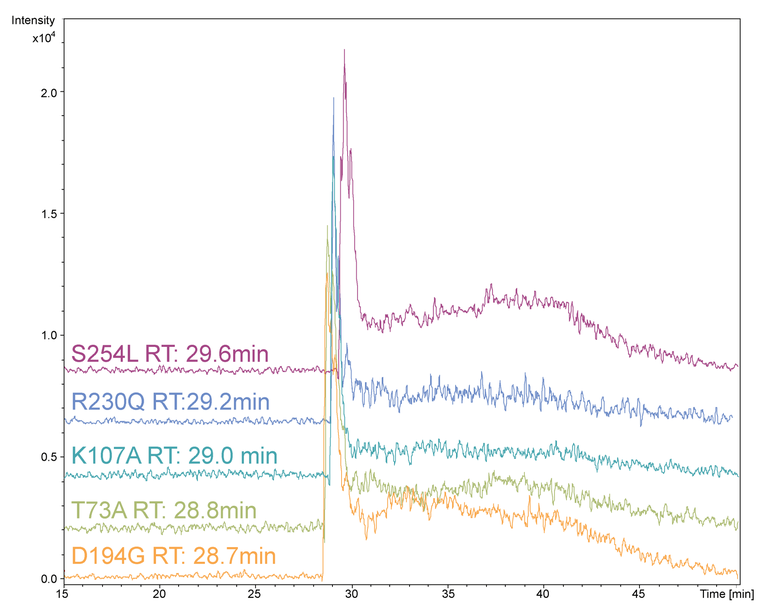


Figure 4. Chromatograms of the different mutant proteins.

By co-injecting two mutant proteins it was possible to achieve baseline separation:

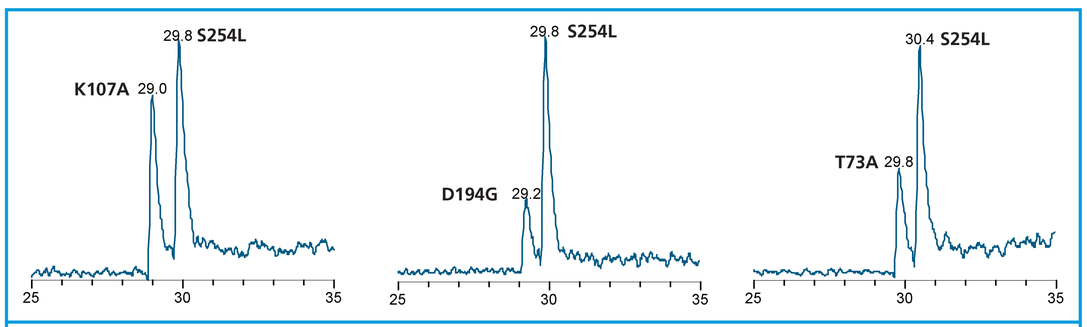


Figure 5. Selected ion chromatograms of the binary protein mixtures.

The shift in retention time can be correlated to the change in hydrophobicity. A hydrophobicity scale published by Cowan and Whittaker was used because it is based on HPLC retention data (R.Cowan and R.G. Whittaker, 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.

LC-MS Data for the Mutated Proteins				
Mutation	Mw (calc.)	Mw (meas.)	RT [min]	Hydrophobicity
R230Q	33036.8	33037.7	28.8	0.57
W287R	33034.8	33035.9	27.9	-2.85
T73A	33034.8	33036.1	28.8	0.62
S254L	33090.9	33092.5	29.6	2.43
D194G	33006.8	33008.8	28.7	2.15
K107A	33007.7	33009.7	29.0	1.89
Y108A	32972.7	32974.7	28.4	-0.04
S136P	33074.9	33251.2	28.5	1.47
G69D	33122.9	33299.0	28.4	-2.15

Table 1. Properties and retention data for the nine mutant proteins.

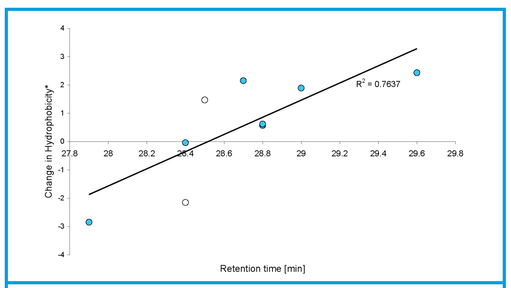


Figure 6. Correlation between the change in hydrophobicity and the retention time.

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 phases. 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.