



University of Pannonia
Chemical Engineering and Material Sciences Doctoral School

**New analytical strategies for separation and
fluorescent labelling of therapeutic proteins in
sodium dodecyl sulfate capillary gel
electrophoresis**

Ph.D. THESIS

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Introduction and objectives

In recent years, biotherapeutics have become increasingly important because of their specificity and less side effects. Comprehensive characterization of therapeutic proteins requires the development of high-performance analytical methods. Capillary electrophoresis is widely used to investigate the purity of proteins and to detect their structural differences, in particular capillary gel electrophoresis (CGE) format, which provides high-efficiency separation. My research aimed to develop novel analytical methods to accelerate and simplify the separation and fluorescent labeling of therapeutic proteins, thus reducing cost and labor.

Our research group is working in close collaboration with the Bio-Nanosystems Research Laboratory at the University of Pannonia, who developed high-yield production of an endoglycosidase enzyme (6His-PNGaseF), which is crucial for the analysis of N-glycosylated proteins. Therefore, my objectives were to compare this enzyme with commercially available enzymes to determine the optimal digestion time and the effect of sample glucose content as a factor influencing enzyme activity. In my research, I used tetrahydroxyborate cross-linked dextran-containing matrices as separation media and aimed to investigate the mechanism of separation of SDS-protein complexes in ultra-dilute and zero (1.00%-0.00%) dextran-containing matrices, with the focus on the separation of a therapeutic monoclonal antibody (daratumumab) and its subunits. A further aim was to introduce a new type of *in migratio* fluorescent labeling method using propidium iodide (PI) for SDS-CGE. The goal of this part of the thesis is to demonstrate the applicability of the new labeling method for the analysis of therapeutic proteins and to investigate the effect of the propidium ligand on the electromigration of SDS-protein complexes and the separation efficiency.

Experimental work

My research endeavor was first focused on the kinetics of N-glycan release during digestion using the in-house made enzyme 6His-PNGaseF including quantification of the products by SDS-CGE to determine the optimal digestion time. I scrutinized the effect of glucose content on the activity of the enzyme and performed a comparative kinetic study between 6His-PNGaseF and a commercial PNGaseF. My results showed that the presence of 1 mg/mL of glucose activated the 6His-PNGaseF enzyme, while the activity of the commercial product was not affected. Slight differences in charge heterogeneity were observed in the cIEF traces, which probably influenced the glucose-mediated changes in enzyme activity.

Next I turned to the investigation of the applicability of ultra-dilute dextran matrices for dextran polymer based gels cross-linked with tetrahydroxyborate, which is the SDS-CGE industry standard for protein separation. Ferguson and reptation plots were used to elucidate the separation mechanism. Highly diluted dextran solutions resulted in linear Ferguson plots for both solute types (cf. Ogston theory) in spite of the fact that this model assumes a rigid pore structure, thus cannot support the separation mechanism in ultra-dilute polymer solutions with no reticulations. The saddle differences between the resolution of the protein standard and the intact/subunit mAb forms in ultra-dilute dextran-borate matrices suggested the importance of shape selectivity, manifested by the adequate separation of the SDS covered intact as well as light and heavy chain subunits of the therapeutic mAb even at zero dextran concentration.

The increase in the number of biotherapeutics requires high-throughput analysis of SDS proteins by capillary and even multicapillary gel electrophoresis. A novel *in migratio* capillary fluorescent labeling technique was developed using propidium iodide (PI) which eliminated the need for the expensive and time consuming chemical derivatization steps. This new method enabled in situ labeling and efficient detection of SDS proteins, which I validated using protein standards as well as therapeutic monoclonal antibodies. The sensitivity and accuracy of the method can be optimized by adjusting the concentration of PI.

New scientific results: Theses

- 1. During the optimization of the digestion time of 6His-PNGaseF enzyme produced by the Bio-Nanosystems Laboratory of the University of Pannonia I observed that the presence of glucose increased the efficiency of the enzymatic digestion process.**

In my work, the deglycosylation kinetics of an in-house produced 6His-PNGaseF enzyme was investigated to determine the optimal digestion time and the effect of sample glucose content as a potential modifier of endoglycosidase activity was studied. The enzyme activity was evaluated by using the Michaelis-Menten model and compared with the activity of a commercially available and widely used PNGaseF enzyme.

- 2. The use of dilute and ultra-dilute dextran solutions in SDS-CGE**

I have investigated the effects of dilute and ultra-dilute (1%-0.01%) as well as zero dextran concentration separation buffer systems for the separation of a protein standard mixture as well as a therapeutic protein and its subunits to study the mechanism of separation. Dilute and ultra-dilute polymer solutions can provide extremely fast separation times for the analysis of therapeutic proteins during development and production.

- 3. Investigation of the separation mechanism in dilute and ultra-dilute dextran-borate matrices**

Ferguson diagrams showed linear characteristics for both samples, despite the fact that reticulation was not expected in ultra-dilute polymer solutions. The differences in resolution between the protein standard and the therapeutic protein sample components indicated the importance of shape selectivity. The intermittent non-sticky bumping of the SDS proteins into the randomly distributed dextran chains probably resulted in pivoting for smaller and rope like hanging for the larger proteins during their electromigration, consequently resulting in some size based separation.

- 4. *In migratio* application of propidium iodide as a fluorescent dye in the analysis of therapeutic proteins**

I introduced the concept of *in migratio* fluorescent labelling of SDS-protein complexes using propidium iodide, which was previously used only for nucleic acid labelling. The method allowed simple *in situ* labelling during the separation process, thus requiring no pre- or post-column derivatization steps, significantly speeding up and facilitating the analysis workflow.

Publications related to theses

Patent

Felicia Auer, András Guttman: Módszer az SDS-fehérjék nem kovalens migrációs fluorofór címkzésére,ellenáramú elektromigrációt használva SDS-CGE során Bejelentés napja: 22. December 2023

Scientific Articles

Felicia Auer, Andras Guttman: *In Migratio* Noncovalent Fluorophore Labeling of Proteins by Propidium Iodide in Sodium Dodecyl Sulfate Capillary Gel Electrophoresis, *Analytical Chemistry* 27, (2024), 10969–10977 DOI: 10.1021/acs.analchem.4c01371

Felicia Auer, Andras Guttman: Size separation of sodium dodecyl sulfate–proteins by capillary electrophoresis in dilute and ultra-dilute dextran solutions, *Electrophoresis* 19-20, (2023), 1607-1614, DOI: 10.1002/elps.202300067

Rebeka Torok, **Felicia Auer**, Robert Farsang, Eszter Jona, Gabor Jarvas, Andras Guttman: The Effect of Sample Glucose Content on PNGase F-Mediated N-Glycan Release Analyzed by Capillary Electrophoresis, *Molecules* 23, (2022), 8192. DOI: 10.3390/molecules27238192

Other scientific publications

Balázs Gyebrovski, András Ács, Dániel Szabó, **Felicia Auer**, Soma Novozánszki, Bernadette Rojkovich, Anna Magyar , Ferenc Hudecz , Károly Vékey, László Drahos, Gabriella SármayThe Role of IgG Fc Region N-Glycosylation in the Pathomechanism of Rheumatoid Arthritis *International Journal of Molecular Sciences* 10 (2022),5828, DOI: 10.3390/ijms23105828

Felicia Auer, Gabor Jarvas, Andras Guttman: Recent advances in the analysis of human milk oligosaccharides by liquid phase separation methods, *Journal of Chromatography B* 1162, (2021),122497, DOI: 10.1016/j.jchromb.2020.122497