Separation of proteoforms under native conditions at semi-preparative scale, using free-flow electrophoresis (FFE)

Michiel Akeroyd, Nicolas Abello, Maurien Olsthoorn

DSM Biotechnology Center, Delft, The Netherlands

FFE Service GmbH

www.ffeservice.com

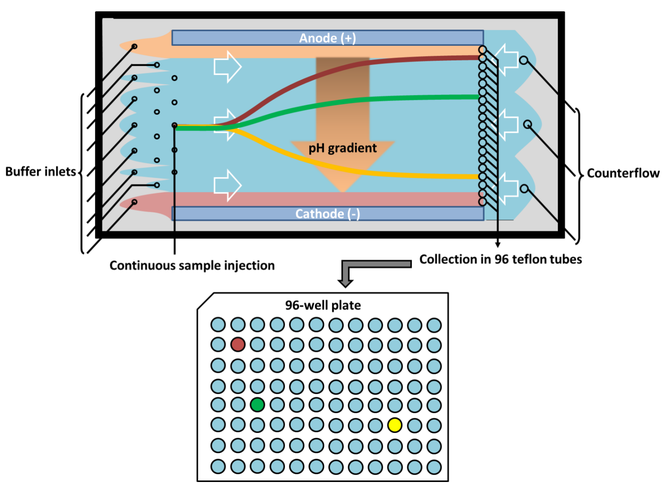


Figure 1. Free-Flow Electrophoresis Free-Flow Electrophoresis was performed at FFE Service in Feldkirchen (Germany). Analytes are separated based on their charge in a flow chamber (left). The separated analytes are directly collected in 96-well plate format, which enables semi-preparative purification of analytes.

Introduction

Bulk-produced enzymes and pharmaceutical proteins often consist of several proteoforms. The different proteoforms originate from naturally occurring post-translational modifications (e.g. glycosylation, disulfide bonds, N-terminal modifications) and protein heterogeneity can be further induced during the production process (due to e.g. proteolytic degradation, deamidation, oxidation, or glycation).

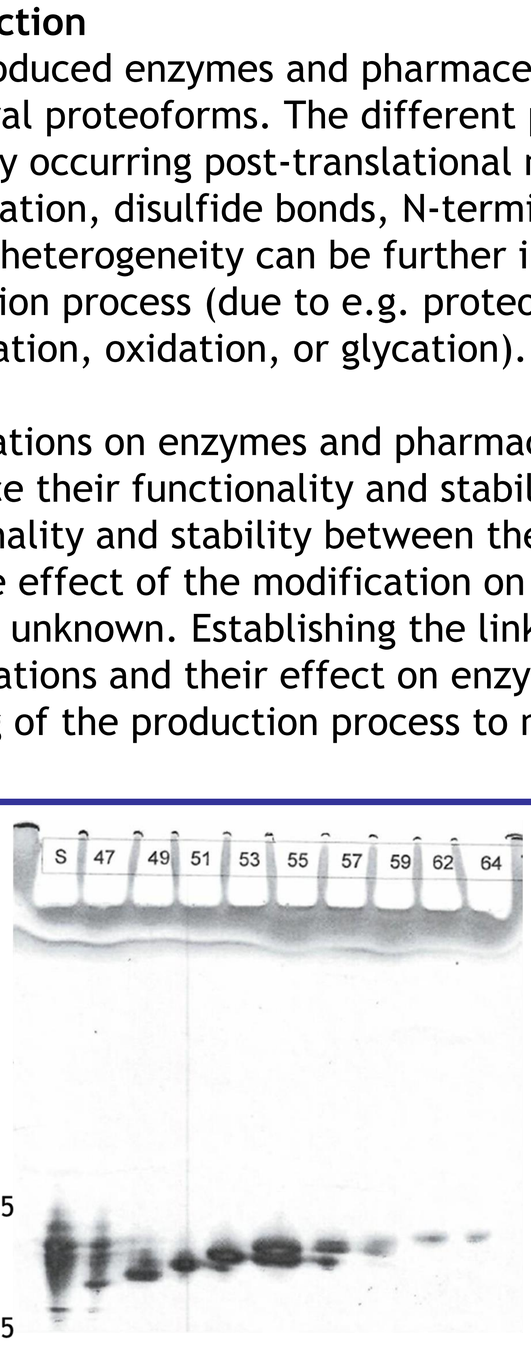
Modifications on enzymes and pharmaceutical proteins can influence their functionality and stability. The difference in functionality and stability between the individual proteoforms (i.e. the effect of the modification on the enzyme activity) often remains unknown. Establishing the link between the molecular modifications and their effect on enzyme performances allows steering of the production process to maximal efficiency.

Results

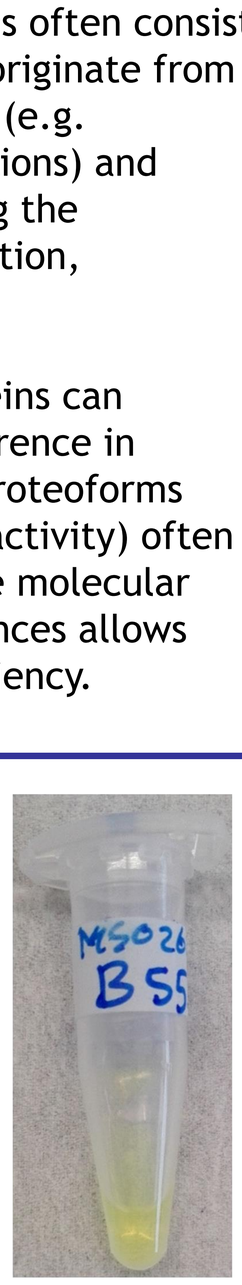
In this study we present the separation of proteoforms at semi-preparative scale of a bulk-produced enzyme for the food industry, using free-flow electrophoresis (FFE, Fig. 1). The different proteoforms were separated with ~0.1 pH unit resolution, shown on Iso-Electric Focussing (IEF) gel (Fig. 2a). We showed that the purified proteoforms are still active, (Fig. 2b) using an enzyme specific substrate.

LC-MS based protein identification showed that the fractions contained almost exclusively the enzyme of interest (Fig. 2c).The free-flow electrophoresis method was optimized to purify two milligrams of proteoforms per hour (Fig. 3). The amounts of purified proteoforms obtainable with this method are sufficient to perform molecular enzyme characterization, enzymatic activity studies as well as downscaled enzyme application tests on the individual proteoforms.

a)



b)



c)

	Area top 3 peptides			Theoretical	
	Sample	Fraction 53	Fraction 55	MW [kDa]	pI
Enzyme of interest	**4.55E+09**	**7.48E+08**	**4.01E+08**	**56.9**	**4.6**
background protein a	1.50E+08			55.2	4.4
background protein b	8.94E+06			41.6	5.9
background protein c	3.87E+07			46.8	4.5
background protein d	5.21E+06			62.5	4.7
background protein e	8.54E+06			58.4	4.9
background protein f	2.88E+06			41.0	4.5
background protein g	6.79E+05			61.3	5.3
background protein h	7.27E+05			43.4	5.6
background protein i	1.03E+06			64.5	5.0
background protein j	1.94E+06		1.11E+06	25.8	4.6
background protein k	9.00E+05			71.3	5.8
background protein l	5.70E+06			68.3	4.4
background protein m	4.45E+06			15.2	4.4
background protein n	1.16E+06			51.8	4.3
background protein o	3.46E+05			32.1	4.8
background protein p	1.51E+06			52.0	4.5
background protein q	1.08E+06			39.9	4.2
background protein r	6.94E+05			72.4	6.1
background protein s	1.66E+06			43.5	5.0
background protein t	3.99E+05			58.9	5.3
background protein u	4.84E+05			90.4	6.4

Figure 2. Analysis of FFE-purified proteoforms. FFE-purified fractions were collected in 96-well micro titer plates. The initial sample (s) and several FFE-purified fractions were put on IEF gel a). Incubation with an enzyme-specific substrate, which turns yellow on conversion, showed that the purified proteoforms were active b). The purified fractions were analyzed with LC-MS based protein identification to show the purity of the fractions c). The table shows the average peak area of the top 3 peptides identified by LC-MS/MS after digestion with trypsin. These results showed that background proteins were removed during purification.

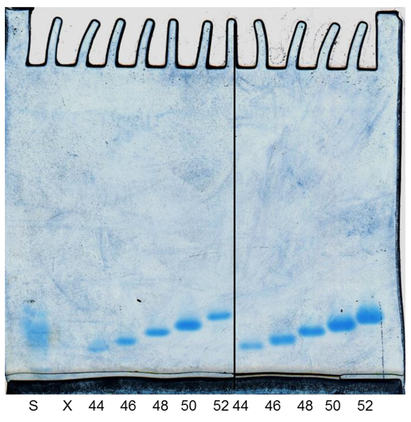


Figure 3. IEF gel of the optimized FFE separation. The initial FFE run showed proof of concept for purification of the proteoforms. However, only ~100 microgram of each of the proteoforms was obtained in a single injection. The FFE method was optimized in order to inject larger amounts of sample and increase the separation resolution even further. This IEF gel shows the separation of two optimized methods capable of purifying two milligram of proteoforms per hour.

Conclusion

- Purification of proteoforms of an enzyme was achieved with FFE at a **resolution of ~0.1 pH unit**.
- **Activity and purity of the proteoforms were confirmed** after purification.
- The FFE purification method was optimized to enable purification of **2 mg proteoforms per hour**.

Acknowledgement

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