

1. Introduction

In most bottom-up proteomics experiments, peptides are generated using enzymatic digestion of the selected proteins, mainly based on trypsin. However, for (absolute) quantitative purposes, tryptic digestion lacks sufficient reproducibility and efficiency, especially when working with low abundant proteins ($\leq 1 \mu\text{g/mL}$). With trypsin being resistant to protein denaturing in organic modifiers like methanol or acetonitrile, the latter were investigated to enhance the trypsinization efficiency but their use is unsatisfactory.

2. Aim

CnBr based chemical cleavage is investigated as an alternative for enzymatic digestion based upon trypsin. Different reaction conditions (P1-6) were evaluated and tested on cystatin C, a 13kDa protein with two disulfide bridges between amino acids 99-104 and amino acids 126-143.

3. Materials and methods

Cystatin C sequence:

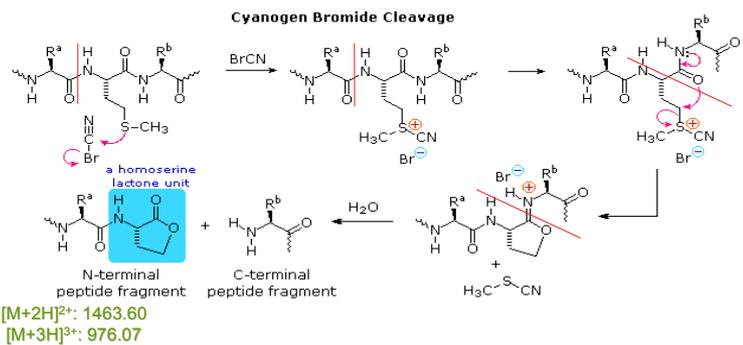
MAGPLRAPLL LLAILAVALAVSPAAGSSSPGKPPRLVGGPM
 signal-peptide
DASVEEEGVRRALDFAVGEYNKASNDMYHSRALQVVRARK
 selected marker peptide
 QIVAGVNYFLDVELGRITCTKTQPNLNDNCPFHDPHLKRRK
 AFCSFQIYAVPWQGTMTLSKSTCQDA

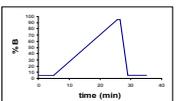
Chicken egg white cystatin sequence (IS):

MAGARGCVLLAAALMLVGAVLGSEDRSRLLGAPVPVDENDEG
 signal-peptide
 LQRALQFAMAEYNRASNDKYSSRVRVISAKRQLVSGIKYILQVE
 IGRITCCKSSGDLQSCFEFHDEPE**MAKYTTCTFVVYSIPWLNQIKL**
 selected marker peptide
LESKQC

$[M+2H]^{2+}$: 1589.9
 $[M+3H]^{3+}$: 1060.27

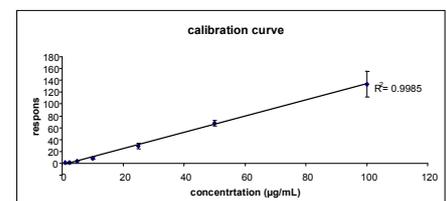
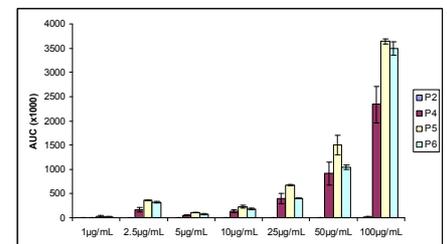
	P1	P2	P3	P4	P5	P6
Trifluoroacetic Acid (TFA)	60%	60%	70%	70%	-	-
Formic Acid (FA)	-	-	-	-	70%	70%
H ₂ O	-	20%	-	15%	-	15%
CnBr 5M, in AcN	40%	20%	30%	15%	30%	15%



LC	MS(/MS)																															
Column: YMC ODS-AQ™ 1.0 x 150 mm, 3 μm (C18) HPLC: Agilent 1100 Eluent A: 0.1 % Formic Acid in millipore H ₂ O Eluent B: 0.1 % Formic Acid in acetonitrile	Micromass Ultima triple quadrupole, ESI + (orthogonal Z-spray®)																															
	<table border="1"> <thead> <tr> <th></th> <th>precursor ion</th> <th>product ion</th> <th>CE</th> <th>dwell time (s)</th> </tr> </thead> <tbody> <tr> <td rowspan="3">cystatin C</td> <td>976.1</td> <td>976.1</td> <td>30.0</td> <td>0.8</td> </tr> <tr> <td>1463.6</td> <td>1463.6</td> <td>30.0</td> <td>0.2</td> </tr> <tr> <td>976.1</td> <td>785.0</td> <td>30.0</td> <td>0.2</td> </tr> <tr> <td rowspan="3">chicken egg white cystatin</td> <td>1060.0</td> <td>1060.0</td> <td>30.0</td> <td>0.8</td> </tr> <tr> <td>1589.9</td> <td>1589.9</td> <td>30.0</td> <td>0.8</td> </tr> <tr> <td>1060.0</td> <td>1469.6</td> <td>30.0</td> <td>0.2</td> </tr> </tbody> </table>		precursor ion	product ion	CE	dwell time (s)	cystatin C	976.1	976.1	30.0	0.8	1463.6	1463.6	30.0	0.2	976.1	785.0	30.0	0.2	chicken egg white cystatin	1060.0	1060.0	30.0	0.8	1589.9	1589.9	30.0	0.8	1060.0	1469.6	30.0	0.2
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4. Results and discussion (compared to tryptic digestion)

- Less complex peptide mixtures due to the natural lower abundance of methionine in human proteins compared to arginine and lysine
- the produced (marker-)peptides contain more amino acids: only one is sufficient as a stoichiometric representative of the protein
- the selected marker peptide can be generated without the need for cystatin C to be denatured, reduced and alkylated, prior to the chemical digestion. (↔ in solution trypsinization requires a minimal 20-fold dilution of the sample to lower the concentration of the used chaotropes like e.g. urea; evaporation under nitrogen to regain the sensitivity is very time-consuming (aqueous characteristics of the used media)).
- Reproducibilities for the highest calibrator (n=6) below 5% for P5 (2.18%) and P6 (4.43%); between 5% and 25 % for P2 and P4
- P1-3 lack good efficiency (not shown in figure)
- Linear calibration curves using P6 from 1 to 100 $\mu\text{g/mL}$ up ($R^2=0.999$)



5. Conclusion

Compared to enzymatic digestion, chemical digestion provides a more desirable methodology, with reproducibilities ranging from 2.53% up to 24.22% dependent on the used reagents, and has a greater ease of use.