Optimization of an in solution tryptic digest protocol for use in quantitative LC/MS of proteins

M. Storme[1], D. Deforce[2] and J. Van Bocxlaer[1]

[1] Laboratory of Medical Biochemistry and Clinical Analysis, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

[2]Labaratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

@ Michael.Storme@UGent.be

1. Introduction

Nowadays, most proteomics applications are purely qualitative or semi-quantitative, e.g. using ICAT-technologies. However, there is a growing need for absolute quantitation of proteïns, in particular due to the growing amount of so-called 'proteïn-medicins' (like Epo and human growth-hormone). Also, the discovery of new proteïn-biomarkers increasingly demands quantitation. Nearly all diagnostic important proteïns are now currently measured by immunoassays. Nevertheless, a certain lack of specificity is an important drawback. The use of mass spectrometry coupled to liquid chromatography could solve some of these problems.

In a new approach we seek to optimize an alternative technique to quantify proteïns with LC-MS. To this end, we enzymatically cleave a given protein, typically with trypsin, and choose a unique set of marker peptides. These peptides, representing the proteïn of interest, are then quantified using LC/MS-MS with an internal standard Our proteïn of interst is Cystatin C, an 146 aa protein of 13 kDa with two disulfid bridges between aa 99-104 and aa 123-143. Cystatin C is choosen as e model but also for his potential as a new marker of kidney failure.

2. Aim

To develop a reproducible in solution tryptic digest procedure, as a first vital step in our marker peptide approach.

3. Materials and Methods

Cystatin C

1 mg/ml solution (in 50 mM tris-HCl /1 mM $CaCl_2$)

Denaturation conditions

- A: 50mMTris-Hcl/6M urea/5mM DTT, 1h 65°
- B: 50mMTris-Hcl/ 6M urea/ 5mM DTT, 20min 95°
- C: 50mMTris-Hcl/ 6M urea/ 10%betaME, 1h 65°
- D: 50mMTris-Hcl/ 6M urea/ 10%betaME, 20min 95°
- E: 50mMTris-Hcl/ 6M urea/ 5mM DTT/ 10%betaME, 1h 65°

HPLC Conditions:

Column: YMC ODS-AQ™ 2 x 150 mm, 5 µm (C18, microbore)

- Eluent A: H_2O with 0.1 % Formic Acid
- Eluent B: Acetonitrile with 0.1 % Formic Acid
- Linear gradiënt from 0 to 50 % eluent B over 45 minutes, flow rate: 0.2 ml/min
- HPLC: Waters Alliance 2695, integrated with Qtof

MS Conditions:

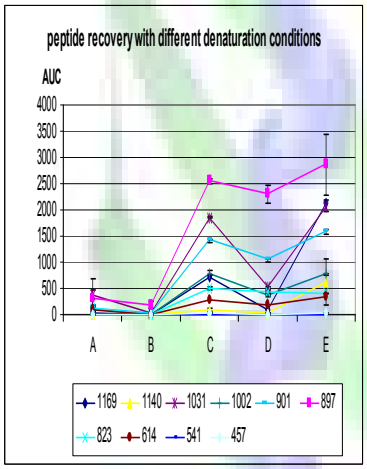
- Mass Spectrometer: Micromass Q-TOF hybrid mass spectrometer, equipped with an orthogonal electrospray source (Z-spray®) in positive ion mode
- Capillary voltage: 2450V
- Cone: 40V

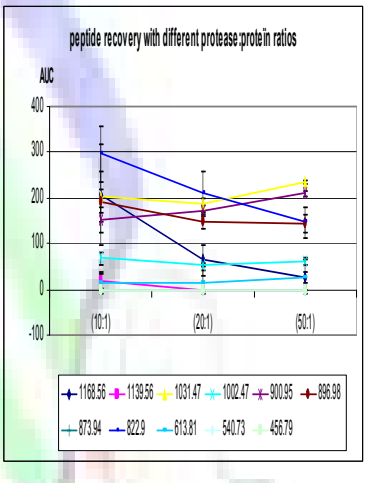
4. Results and Discussion

We started from the standard Promega in solution trypsin protocol and evaluated the optimum time and duration of the denaturation process. We found 1 h at 65° to be the best condition. After that, DTT vs beta-mercaptoethanol was investigated. To that end, 5 different denaturation solutions were investigated. A combination of both reducing agents (condition E) proved to give the best results. Adding iodoacetamide to the protocol, to protect the free SH groups generated, dramatically increased the sequence coverage and peptide recovery.

- As a last step, the optimum protease:protein ratio was investigated. Three different ratios (10:1 / 20:1 / 50:1) were compared. In general, the tested ratios didn't effect the sequence coverage.

		AUC (n=3) with denaturation under condition (same protease:protein ratio):					AUC (n=3) with protease:proteïn ratio (condition D):		
AA sequence (IA=iodoacetamide)	$[M+H]^{2+}$	A	B	C	D	E	10	20	50
AFCSFQIYAVPWQGTMTLSK + IA	1169	18	10	731	96	2119	209	63	26
AFCSFQIYAVPWQGTMTLSK	1140	8	0	89	23	621	20	0	0
TQPNLDNCPFHDQPHLK + IA	1031	375	33	1847	538	2074	204	186	234
TQPNLDNCPFHDQPHLK	1002	0	0	782	365	768	68	54	61
LVGGPMDASVEEEGVRR	901	119	22	1424	1048	1598	153	170	209
QIVAGVNYFLDVELGR	897	312	180	2549	2303	2860	193	147	145
LVGGPMDASVEEEGVR	823	127	0	498	439	406	297	212	146
ALDFAVGEYNK	614	91	0	276	196	350	12	15	27
ASNDMYHSR	541	0	0	0	0	0	0	0	0
ALQVVRAR	457	0	0	22	0	29	0	0	0





5. Conclusion

By optimization of the in solution tryptic digest protocol we reached a 91.92 % sequence coverage (only peptides with a m/z larger then 400 are taken into account). Also, an increase in peptide recovery with a factor 10 is noticed. To that end, we denaturate the protein solution (55µl) for one hour at 65° with a solution of 50mMTris-HCl/ 6M urea/ 5mM DTT/ 10%betaME (145µl). After diluting with 50 mM tris-HCl /1 mM $CaCl_2$ (600µl), to get the urea concentration below 2M, we add iodoacetamide to a final concentration of 20 mM (80µl of a 200mM stock-solution). As a last step, trypsin is added in a protease:protein ratio between 1:10 and 1:50 to start the trypsinisation process. (overnight, 37°):

LVGGPMDASVEEEGVR//R//ALDFAVGEYNK//ASNDMYHSR//ALQVVRAR//K //QIVAGVNYFLDVELGR//TTCTK//TQPNLDNCPFHDQPHLK//R//K// AFCSFQIYAVPWQGTMTLSK//STCQDA

(Cystatin C sequence

___: peptides detected by LC/MS ___: peptides not detected by LC/MS)

6. Acknowledgements

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