

High-Performance Liquid Chromatographic Assays for the Adenovirus Type 5 Proteome

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Overview

The objective of our study was to evaluate the performances (sensitivity and attainable protein sequence coverage) of one-dimensional (1D) and two-dimensional (2D) LC-MS/MS analysis of the adenovirus proteome. The adenoviral proteome is a very appropriate test system for such comparisons: it is fairly complex but well understood and characterized. It consists of approximately 2500 molecules of at least 11 protein species, with quantitative information available on copy numbers based both on electron microscopy results with symmetry considerations and direct measurements with various other methods. As there are large differences in the copy numbers of the structural proteins (covering almost 2 orders of magnitude), the selectivity, sensitivity and dynamic range of the different 1D and 2D LC-MS/MS approaches can be assessed using the adenovirus as a test system.

Introduction

Recombinant adenovirus preparations are used for gene delivery in a growing number of clinical development programs in gene therapy. In support of the anticipated commercial scale production of recombinant adenovirus, analytical tools have to be developed to define the virus product. We compared detection sensitivity and protein sequence coverage achievable by liquid chromatography and tandem mass spectroscopy (LC/MS/MS) using three sample preparation and clean up methods.

Methods

HPLC separation of viral proteins 1.63x10¹² virus particles were injected onto a C4 column (Phenomenex), for fraction collection and proteins were separated using a gradient of 20–60% acetonitrile in 0.1% formic acid over 95 minutes.

Reduction, alkylation and digestion: 3 mM DTT; alkylation: 7 mM iodoacetic acid; quenching: 14 mM DTT; digestion: 1:50 trypsin:protein ratio and incubation overnight at 37°C.

1D LC-MS/MS separation of tryptic peptides on a 75 µm C18 packed tip (New Objective) at a flow rate of 100 nl/min. Gradient: 0–60% acetonitrile in 0.1% formic acid over 60 minutes for analysis of the HPLC fractions and 480 minutes for analysis of the whole virus digest. MS/MS: Thermo Finnigan LCQ™ Deca XP ion trap mass spectrometer equipped with a NanoSpray ionization source. One full MS was followed by 3 MS/MS with spray voltage of 2.0 kV.

2D LC-MS/MS fully automated 2D LC-MS/MS system ProteomeX™ (Thermo Finnigan) first dimension: SCX (0.32 mm x 100 mm, Thermo Hypersil-Keystone) 0, 50, 100, 200, 300, 400, and 500 mM ammonium chloride salt steps over 20 minutes each at a flow rate of 2 µl/min. second dimension: BioBasic-C18 (0.18 mm x 100 mm, Thermo Hypersil-Keystone) gradient from 5 – 60% acetonitrile in 0.1% formic acid over 30 minutes at a flow rate of 2 µl/min. MS/MS: Thermo Finnigan LCQ Deca XP ion trap mass spectrometer - One full MS was followed by 3 MS/MS with spray voltage of 2.6 kV.

Results

1. Analysis of collected RP-HPLC fractions

FIGURE 1: Reversed-phase HPLC chromatogram of the adenovirus type 5 proteome. 5x10¹⁰ viral particles were injected into the column and chromatograms were obtained at 214 nm.

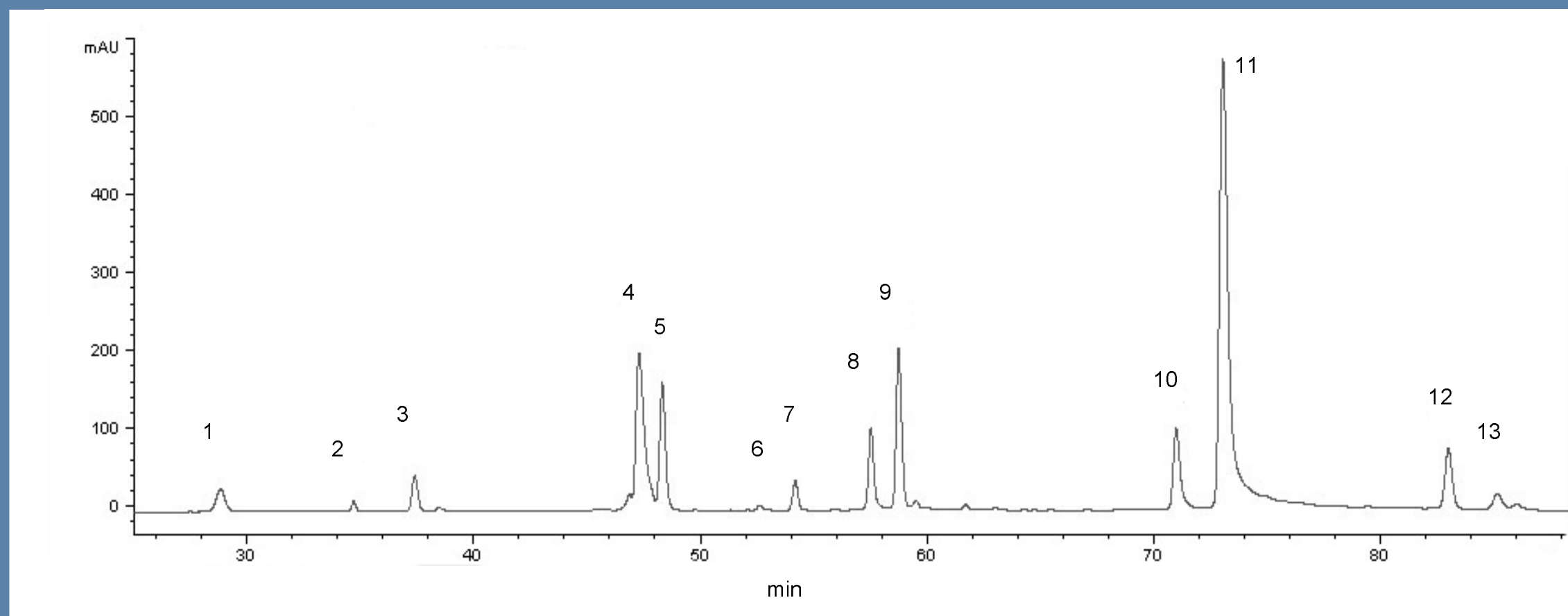


TABLE 1: Identification of viral proteins from the collected reversed-phased HPLC fractions in Figure 1. The numbering of the fractions is the same as in Figure 1.

Fraction	Protein ID	Number of peptides identified	Sequence coverage [%]	Model or biochemical copy numbers ^g
1	ND	-	-	-
2	IX	1	9	240
3	N-terminal fragment of VIII after processing by the 23 kD protease ^{a,b}	2	17	-
4	mature VII ^{a,c}	8	30	833
5	V ^{a,c}	9	30	157
6	VIII	3	20	-
7	C-terminal fragment of VIII after processing by the 23 kD protease ^{a,d}	1	20	-
8	III ^a , 23 kD protease	17, 8	29, 47	60, 10
9	mature VI ^a , 23 kD protease	7, 8	36, 47	360, 10
10	mature IIIa ^a	10	26	60
11	II ^a	22	37	720
12	mature IX ^a , II ^f	8	54	240
13	L1, II ^f	4	10	-

^a the protein identified is the same as assigned to the corresponding RP-HPLC peak in the study reported by Lehmberg *et al.*¹

^b the peptides ILLEQAAITTPR and NNLNPR were detected from the N-terminal fragment of the full length protein VIII which is processed by the 23 kD viral protease at G₁₁₁.¹ This processing site was taken into account for the sequence coverage calculation.

^c the collected samples showed traces of proteins in both fractions. The sequence coverage is given for the major protein detected in the peak

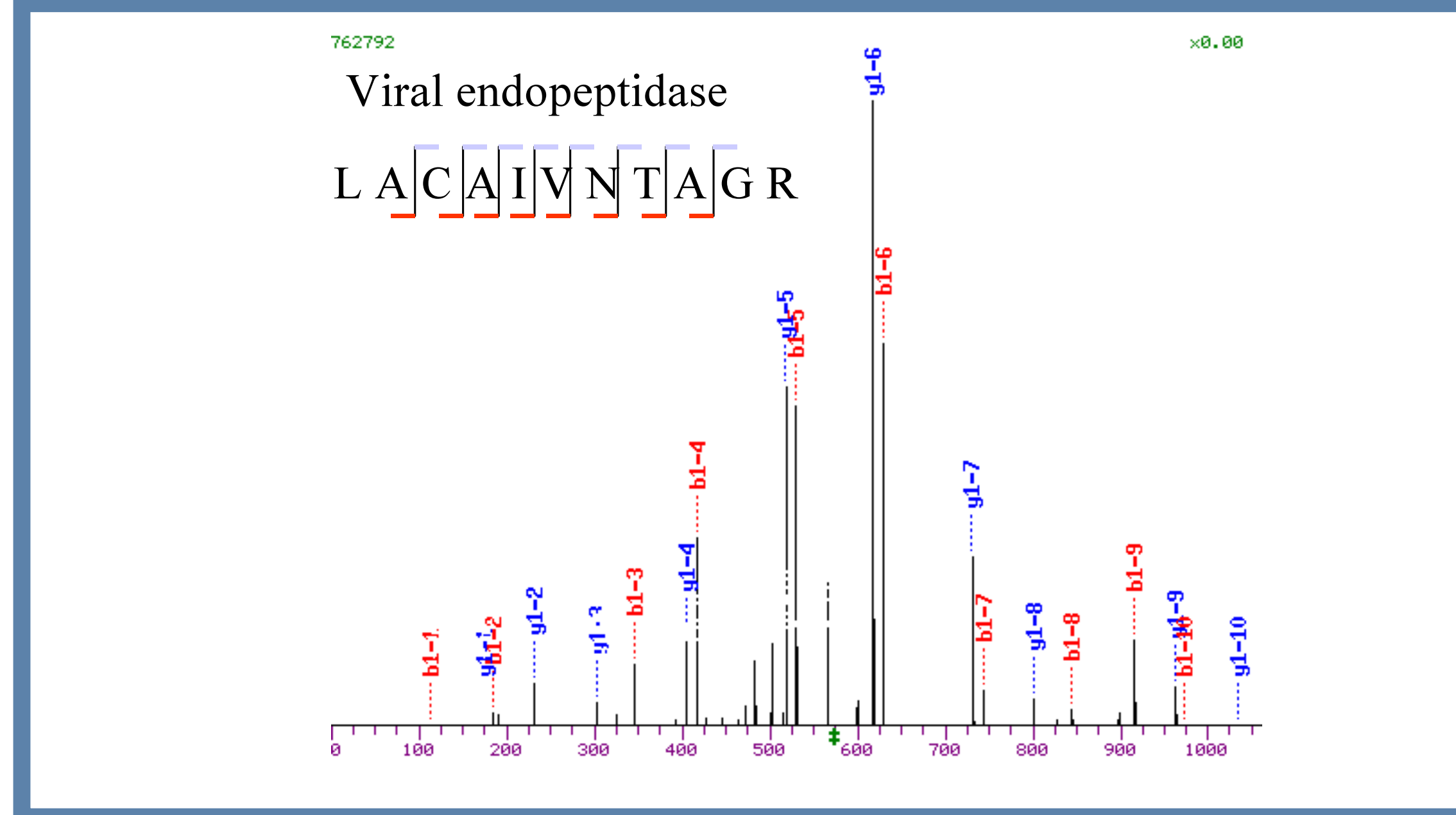
^d the peptide QAILTLQTSSEPR was detected from the C-terminal fragment of the full length protein VIII, starting from the site processing site by the 23 kD viral protease at N-terminus of G₁₅₇.¹ This processing site was taken into account for the sequence coverage calculation.

^e sequences for the 23 kD viral protease were detected in both fractions 8 and 9; 5 peptides in fraction 8 (LACAIVNTAGR, FPGFVSPHK, NQEQLYSFLER, DLGCGPYFLGTYDK, QVYQFEYELLR) and with 6 peptides in fraction 9 (LACAIVNTAGR, FPGFVSPHK, NQEQLYSFLER, TCYLFEPFGFSDQR, SAIASSPDRICITLTK and SATSFCHLK). The sequence coverage (47%) was calculated for all detected peptides in the two fractions.

^f traces of protein II were detected with several identifiable peptides in fractions 12 and 13, likely due to tailing. Sequence coverage for protein II was not reported in these fractions.

^g copy numbers were obtained from Stewart *et al.*²

FIGURE 2: Tandem mass spectrum derived by collision induced dissociation of the (M + 2H)²⁺ precursor ion, m/z 574.5.^h



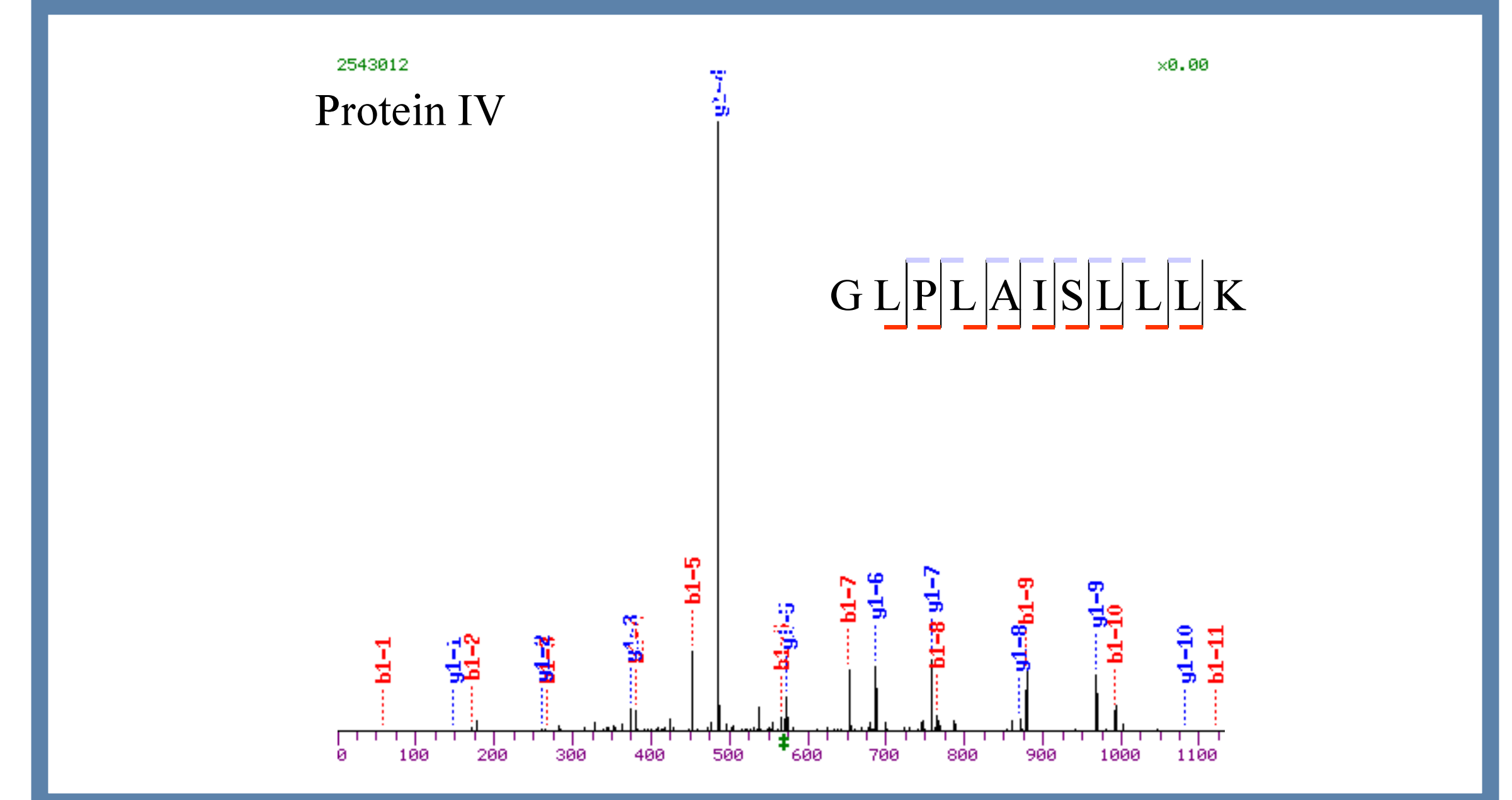
^h Fragment ions in the spectrum represent mainly single-event preferential cleavage of the peptide bonds resulting in the sequence information recorded from both N (b-ions) and C (y-ions) termini of the peptides simultaneously. SEQUEST searches (Eng *et al.*, 1994) of the MS/MS data against homospisians and adenovirus database downloaded from National Center for Biotechnology (NCBI) revealed the peptide identification LACAIVNTAGR, which is a tryptic peptide of viral endopeptidase (FIGURE 2) and the peptide identification GLPLAISLLK, which is a tryptic peptide of viral protein IV (FIGURE 3).

2. Analysis of the whole virus digest

Table 2: Identification of viral proteins from whole virus digests using one-dimensional (1D) or two-dimensional (2D) chromatographic separation of the peptides and analysis by mass spectrometry.

Protein ID	1D analysis		2D analysis		Copy number
	Number of peptides identified	Sequence coverage [%]	Number of peptides identified	Sequence coverage [%]	
II	8	11	2	2	720
III	8	19	10	22	60
IIIa	15	34	17	33	60
IV	3	10	3	3	36
V	12	34	9	28	157
VI	6	36	3	11	360
VII	7	40	5	29	833
VIII	2	11	2	11	-
IX	4	38	3	32	240
L1	7	23	8	19	-
23 kD protease	-	-	1	4	10

FIGURE 3: Tandem mass spectrum derived by collision induced dissociation of the (M + 2H)²⁺ precursor ion, m/z 569.9.^h



Conclusions

A total of 11 protein species were identified from 154 peptides. All major viral proteins were found. In addition, two minor proteins, the 23 kDa viral protease and late L1 protein, were identified for the first time by chromatography based assays. The 23 kDa viral protease, present at only 10 copies per virus, and representing 0.2% of the protein content of the virus, was detected by the 2D LC-MS/MS analysis of the whole virus digest from a sample containing only 70 femtomoles of the protein. This demonstrates the high sensitivity and selectivity of the method. The 2D LC-MS/MS analysis of the whole virus digest was also able to detect all viral proteins with copy numbers at or above 10/virus particle, with broad coverage of the amino acid sequences. Coverage ranged from 2 to 54%, a majority between 20 – 35%, suggesting the possibility of using this analysis to assess the purity of the virus preparations. This broad coverage may also provide a useful approach to identify post-translational modifications on the structural proteins of the adenovirus.

References

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