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Proteomic study of recombinant adenovirus 5 encoding human p53 by matrix-assisted laser desorption/ionization mass spectrometry in combination with database search

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Dedicated to Professor Franz Hillenkamp on the happy occasion of his 65th birthday.

Abstract

The clinical application of recombinant adenoviruses as vectors for gene therapy brings about the need to develop new analytical methodologies for monitoring the quality of the viral production as well as establishing structure–function relationships. A mass spectrometry-based assay has been developed for the characterization of structural proteins of the recombinant adenovirus type 5 vector encoding human p53 tumor suppressor gene. The fingerprinting of the viral proteome was accomplished by integration of MALDI-MS and/or MALDI-PSD-MS with SDS–PAGE and RP-HPLC, followed by database search using MS-Fit and MS-Tag algorithms. Viral proteins (molecular weights ~10,000–100,000 Da) corresponding to more than 95% of total protein mass were resolved and identified, which include hexon (II), penton base (III), peripentonal hexon-associated protein (IIIa), minor core protein (V), major core protein (VII), and other hexon-associated proteins (VI and VIII). An important finding of our studies was the identification of some precursor proteins (i.e., pVIII) and propeptides of precursor proteins (pVIII, pX, and pVI) present in the adenovirus sample. The information obtained allows direct and accurate assessment of the quality of recombinant adenoviruses.

Keywords: Recombinant adenovirus; Gene therapy; MALDI-MS; MALDI-PSD-MS; MS-Fit; MS-Tag; SDS–PAGE; RP-HPLC

1. Introduction

Recent advances in gene therapy technology have provided new directions for combating cancers and other serious diseases by delivering therapeutic genes to target cells. Recombinant adenovirus, an icosahedral, nonenveloped and double-stranded DNA virus, is a preferred vector for gene therapy [1,2]. The advantage of an adenovirus-based delivery system is its ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in

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target cells [3]. The adenovirus infects a broad range of mammalian cell types without undesired integration of the therapeutic gene into the host cell genome [4]. Traditionally, CsCl density gradient centrifugation is a primary method for the adenovirus purification, which is not suitable for large-scale viral production [5,6]. With the growing use of the adenovirus in clinical applications, new chromatographic methodologies were developed for the purification of the adenovirus [7–9]. To monitor the quality of the purified adenovirus and to better understand viral structure–function relationships, rapid and accurate analytical assays are in demand to define the recombinant adenovirus at the proteome level for its use as a therapeutic entity.

Even though the structural information of the adenovirus can be obtained through electron microscopy [10] and X-ray crystallography [11] studies, little information is available at the molecular level of the proteome that makes up of an adenoviral particle. The 200×10^6 dalton (Da) adenoviral particle has more than 11 distinct structural proteins (polypeptides II–IX, IIIa, μ, and terminal) present in multiple copies and held together by non-covalent interactions [12]. These viral proteins, ranging from less than 10,000 Da to more than 100,000 Da, directly or indirectly contain nuclear targeting information. The assembly of these structural proteins to form a complete virion is critical for the infectivity of the virus and may contribute to the immunogenicity observed with the adenovirus gene therapy. In the previrion steps, structural proteins including hexon (II), penton-base (III) and fiber (IV) form the empty capsid. Even though virus-like particles are produced at this stage, these previrions with the absence of internal proteins V and VII are essentially non-infectious, since the DNA molecules they encapsidate are still hanging outside of the particles. The addition of internal precursor protein pVII and activation of a 23-kDa viral proteinase lead to the formation of virion. Activated at a later stage of virus formation, the viral proteinase cleaves the precursor proteins (pVI, pVII, pVIII, pIIIa and pX) to form their respective mature forms [13,14]. This action of the adenoviral proteinase is essential for virion maturation and hence its infectivity. Some post-translational modifications of adenoviral proteins, such as phosphorylation of IIIa, V, and VI, were also reported in the literature [13].

Recent advancement in mass spectrometry (MS) has made the detailed study of the proteome of adenovirus possible. With the advent of new ionization techniques of matrix-assisted laser desorption/ionization (MALDI) [15,16] and electrospray ionization (ESI) [17], MS has become a powerful tool in structural characterization of large biomolecules. Much of the progress in biological MS has been highlighted by its application to the rapidly evolving field of proteomics that correlates proteins and their genes [18,19]. The key steps in proteomic studies by MS methods usually involve separation of proteins in complex mixtures, followed by characterization using MALDI-MS and/or ESI-MS in combination with database searches [20–26]. MALDI-MS, with its speed, sensitivity, and tolerance toward contaminants, is playing a very important role in characterization of proteins supporting a broad range of programs that cover genomics and proteomics [27,28]. The paradigm of proteome analysis by MS also has many applications in biotechnology that include monitoring the progress of manufacturing processes for quality control, as well as solving problems for process improvement of the biological products [29].

Some studies on viruses by MS have been reported in the literature, including characterization of capsid proteins of rhinovirus [30], detection of intact MS2 virus capsid [31], proteolytic ^{18}O-labeling for comparative proteomics of two serotypes of adenoviruses [32], and characterization of polypeptides of recombinant adenovirus [33–35]. Many of these studies focused on the dynamics of viruses and were limited in scope. In this article, we describe the development of a MALDI-MS-based assay for rapid identification of viral proteins (including mature forms, precursor forms and their propeptides) of the recombinant adenovirus generated for p53 gene therapy. The recombinant adenovirus in this study was derived from serotype 5 virus that has had the E1 coding sequences replaced with a 1.4-kb full-length human p53 cDNA (a tumor-suppressor gene mutated in 50% of all

human cancers) [36]. Our assay combines the use of multi-dimensional analytical techniques that include SDS–PAGE, chromatography, mass spectrometry (MALDI-MS and tandem mass spectrometry) and database search. It involves dissociation of intact viruses, separation of viral proteins by RP-HPLC and/or SDS–PAGE, enzymatic digestion of separated proteins, followed by MALDI-MS, MALDI-post source decay (PSD)-MS and database search.

2. Experimental

2.1. Recombinant adenovirus

Replication-deficient recombinant adenoviruses derived from type 5 expressing human p53 transgene were produced in HEK293 cells growing in serum-containing medium [36]. Viral vectors were purified from virus lysates through a two-stage column chromatography of anion-exchange and size exclusion as described previously [7].

2.2. SDS–PAGE separation of adenovirus structural proteins

Adenovirus structural proteins dissociated from $\sim 1.0 \times 10^{12}$ particles/mL of virus were loaded onto each well of Novex precast gradient 4–20% acrylamide gels (San Diego, CA, USA) and stained with Coomassie Blue (Novex, San Diego, CA, USA).

2.3. RP-HPLC separation of adenovirus structural proteins

Preparative RP-HPLC separation of viral proteins was performed as described by Lehmberg et al. [33] with minor modifications [37]. Approximately 6 mL of the column-purified virus preparation ($\sim 1 \times 10^{12}$ particles/mL) was loaded onto a Jupiter C4 column (4.6 × 250 mm, Phenominex, Torrance, CA, USA) equilibrated at 50 °C for viral protein dissociation and separation. Each chromatographic peak was collected manually for further mass spectrometric characterization.

2.4. Tryptic digestion of recombinant adenovirus structural proteins

After separation by SDS–PAGE, each band was excised from the Coomassie Blue-stained gel and destained with 50% acetonitrile/25 mM ammonium bicarbonate before being subjected to an overnight tryptic digestion at 37 °C (modified sequencing grade bovine trypsin; Roche Diagnostics, Indianapolis, IN, USA). Peptides were subsequently extracted from the gel with 60 μL of 75% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The final volume of the peptide extraction was brought down to near dryness using a SpeedVac concentrator (ThermoQuest, Holbrook, NY, USA) and resuspended in 12 μL of 10% acetonitrile containing 0.1% TFA for MALDI-MS analysis.

The dried adenoviral protein fractions collected from preparative RP-HPLC were reconstructed in 100 mM ammonium bicarbonate and subjected to trypsin digestion. Trypsin was added to the protein solution at an enzyme:substrate ratio of 1:20 (w/w). The solution was incubated at 37 °C overnight before MALDI-MS analysis.

2.5. MALDI-MS and database search

A PerSeptive Biosystems Voyager DE-STR MALDI time-of-flight (TOF) mass spectrometer (Applied Biosystems, Framingham, MA, USA) was used to acquire mass spectra data. The instrument was equipped with nitrogen laser (337 nm, 3 ns wide output pulse at 20 Hz) and a high current detector. For protein molecular weight (MW) detection, the instrument was operated in the linear mode. Sinapinic acid (Sigma–Aldrich, Milwaukee, WI, USA), saturated in 50% acetonitrile aqueous solution containing 0.1% TFA, was used as a matrix for MALDI-MS analysis of proteins.

For tryptic peptides, saturated α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich Milwaukee, WI, USA) in 50% acetonitrile aqueous solution–0.1% TFA was used as the matrix. The mass spectra of tryptic peptides of viral proteins were acquired in the reflectron

mode with an acceleration voltage of 20 kV, where isotopic peptide masses were obtained. The spectra were internally calibrated with trypsin autolysis peptides at *m/z* 659.384 and 2163.057 to ensure a mass accuracy of 30 ppm as database search criteria. Selected by a time ion selector (TIS), MALDI-PSD experiments were performed on the precursor peptide ions with a single stage reflectron operated at a series of descending mirror ratios from 1 to 0.1. This progressive decrease of applied potential to the reflectron is necessary to acquire a complete product–ion spectrum by concatenation of several sections. Calibration of product–ion spectrum was achieved by one-point calibration using the mass-to-charge value of the precursor ion (reflectron mirror ratio at 1). More than 100 laser shots were averaged for each PSD spectrum before generating the product–ion spectrum.

To identify adenovirus structural proteins, peptide mass fingerprints were searched against SwissProt non-redundant database using the MS-Fit algorithm, accessible at http://prospector.ucsf.edu/. For those search results with low MOWSE score or no hit on adenoviral proteins, MALDI-PSD-MS experiments were carried out to obtain peptide sequence tags that can be searched using MS-Tag program (http://prospector.ucsf.edu/).

3. Results and discussion

The major proteins of adenoviruses are hexon (II, MW 107,876 Da), penton base (III, MW 63,292 Da), peripentonal hexon-associated protein (IIIa, MW 63,501 Da), minor core protein (V, MW 41,446 Da), major core protein (VII, MW 19,412 Da), and other hexon-associated proteins (VI, MW 22,100 Da; VIII, MW 12,580 Da). In our process, traditional approaches such as SDS–PAGE with N-terminal amino acid sequencing were applied to fingerprint the adenoviral proteins for monitoring the quality of recombinant adenoviruses produced from purification steps, in-process and side fraction virus preparations, as well as various virus constructs [7,8]. However, it was found that most of the adenoviral proteins were N-terminally blocked. To obtain a chemically well-defined characterization method of viral particles, we developed a mass spectrometric assay to correlate viral structures with our process development. As shown in Fig. 1, the analytical strategy employed in this study is to combine SDS–PAGE and RP-HPLC with MALDI-MS and/or MALDI-PSD-MS in conjunction with database searches. In general, the dissociated viruses and/or intact viruses are first separated either by SDS–PAGE or RP-HPLC. Then, the separated viral proteins (gel bands from SDS–PAGE or fractions collected from RP-HPLC) are subjected to tryptic digestion, followed by MALDI-MS for peptide mass mapping. The identification of proteins is carried out by using MS-Fit search. If MOWSE score is insignificant or generating no identification, MALDI-PSD-MS experiments are performed to obtain peptide sequence tags with further MS-Tag search for the identification of the protein. This approach ensures complete characterization of viral proteins during the assembly process of viral production.

3.1. Identification of SDS–PAGE separated adenoviral proteins

As previously reported in our studies, the recombinant adenoviral proteins were initially extracted from gel bands of SDS–PAGE for the determination of their MWs by MALDI-MS [38]. This step provided mass measurements of viral proteins with far better accuracy than those obtained from SDS–PAGE (a mass accuracy of 0.1% was normally obtained for MALDI-MS). However, depending on the stage of maturation of the adenovirus, there are possibilities of the presence of precursor proteins, propeptides and post-translational modified proteins in the viral particle. To gain detailed structural information of viral proteins, tryptic digestion of each gel band followed by peptide mass mapping and protein identification through database search was carried out to provide additional information on the primary structures of these proteins.

All the proteins separated by SDS–PAGE (Fig. 2), with MWs ranging from 10,000 to 100,000 Da, were

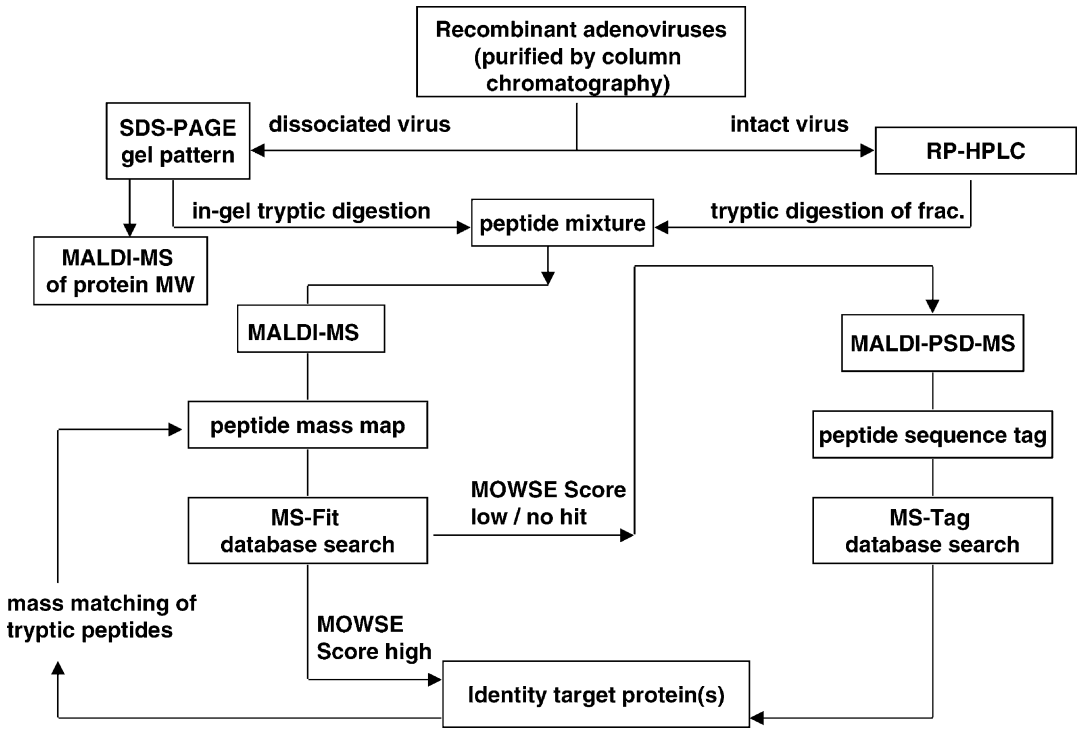

Fig. 1. Schematic diagram of multi-dimensional analysis of recombinant adenoviral proteins.

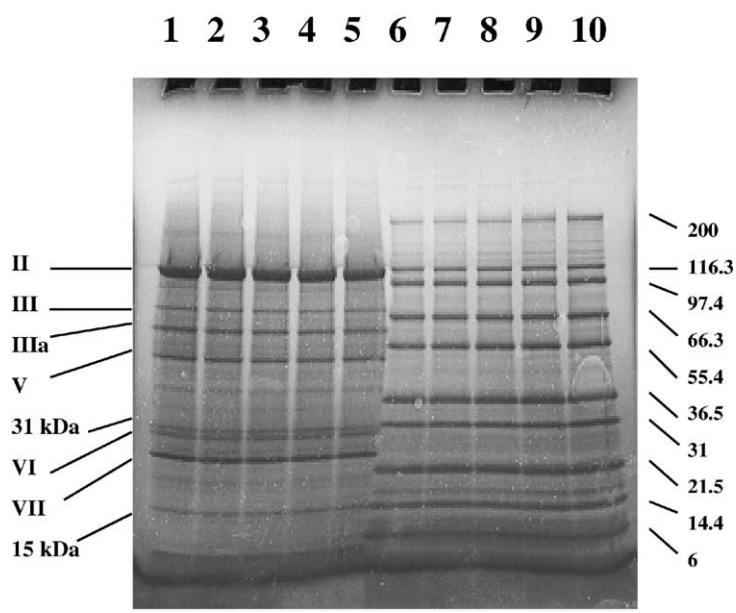

Fig. 2. Coomassie-Blue stained SDS–PAGE gel (4–20% gradient). Lanes 1–5 are adenoviral proteins, and lanes 6–10 are MW markers (values in kDa).

Table 1
Adenoviral protein identification from SDS–PAGE by MALDI-MS

Gel band	Calculated MW (Da)	Measured MW (Da)	MS-Fit search sequence coverage (%)
II	107,876	108,074	45
III	63,292	63,338	38
IIIa	63,501	63,526	50
V	41,446	41,518	43
pVIII (31 kDa)	24,687	24,620	43
VI	22,100	22,123	32
VII	19,412	19,482	52
Propeptide of pVIII (15 kDa)	12,114	12,058	52

identified as adenoviral proteins from the SwissProt database when searched against all taxonomy. Mature viral proteins, II, III, IIIa, V, VI, and VII, were characterized, and their presence was confirmed for the first time by MS. In addition, precursor protein pVIII as well as the propeptide of pVIII were detected. The amount of proteins loaded on the gel was ~2.5 μg dissociated from 1.0×10^{12} particles/mL of virus. The results are summarized in Table 1.

It is important to note that the 15 kDa gel band has been previously referred to as the mature form VIII of the precursor protein pVIII, located on the inner surface of the capsid. However, peptide mass mapping with MS-Fit search of this gel band identified the protein to be pVIII (MW 24,687 Da), rather than the originally predicted protein VIII. The database search results revealed that the amino acid coverage for the propeptide domain of pVIII (1–111, MW 12,125 Da) and the mature VIII (112–227, MW 12,579 Da) was 52 and 0%, respectively. Thus, combining mapping data with the MW information obtained from MALDI-MS measurement as shown in Table 1, the 15 kDa band was determined to be the propeptide of pVIII instead of protein VIII. Even though no adenoviral proteinase could be directly detected by SDS–PAGE, because of its extremely low copy number [13], the identification of the propeptide of precursor protein pVIII indicates the activity of the adenoviral proteinase. The presence of propeptide of pVIII may represent certain intermediate assembly state of the adenoviral particles during viral production.

In the SDS–PAGE assay of the purified adenovirus, a gel band located in between band V and band VI was observed and labeled as the 31 kDa band (Fig. 2). This band is one of the three major bands (II, IIIa and 31 kDa) observed only in the empty capsids, which was not detected in complete viral particles [37]. Previous studies have shown that the empty capsids are early assembly forms of the complete viral particles, which may contain precursor proteins, such as pVI and pVIII [39,40]. The 31 kDa band was previously referred to as the precursor of VI (pVI), solely based on the MW information derived from the mobility of the protein on the gel. Furthermore, due to a possible blockage of its N-terminus, the identity of this protein could not be confirmed by Edman sequencing. MALDI-MS analysis of trypsin digested 31 kDa band followed by database search using MS-Fit indicated that the tryptic peptides generated from the 31 kDa band actually matched the sequence of pVIII with 43% sequence coverage (Figs. 3 and 4). In Fig. 3, the signal with asterisk (∗) corresponded to the tryptic peptides from pVIII. The remaining signals represented peptides from trypsin autolysis and background ions. The measured MW of this protein at 24,620 Da is consistent with the identity of this band as pVIII.

The identification of this band has several important implications in virus purification and mechanism studies. The presence of pVIII in empty capsid confirms that the viral particle is at an early stage of virus assembly. Based on this finding and the uniqueness of pVIII to the empty capsid of the adenovirus, an assay has been developed to quantify the empty capsid contaminants by measuring the amount of pVIII detected in SDS–PAGE during our recombinant adenovirus preparation [37]. This assay helped to demonstrate that with a single cell line (HEK293) and a single recombinant adenovirus serotype, the amount of empty capsid concentration could vary significantly [37]. This new finding was quite different and unexpected from those earlier reports [6,40].

In addition to protein processing discussed above, post-translational modifications, such as phosphorylation of adenoviral proteins IIIa, V and VI, have been studied previously [13,34]. In the SDS–PAGE assay

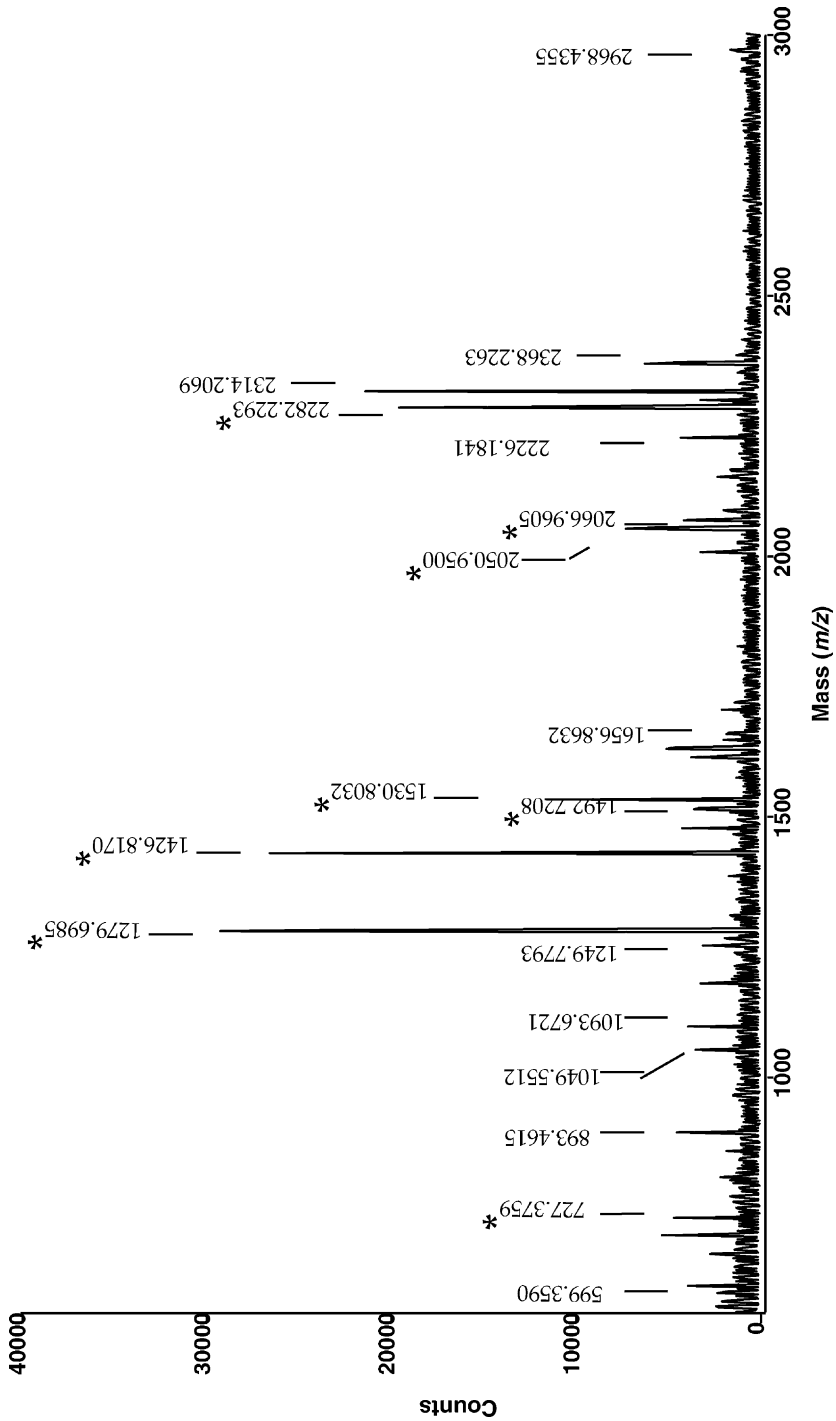

Fig. 3. Peptide mass mapping of in-gel trypsin digested 31 kDa band by MALDI-MS.

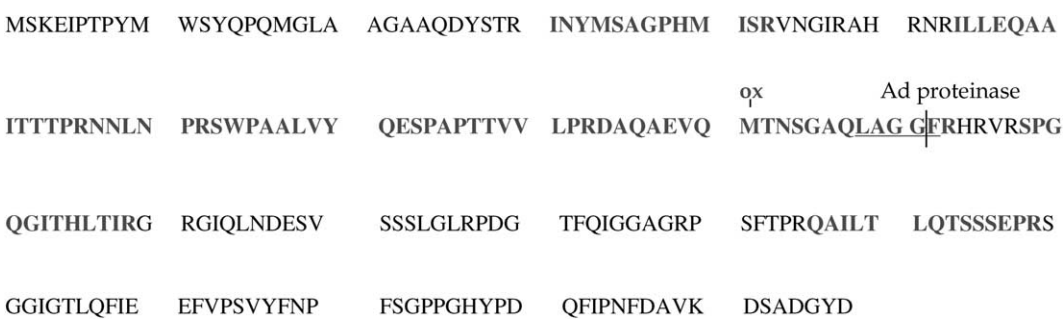

Fig. 4. Sequence and sequence coverage of pVIII (the matched sequences are highlighted in bold face). The adenovirus proteinase cleaved at the consensus sequence of LAGG/F ("/" designates the cleavage) to generate the propeptide of pVIII (1–111) and mature domain of pVIII (112–227). Oxidation of Met^{101} was detected by peptide mapping.

of adenoviral proteins (Fig. 2), bands III and IIIa are not in the normal gel running order according to their MWs obtained from MALDI-MS analysis (63,338 Da for band III and 63,526 Da for band IIIa) (Table 1). The band III with a lower MW showed a higher mobility on the gel electrophoresis. In order to explain this phenomenon, a separate study was conducted by LC/ESI-MS, which showed that protein IIIa is mono- and diphosphorylated (data not shown). The phosphorylation of IIIa is very likely to affect its mobility on the SDS–PAGE.

3.2. MALDI-MS of recombinant adenoviral proteins separated by RP-HPLC

Under RP-HPLC condition, intact adenoviral particles were dissociated into individual structural components (proteins and DNA) that were separated to yield a characteristic fingerprint [33]. Fig. 5 exhibits a typical RP-HPLC fingerprint of dissociated adenoviruses along with 17 identified adenoviral proteins. Compared with SDS–PAGE assay of the recombinant adenovirus, RP-HPLC is highly sensitive in providing faster and reproducible results. Most importantly, it can capture the information of smaller adenoviral polypeptides, generated during the maturation process of the virus, with MWs less than 10,000 Da. After MW measurements by MALDI-MS or LC/ESI–MS (data not shown), individual fractions collected from RP-HPLC were subjected to proteolytic digestion with trypsin for protein identification. All the bands previously observed from SDS–PAGE could be correlated and identified in the RP-HPLC assay. However, for the polypeptides captured in the early fractions of the RP-HPLC separation, peptide mass mapping alone may present a challenge for protein identification. Most of these polypeptides were generated from processing of viral proteins, possibly by adenoviral proteinase, which are smaller in size compared with their precursor protein database entries. Search of database with MS-Fit on these polypeptides normally generates non-significant protein IDs, either with low MOWSE score or no hit on adenoviral proteins. In such cases, peptide sequence tags from MALDI-PSD-MS studies were applied to search database using MS-Tag algorithm for protein identification. The MS-Tag search is well-documented to be successful for identifying proteins, especially in cases where unfavorable digestion conditions or modifications have made the digested peaks unidentifiable by a peptide mass mapping approach [18].

The following examples demonstrated the advantage of MALDI-MS methodology in the identification of low MW components and thus, its implications in correlating the quality of virus. Fraction 2 of RP-HPLC was isolated for MS characterization. The MW of this component was established to be 3037 Da. When interrogated against all taxonomy, as shown in Fig. 6 and Table 2, MS-Fit search of the tryptic

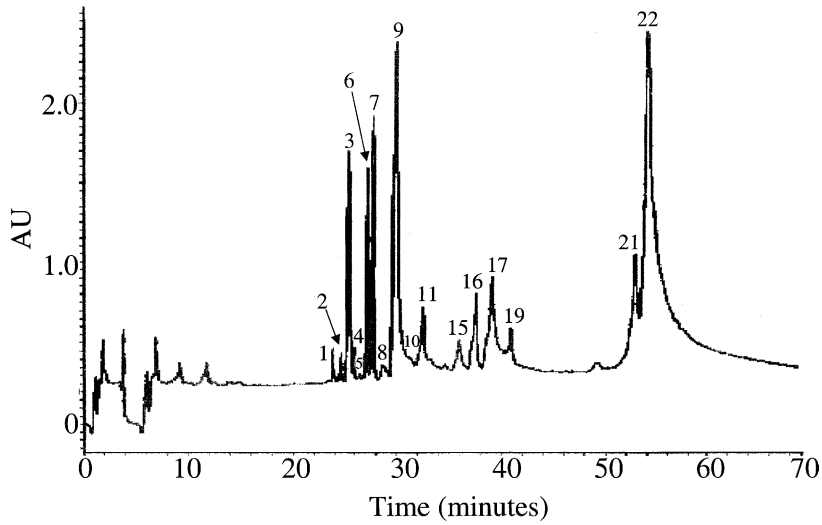

Fig. 5. RP-HPLC/UV chromatogram of dissociated polypeptides of recombinant adenoviruses. Seventeen adenoviral polypeptide peaks were identified: 1 = VI (240–250); 2 = pX propeptide (2–27); 3 = VIII (132–156), VIII (132–157), and pX propeptide dimer ((2–27)-SS-(2–27)); 4 = IIIa (571–585); 5 = pVIII (23–111); 6 = pVI propeptide (1–33); 7 = pVIII (22–131); 9 = VII; 11 = V; 15 = pVIII; 16 = VIII (157–227); 17 = VI; 19 = III; 21 = IIIa; and 22 = II.

peptides resulted in four protein identification with low and undistinguishable MOWSE scores. The third entry is the only protein correlated to an adenovirus related core protein precursor, pX. However, this identification could be regarded as a random hit. The search algorithm matched four peptides with 15% amino acid sequence coverage for pX, which is far less than the acceptable coverage (about 30%). In order to confirm this identification, sequence tags obtained through MALDI-PSD-MS studies of a tryptic peptide at *m/z* 819.46 were recorded (Fig. 7) and searched against SwissProt database using MS-Tag program. Adenoviral protein pX was unambiguously identified. Clearly, information obtained from MALDI-PSD-MS eliminated spurious protein fits and complemented peptide mass mapping information. Note that the identified pX protein is only available for adenovirus type 2 in the database and we assume a homology of adenovirus type 5.

Examination of the amino acid sequence coverage and the MW of the polypeptide in fraction 2 revealed that the adenoviral polypeptide covers the N-terminal portion of the authentic propeptide region of pX (1–32), from amino acid residues 2 to 27 (Fig. 8).

Table 2
MS-Fit search results of RP-HPLC fraction 2

Rank	MOWSE score	Protein MW (Da)/pL	Species	SwissProt Accession #	Protein name
1	77.6	37832.2/8.23	CHVP1	Q84424	MRNA capping enzyme
2	57.2	48778.4/8.39	HAEIN	P44856	NADH dehydrogenase
3	48.9	59428.2/7.07	ORENI	P70091	Cytochrome P450 19A133
3	48.7	8845.7/12.88	ADE02	P14269	Late L2 MU core protein precursor (11 kDa core protein) (protein X)
4	45.4	23123.2/9.06	BRUCA	Q45110	25 kDa outer-membrane immunogenic protein precursor

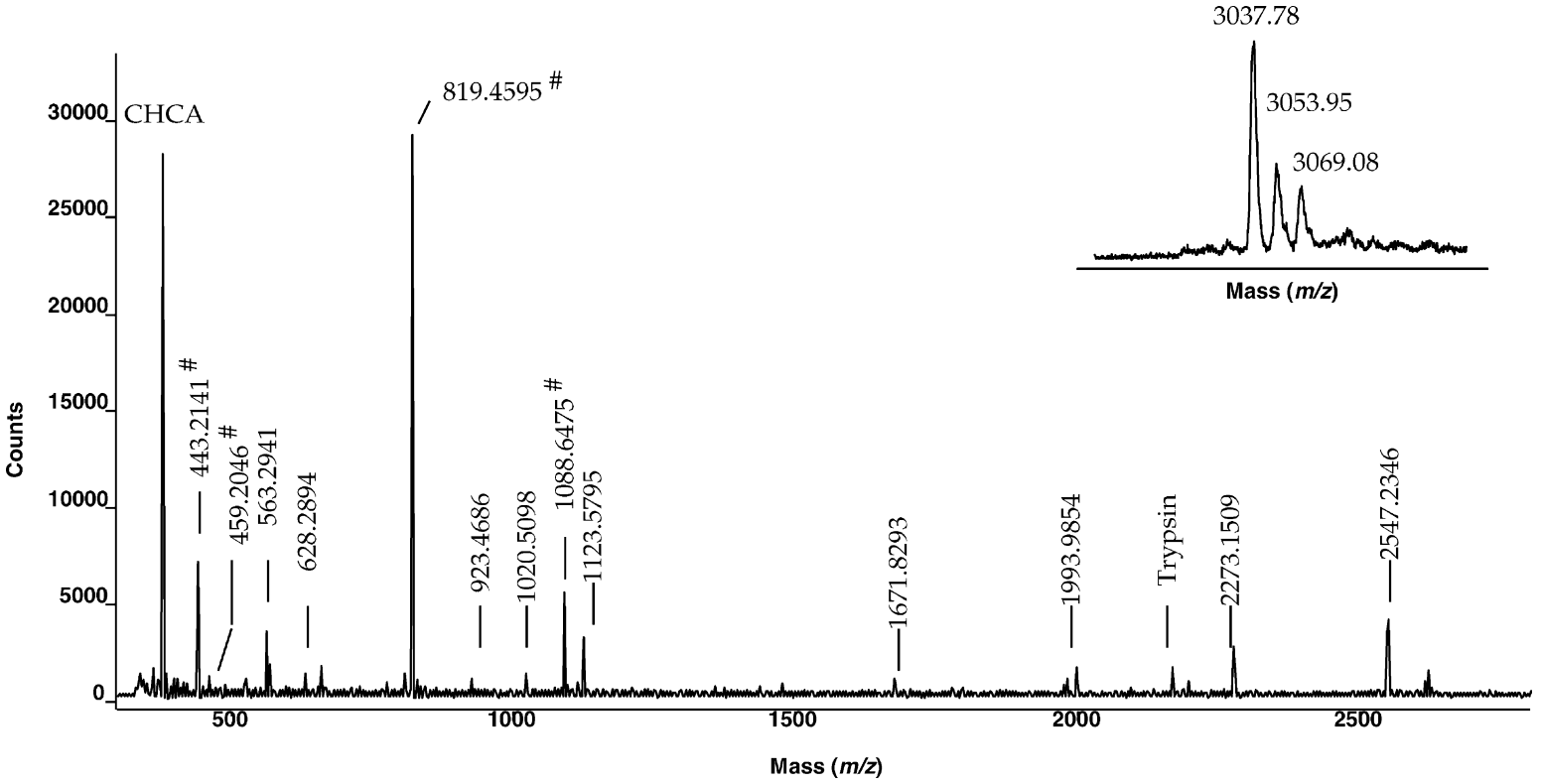

Fig. 6. Peptide mass mapping of trypsin digested RP-HPLC fraction 2 by MALDI-MS. The “#” indicates the MS-Fit matched tryptic peptide ions of pX.

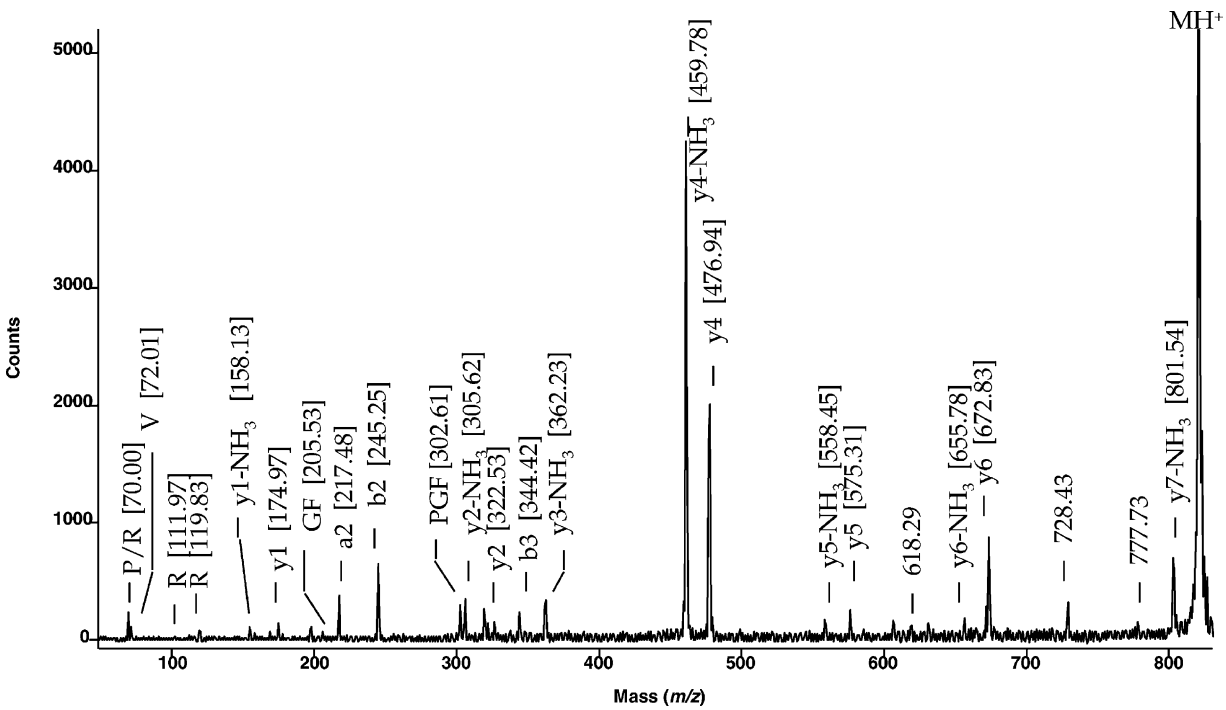

Fig. 7. MALDI-PSD-MS of tryptic peptide ion at *m/z* 819.5 from RP-HPLC fraction 2.

The cleavage at positions 27/28 (MAGH/G, "/"[designates cleavage site) conforms to one of the viral proteinase cleavage consensus sequences, which is (M, I, L)XGX/G (X represents any amino acid). This novel cleavage site of pX by adenoviral proteinase was not reported until very recently [34]. The detection of propeptide of pX confirms the adenoviral proteinase activity. The pX is a viral polypeptide tightly associated with DNA that has a role of facilitating DNA packaging into the virion. The information obtained from the MALDI-MS and MALDI-PSD-MS is valuable for understanding the virion packaging and adenoviral proteinase activity. With the additional sequence information, a manual matching of the tryptic peptides detected in Fig. 6 to the novel N-terminal propeptide of pX increased the sequence coverage

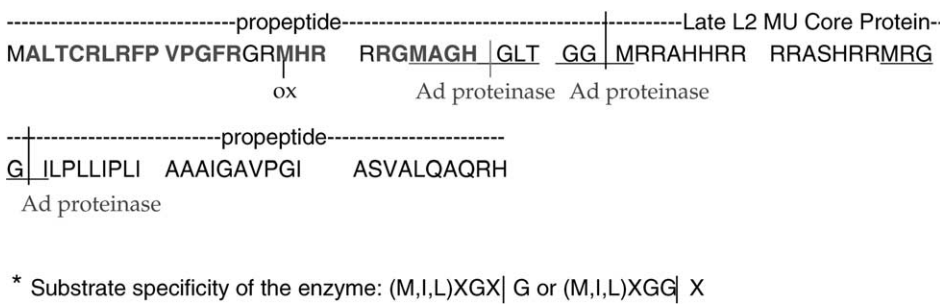

Fig. 8. Adenoviral proteinase cleavage sites of pX and the sequence coverage of pX by MALDI-MS and MALDI-PSD-MS studies (the matched sequences are highlighted in bold face).

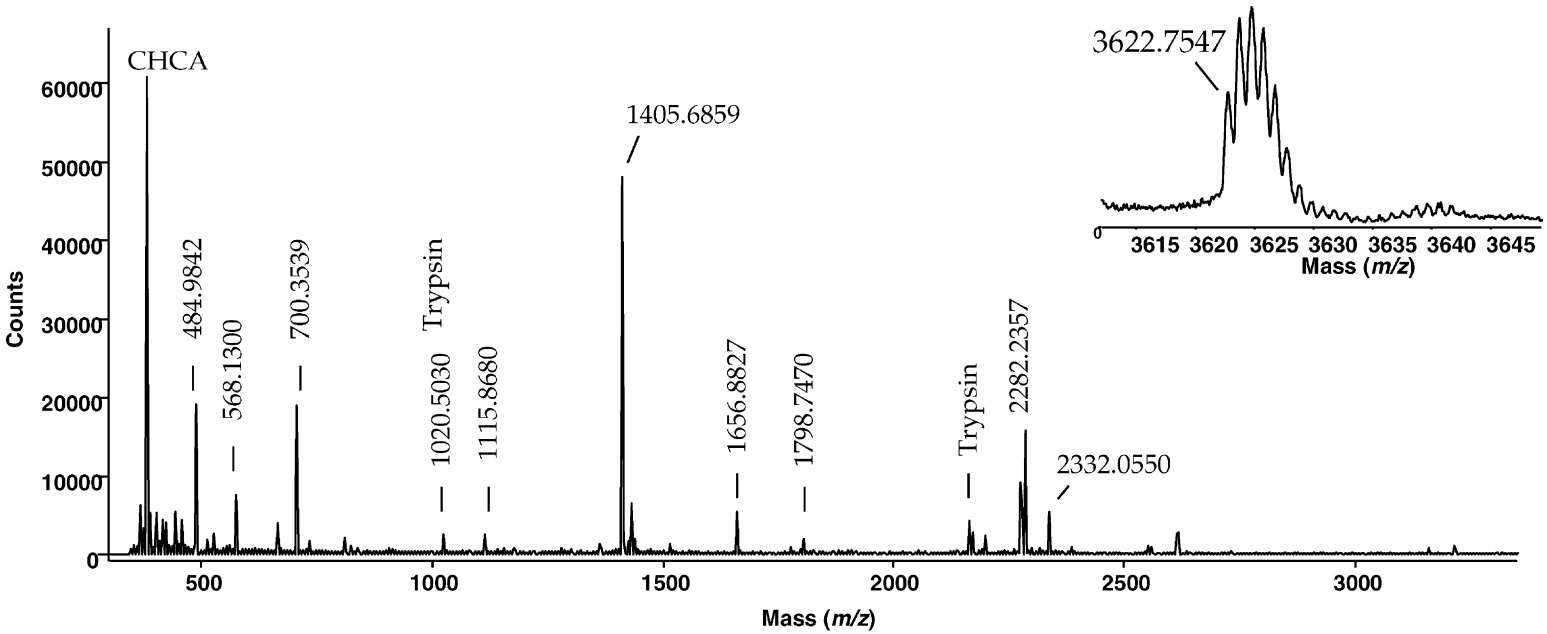

Fig. 9. Peptide mass mapping of trypsin digested RP-HPLC fraction 6 by MALDI-MS.

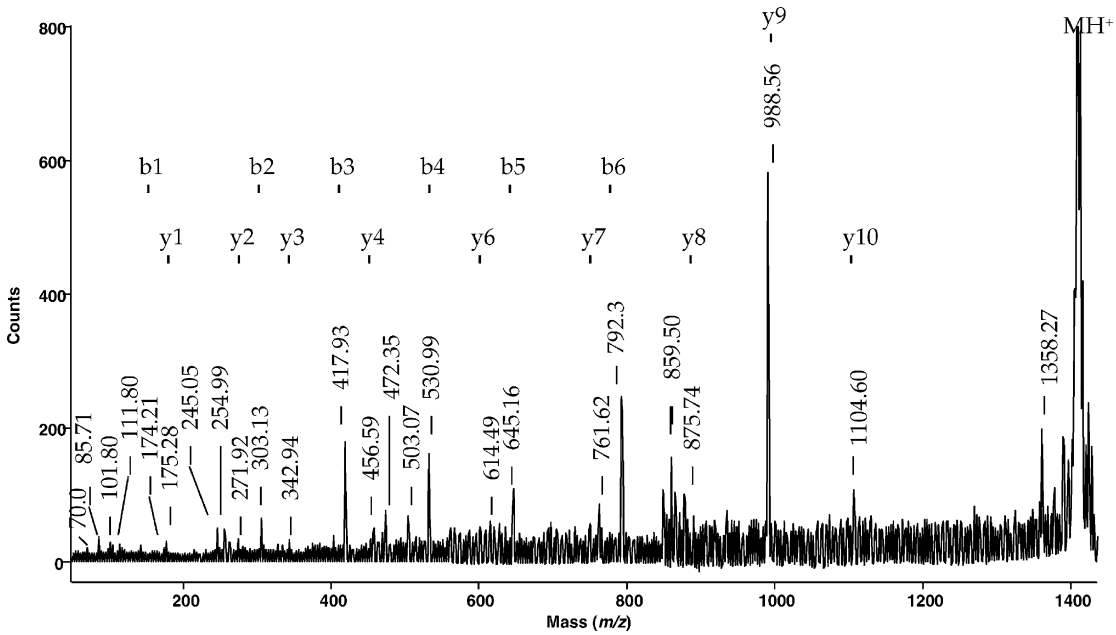

Fig. 10. MALDI-PSD-MS of tryptic peptide ion at *m/z* 1405.7 from RP-HPLC fraction 6. The individual fragment ions were indicated as "b" or "y" ions.

of the propeptide to 88%, consistent with the result of protein identification by sequence tags. Methionine oxidation was also detected on the propeptide of pX (-RM(-oxidation)HR-, *m/z* 443.2141 vs. *m/z* 459.2046) (Fig. 6).

Sequence tag information obtained from MALDI-PSD-MS alone would make reliable identifications of adenoviral propeptides using MS-Tag search, as illustrated in the case of identification of fraction 6. MALDI-MS of the polypeptide in fraction 6 detected a polypeptide with MW of 3622 Da. MS-Fit search of its tryptic peptide map led to no identification of adenoviral proteins. Except for the base peak at *m/z* 1405.6859 in the spectrum, most of the peptide signals were generated from trypsin autolysis (Fig. 9). The unidentifiable peptide ion at *m/z* 1405.6859 was selected for MS/MS studies by MALDI-PSD-MS and the spectrum is shown in Fig. 10. The database search using MS-Tag identified the polypeptide in fraction 6 as the minor capsid protein VI precursor (pVI). The sequence of the tryptic peptide at *m/z* 1405.6859 was interpreted to be a N-terminal acetylated peptide, Ac-MEDINFASLAPR, located at the N-terminus of pVI. Combining this sequence information with the MW of the polypeptide, propeptide of pVI (1–33) was confirmed to be present in fraction 6. This is another indication of the adenoviral proteinase activity.

4. Conclusions

Our study of the proteome of the recombinant adenovirus type 5 vectors demonstrated an important application of MALDI-MS techniques in the biopharmaceutical industry. With completely sequenced adenovirus genome available, MALDI-MS in combination with separation techniques (SDS–PAGE and RP-HPLC) and database searches provided a chemically well-defined method of characterization of structural proteins of recombinant adenoviral vectors. The information of protein MWs, tryptic peptide

mass mapping, and sequence tags of tryptic peptides derived from MALDI-MS and/or MALDI-PSD-MS resulted in the identification of 17 adenoviral proteins/polypeptides in the purified virion. Proteolytic processing of precursor proteins (pVI, pVII, pVIII and pIIIa) into their mature forms by adenoviral proteinase was clearly established by MS studies. In addition, precursor protein pVIII, propeptides of precursor proteins pVI, pVIII, and pX, as well as polypeptides derived from further processing of pVIII or VIII were identified in the recombinant adenovirus. Furthermore, a novel adenoviral proteinase cleavage of pX was confirmed by tandem mass spectrometry for the first time. Post-translational modifications and/or purification related modifications, such as N-terminal acetylation of propeptide of pVI and oxidation of propeptide of pX, were also revealed from MALDI-MS and MALDI-PSD-MS studies. The rapid and accurate identification of viral proteins from recombinant adenoviruses in this study is significant since it provides direct evidence of the maturation stage of adenoviruses, which is closely related to viral infectivity and efficacy in gene therapy.

The findings of this investigation have significant implications in the development of biological assays as well as infection mechanism studies of the adenovirus. Based on the identification of pVIII and the measurement of the quantity of pVIII in empty capsids, an assay has been developed to quantify the amount of empty capsid present in the adenoviral particles purified from column chromatography. Protein fingerprints of adenoviruses and their post-translationally modified counterparts have been used to monitor structural changes of adenoviruses for process development and quality control of the recombinant products.

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