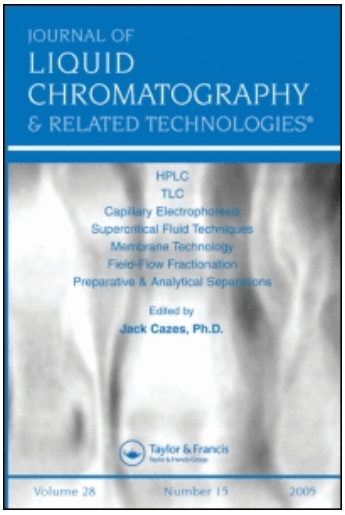

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bhown, Ajit S. , Bennett, J. Claude , Cornelius, Thomas W. and Hunter, Eric(1983) 'Exclusive Use of High Pressure Liquid Chromatography for the Determination of the Complete Amino Acid Sequence of the 12K Fragment of Avian Sarcoma Virus Structural Protein p27', Journal of Liquid Chromatography & Related Technologies, 6: 2, 249 — 258
To link to this Article: DOI: 10.1080/01483918308066886
URL: http://dx.doi.org/10.1080/01483918308066886

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 249-258 (1983)

EXCLUSIVE USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF THE COMPLETE AMINO ACID SEQUENCE OF THE 12K FRAGMENT OF AVIAN SARCOMA VIRUS STRUCTURAL PROTEIN p27

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ABSTRACT

Using exclusively high pressure liquid chromatography for the protein and peptide separation complete primary structure of the 12,000 molecular weight (12K) amino terminal (1-87 residues) fragment obtained by mild acid hydrolysis of p27 (Avian Sarcoma Virus structural protein) has been determined. The sequence was established by direct degradation of the native molecule and its (12K) peptides isolated by molecular exclusion and reverse phase HPLC.

 0148-3919/83/0602-0249$3.50/0

INTRODUCTION

Avian Sarcoma viruses (ASV) possess a major internal structural polypeptide (p27) of 27,000 daltons, which forms the inner capsid shell of the virion (1,2). This protein, p27, is highly conserved and carries group specific antigenic determinants, that characterize the avian sarcoma viruses (ASV). The relatedness of the conserved or interspecies antigenic determinants of a viral protein can be considered indicative of the taxonomy of the viruses (3,4). The present study was undertaken to delineate the total primary structure of p27 to help locate the conserved areas of amino acid sequences responsible for its antigenicity.

The study of protein structure presents unique problems that require innovative approaches. The limiting factor in achieving the total primary structure of a protein molecule is the availability in sufficient amounts of pure homogeneous sample. The classical methods of protein purification involving gravity fed open column chromatography suffer with numerous major drawbacks. These include sample losses and larger volumes not to mention the time of separation which frequently extends into days. HPLC has emerged as an excellent alternative to overcome these otherwise insurmountable problems of the protein chemist.

Earlier methods (5) of separation of p27 by gel filtration utilizing 6 M quanidine hydrochloride were time consuming and involved desalting which frequently resulted in significant losses. Ion exchange chromatography, a commonly used technique for peptide separation, is nearly always associated with low recoveries. In addition all of these techniques are time consuming. By utilizing selective methods of chemical and enzymatic cleavages followed by their size and reverse phase separation by HPLC, it is feasible to obtain structural

information on protein and peptides on small amounts of sample. Furthermore, the speed of separation, high yields and use of volatile buffer system makes the technique even more attractive for structural analysis.

p27, when subjected to mild acid catalyzed hydrolysis, generates two fragments with 12,000 (12K) and 15,000 (15K) molecular weights as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Earlier (6) we have reported the alignment of these two peptides in p27, in the present study, we report the determination of complete primary structure of the 12,000 daltons (12K) fragment (6) employing only HPLC and volatile buffer systems.

MATERIALS AND METHODS

Chemicals: Sequencer grade chemicals were purchased from Spinco Division, Beckman Instruments (Palo Alto, CA). Methanol (Omnisolv) for high pressure liquid chromatography was the product of MCB Manufacturing Chemicals Inc. (Cincinnati, OH). All other chemicals were of highest purity grade and were obtained from Pierce and/or Fisher.

Virus: Rous Sarcoma virus (RSV-PRC) was propagated in chick-helper factor-negative C/E chicken embryo cells as described earlier (6)

Purification of p27: The viral protein p27 was purified by high pressure liquid chromatograph (HPLC) from Rous Sarcoma virus (RSV-PRC) as described by Bhown et al. (7) except the sample was not reduced and alkylated.

Amino acid analysis: For amino acid analysis protein samples were hydrolyzed at 110°C with constant boiling HCl in sealed evacuated ampules for 24 hrs and analyzed on a Durrum D-500 analyzer.

Acid Digestion: Acid catalyzed hydrolysis of the single aspartylprolyl bond in p27 was performed as described by Bhown et al. (6).

Separation of the products of acid digestion: Acid cleaved fragments were separated by HPLC (Waters Associates, Milford, Mass.) equipped with two 6000A solvent delivery pumps, a U6K universal septumless injector, model 440 dual channel absorbance detector and four I-125 columns (Part #84601 Waters Associates) attached in series. A mixture of acetic acid: propanol: water (20:15:65) was employed as a mobile phase, at a flow rate of 0.2 ml/min. The effluent was monitored at 280 nm, the peaks were collected manually and lyophilized.

Cyanogen bromide digestion: The low molecular weight fragment (12K) was digested with 30 fold excess of cyanogen bromide in 70% formic acid for 4 hrs at room temperature. The digest was diluted with water and lyophilized.

Separation of the products of cyanogen bromide digestion: The products of CNBr digestion were fractionated by HPLC employing two I-60 columns (Part #85250 Waters Associate) in series and acetic acid: propanol: water (20:15:65) as mobile phase at 0.2 ml/min flow rate. Peak fractions were manually collected and lyophilized.

Acetylation: The 12K fragment was dissolved in 0.2 M N-ethylmorpholine acetate pH 9.0 and 10 fold molar excess of acetic anhydride over protein was added during constant vortexing. The mixture at this stage develops a cloudy appearance. The sample was lyophilized.

Trypsinization; The acetylated 12K fragment was solubilized in 1% ammonium-bi-carbonate and digested at 37°C for 4 hrs with TPCK trypsin (Worthington Biochemical Corp. Freehold, N.J.) solubilized in 1mM HCl, using an enzyme:protein ratio of 1:50. The trypsin digest was lyophilized and peptides fractionated on reverse phase HPLC.

Tryptic peptide isolation: Peptides resulting from trypsin digestion of acetylated 12K were chromatographed on a Waters C_{18} μBondapak column (0.46 x 25cm part #) as follows: Solvent "A" was 0.1% TFA and "B" 60% acetonitrile containing 0.1% of TFA. The column was developed with a 60 min linear gradient of 0% B to 100% B at a flow rate of 2.0 ml/min with a 10 min delay between start of the gradient and sample injection. The effluent was monitored at 206 nm in an LKB absorbance detector (Uvicord model #2138).

Amino acid sequence analysis: Sequential degradation of proteins and peptides was achieved on a modified Beckman 890C automated sequenator as described by Bhown et al. (8). Phenylthiohydantoin (PTH) derivatives of cleaved amino acids were identified and quantitated by HPLC as described earlier (8).

RESULTS AND DISCUSSION

Separation and amino acid sequence of 12K: In order to obtain large peptides which could be easily, separated by molecular exclusion HPLC and sequenced p27 was subjected to mild acid catalyzed hydrolysis as reported earlier (6). This chemical cleavage yields two major fragments with 12,000 (12K) and 15,000 (15K) molecular weight. Separation of these products (80-90% yield) was carried out by gel permeation HPLC (Figure 1). The amino terminal amino acid sequence analyses of these fragments confirmed that the 12K fragment orginated from the amino terminus of the parent molecule (6).

Amino terminal sequence: Quantitative recovery (>90% yield) of p27 by gel permeation HPLC (7) have permitted assignment of 36 residues from the amino terminus as shown in figure 4.

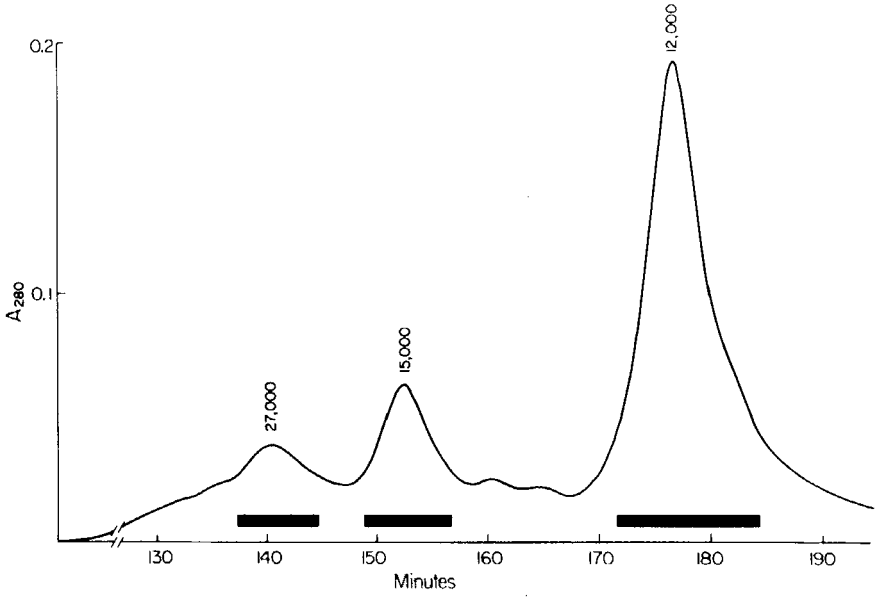

Figure 1. Molecular exclusion HPLC pattern of the products of acid catalyzed hydrolysis of p27. Horizontal bars indicate the peak area pooled. For details, see text.

Separation of the products of cyanogen bromide digest of the 12K fragment: Amino acid analysis (not shown) of this fragment (12K) revealed the presence of 4 methionines. Cyanogen bromide digestion of 12K fragment was attempted next which produced three major peptides. These peptides were separated on a low molecular weight exclusion column (I-60) on HPLC. Separation of the peptides is shown in Figure 2. Amino acid sequence analysis of second and third fraction, (in order of elution) helped in establishing the total primary structure of the 12K fragment.

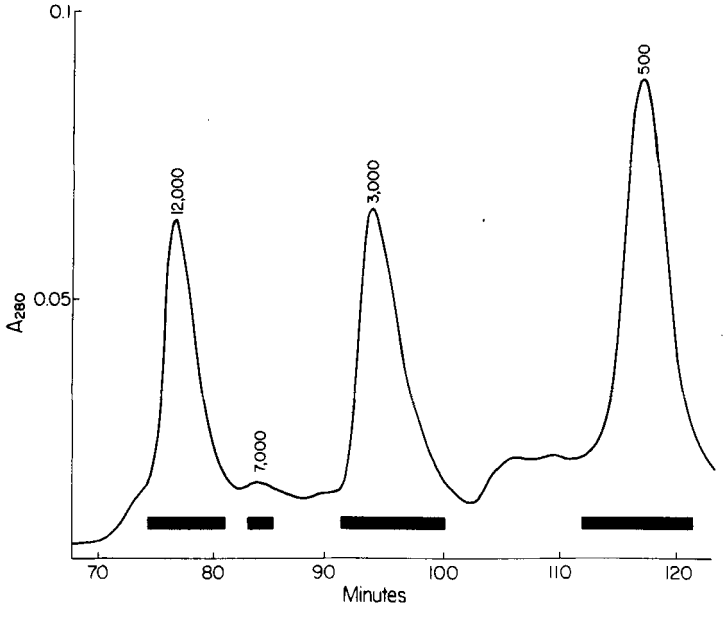

Figure 2. Gel filtration of cyanogen bromide digest of the 12K fragment on I-60 columns attached in tandem. Horizontal bars indicate the peak area pooled. For details, see text.

Separation of tryptic peptides: In order to restrict the trypsin cleavage sites lysine residues of the 12K fragment were irreversibly blocked with acetic anhydride. The separation of tryptic peptides was achieved on reverse phase HPLC and is shown in Figure 3. Tryptic peptides nine and fourteen on amino acid sequence analysis provided the necessary data to complete the structural studies of this fragment.

Amino acid sequence analysis: Peptides (2-4 nmols) obtained by cyangen bromide and trypsin digest were separated by HPLC and sequenced wih an average repetitive yield of 95%. The results are summarized in figure 4.

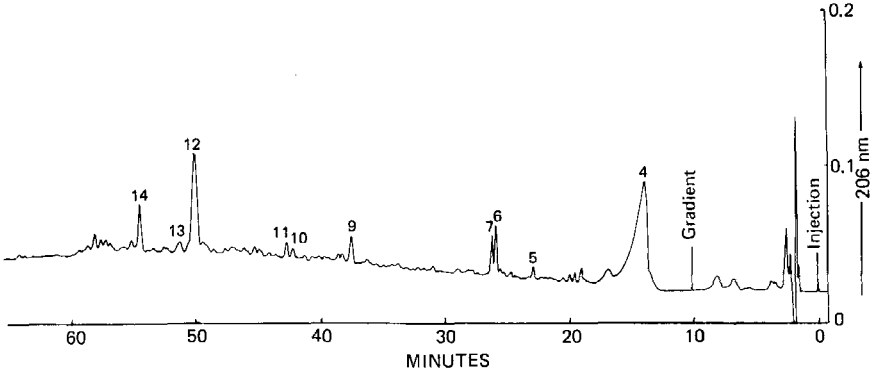

Figure 3. Reverse phase HPLC of tryptic peptides of the acetylated 12K fragment.

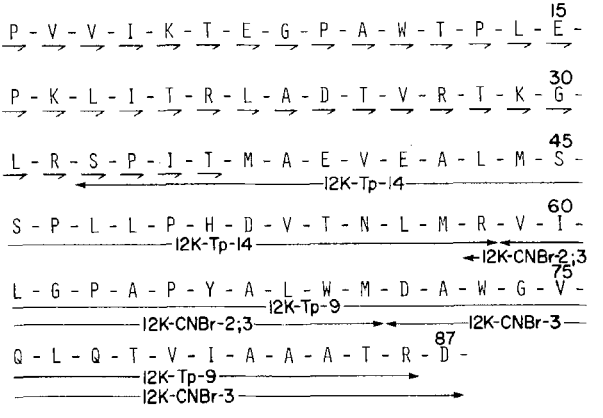

Figure 4. Amino acid sequence of the 12K fragment.

Our HPLC techniques have allowed us to obtain p27 in highly purified form and in high yields that have prompted us to undertake detailed studies of this molecule. The presence of a single aspartylprolyl bond, susceptible to dilute acid almost in the center of the molecule between residues 87-88 simplified the inital approach to sequence analysis. By employing both gel permeation HPLC and reverse phase HPLC we have successfully isolated the necessary cleavage fragments to complete the total primary structure of a 12,000 molecular weight fragment of p27, thus establishing the amino acid sequence of residues 1-87 (Fig. 4). This is in total agreement with that predicted from DNA sequence studies (D. Schwartz, Harvard University, personal communication).

ACKNOWLEDGEMENTS

The authors wish to thank Thomas Cornelius, Jane Ellen Robbins, Karen Buttler, Lisa Kallman and Edgar Hill for their expert assistance, and to Brenda Gosnell and Melissa Ham for typing the manuscript. This work was supported by grants AM 03555, AM 20614, CA-13148 and CA 29884.

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