[1] Purification of Viral Polymerases: General Considerations

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Introduction

Viruses are obligate parasites that can infect most prokaryotic and eukaryotic cells in nature. Viruses contain either DNA or RNA as their genomic material. Essentially, the viral genome is made up of three types of genes: (a) genes encoding enzymes required for the replication of the viral genome; (b) genes encoding proteins involved in regulatory processes; and (c) genes that encode the viral structural proteins such as capsid and envelope proteins. The smaller DNA viruses such as polyoma and SV40 (parvoviruses and papovaviruses), which are similar to phages Ff and ϕX174 in size (5-kb genome size), produce virulent infections and rely almost entirely on the host replication machinery. The intermediate size viruses, such as λ phages (48-kb genome size), induce the production of a few enzymes of their own but depend primarily on host enzymes. However, larger viruses, such as herpes and vaccinia viruses (100- to 300-kb genome size) and phages such as T4 and T7, contain several genes encoding multiple enzymes that direct the synthesis of precursor proteins as well as a relatively complete and independent replicative apparatus.[1,2]

With the availability of primary sequences for DNA- and RNA-dependent DNA polymerases, it has become possible to compare and delineate some of the highly conserved regions found in polymerases from diverse origins. By such sequence comparisons it has been shown that the amino acid sequence of the DNA polymerase from bacteriophage SPO2 is homologous to the polymerase domain of the Klenow fragment of DNA polymerase I from *Escherichia coli*. The same regions are also related to the enzyme from other species, such as *Staphylococcus pneumoniae, Thermus aquaticus,* and bacteriophages T7 and T5.[3,4] The sequence alignments among these polymerases indicate at least three conserved motifs in these proteins. Similarly, sequence alignments of various RNA-directed polymerases have revealed four conserved motifs. Two of these motifs are common among

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DNA-Dependent Polymerase Motifs

	Motif A	Motif B	Motif C
Adenovirus	PLYVYDICGMYASAL.THPMPWGPP	TLRSIAKLLS.NALYGSFATKLDNKKIV	PLKSVYGDTDSLFVT
CMV	PVAVFDFASLYPSIIMAHNLCYSTL	KEQMALKVTC.NAFYGFTGVVNGMMPCL	EARVIYGDTDSVFVR
Epstein Barr	PVLVVDFASLYPSIIQAHNLCYSTM	KQQLAIKCTC.NAVYGFTGVANGLFPCL	QLRVIYGDTDSLFIE
Herpes	PVVVFDFASLYPSIIQAHNLCFSTL	KQQAAIKVVC.NSVYGFTGVQHGLLPCL	SMRIIYGDTDSIFVL
Vaccinia	NVLIFDYNSLYPNVCIFGNLSPETL	SMQYTYKIVA.NSVYGLMGFRNSALYSY	RFRSVYGDTDSVFTE
Varicella	PVVVLDFASLYPSIIQAHNLCFTTL	KQQAAIKVVC.NSVYGFTGVAQGFLPCL	EVKVIYGDTDSVFIR
Human Pol α	FILLLDFNSLYPSIIQEFNICFTTV	IRQKALKLTA.NSMYGCLGFSYSRFYAK	NLEVIYGDTDSIMIN
Klenow	VIVSADY.SQIELRIMAHLSRDKGL	EQRRSAKAINFGLIYGMSAFGLARQLNI	RMINQVHD.ELVFEV
Spo2	EFYVSDF.SAIEARVIAWLAGEEWR	PLRQKGKVAELALGYQGGKGALIQMGAL	KTVMHVHD.EAVLLV
Staph	VLLSSDY.SQIELRVLAHISKDEHL	NDRRNAKAVNFGVVYGISDFGLSNNLGI	KMLLQVHD.EIVLEV

RNA-Dependent Polymerase Motifs

	Motif 1	Motif 2	Motif 3	Motif 4
Flu	EISFTITGDNTK.WNEN	LIDGTASLSPGMMMG..MFN.MLSTVLG	GLQSSDDFALI	LLGINM.SKKKSY
Hepititis B	SNLSWLSLDVSAAFYHL	ILGFR.KIPMGVGLSPFLLAQFTSAICS	AFSYMDDVVLG	SLGIHL.NPNKTK
HIV1	KKKSVTVLDVGDAYFSV	RYQYN.VLPQGWKGSPAIFQSSMTKILE	IYQYMDDLYVG	RWGLTT.PDKKHQ
HIV2	KKRRITVLDVGDAYFSI	RYIYK.VLPQGWKGSPAIFQHTMRQVLE	IIQYMDDILIA	GLGFST.PDEKFQ
Polio	MEEKLFAFDYTG.YDAS	TYCVKGGMPSGCSGT.SIFNSMINNLII	MIAYGDDVIAS	DYGLTMTPADKSA
Rhinovirus	MDGHLMAFDYSN.FDAS	IYVVEGGMPSGCSGT.SIFNSMINNIII	ILAYGDDLIVS	NYGLTITPPDKSE
RSV	RGWPLMVLDLKDCFFSI	RFQWK.VLPQGMTCSPTICQLVVGQVLE	MLHYMDDLLLA	RAGFTI.SPDKVQ

both DNA- and RNA-directed polymerases.[5] Figure 1 depicts these conserved motifs for a number of DNA- and RNA-dependent polymerases from different sources. Even though these motifs encompass only a few invariant amino acids, the validity of these alignments is generally supported by genetic studies that have demonstrated a critical role for these residues in the catalytic function of these polymerases.[6–10] For example, the invariant DD in motif 3 of RNA-dependent DNA polymerases is thought to be at the polymerase active site.

The primary sequence of human DNA polymerase α deduced from the full-length cDNA also contains regions of similarity to sequences in replicative DNA polymerases from E. coli phages PRD1 and T4, Bacillus phage f19, yeast DNA polymerase 1, herpes viruses, vaccinia virus, and adenovirus.[11–13] Furthermore, the conservation of these homologous regions across a vast phylogenetic expanse indicates that these prokaryotic and eukaryotic DNA polymerases may have evolved from a common primordial gene.[12] These conserved consensus sequences are suggested to define potential sites that serve essential roles in the DNA polymerase-catalyzed reactions. By a careful comparison of the viral genome sequences with the existing known motifs, it is possible to delineate the region encoding for the specific polymerase. For example, by using this approach, RNA polymerase from hepatitis C virus[14] and reverse transcriptase from duck

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FIG. 1. Multiple alignments of a few DNA- and RNA-dependent polymerases. The three motifs A, B, and C in DNA-dependent polymerases and the four conserved motifs numbered 1 to 4 in RNA-dependent polymerases have been highlighted. These motifs are contiguous in their primary sequences within each polymerase. The number of first amino acid is 538, 712, 579, 717, 519, 677, 858, 700, 381, 648, 297, 410, 257, 286, 1973, 1944, and 99 for adeno, CMV, Epstein–Barr, herpes, vaccinia, varicella, human Pol α, E. coli, SPO2, S. pneumoniae, Flu, HepB, HIV-1, HIV-2, polio, rhino, and RSV, respectively.

hepatitis B virus[15] have been identified, expressed, and purified. Further evidence for the close structural relatedness of polymerases from diverse sources comes from X-ray crystallography studies. A pattern of structural similarity has been identified in the crystal structures of HIV-1 reverse transcriptase[16] and the Klenow fragment of DNA polymerase I[17] and T7 RNA polymerase.[18]

Although there are a few conserved amino acid residues in different motifs of these polymerases and although polymerases may share common structural and functional features, no single method can be generalized to purify all polymerases. However, the requirement for polymerases to bind either DNA or RNA (the template) has been extensively used in their purification and in the purification of many other DNA-binding proteins. In addition, substrates and specific inhibitors have been used as affinity ligands for the purification of polymerases. For related polymerases (from the same family of viruses), published procedures can be used as a starting point and modifications be made along the way to improve the specific activity, purity, and recovery of the enzyme.

Purification of Polymerases: A General Strategy

The first DNA polymerase (pol I) was discovered and purified almost four decades ago[19] from *E. coli.* The original procedure of purification included several fractionation steps such as streptomycin precipitation, DNase digestion, adsorption to alumina gel, and ammonium sulfate precipitation followed by chromatography on DEAE-cellulose. Since then, many polymerases and replication proteins have been identified. Isolation and purification of viral polymerases, in general, can be achieved with conventional column chromatographic techniques.[20–23] While detailed purification procedures for individual viral DNA or RNA polymerases have been described in this volume, only a general summary of salient features is provided that is specific for polymerases.

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General Considerations

Polymerases have been purified from virions, tissue culture cells, rabbit reticulocyte lysates, *E. coli,* yeast expression systems, and eukaryotic expression systems such as vaccinia virus and baculovirus.[14,15,24-32] For isolating the most active and stable form of the enzyme it is sometimes necessary to compare purified recombinant enzyme from different sources. It is best to start with a source that produces a decent amount of the required enzyme in a soluble form. Expression of proteins in bacteria does not always produce the enzyme in its native, soluble, and biologically active conformation. Instead, overproduction of the enzyme of interest may result in the formation of insoluble aggregates (inclusion bodies) within the host cell.[21,33] Isolation of enzymes from inclusion bodies requires a denaturation–renaturation step and thus can introduce uncertainties regarding proper folding of the enzyme. If expression in a bacterial system results in the formation of inclusion bodies, factors such as host strain, incubation temperature, and length of incubation can be varied. Sometimes simply lowering the temperature and decreasing the incubation time can result in a soluble enzyme.

In order to maintain the stability of an enzyme during purification, it is essential to include ingredients in the buffer that would stabilize the enzyme and minimize the conditions that would denature it. In general, operations should be carried out at 0–4° and protease inhibitors should be included in buffers, at least during the initial steps when the crude cellular extract contains a wide variety of proteolytic activities. A list of commonly used protease inhibitors is shown in Table I. It is important to note that some of the protease inhibitors such as phenylmethylsulfonyl fluoride have a short half-life and should be replenished during the purification.

The manner in which cells are disrupted to prepare the cellular extract can have an immense effect on further purification protocols. Conditions that ensure maximum yield while maintaining the integrity of the native

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TABLE I
LIST OF COMMONLY USED PROTEASE INHIBITORS IN PROTEIN PURIFICATION

Inhibitor	Protease type inhibited	Effective concentration
Antipain	Cysteine/serine protease	1 µg/ml
Aprotinin	Serine protease	5 µg/ml
Benzamidine	Serine protease	1 mM
Chymostatin	Cysteine/serine protease	10–100 µM
EDTA/EGTA	Metalloprotease	0.1–1 mM
Leupeptin	Cysteine/serine protease	1–100 µM
Pepstatin	Metalloprotease	1 µM
1,10-Phenanthroline	Metalloprotease	1 µM
Phosphoramidon	Metalloprotease	1 µM
Phenylmethylsulfonyl fluoride	Cysteine/serine protease	0.1–1 mM

enzyme and its activity should be employed. Extraction volumes should be kept minimal and the composition of the buffer should preserve enzyme activity. The method used for the lysis of cells largely depends on the nature of cells. Animal cells grown in tissue culture can be lysed with hand-held or motor-driven homogenizers in hypotonic buffers. Bacterial and yeast cells can be disrupted by agitation with glass beads. For small volumes (<1 ml), sonication is a convenient method. Pressure cells such as French Press (~50 ml), Microfluidizer, and Manton-Gaulin Press (150–1000 ml) are efficient means of lysis. It is always a good idea to monitor the extent of cell breakage through direct microscopic examination and by monitoring enzyme activity. The enzyme recoveries can be dramatically improved if the lysis step is efficient and complete.

The initial fractionation step of the cellular lysate should be fast, thus removing some of the contaminating proteases that can result in the loss of polymerase activity. This is usually achieved by a salting out procedure such as ammonium sulfate, polyethyleneglycol, polyethyleneimine precipitation, or an adsorptive chromatographic step such as batch ion-exchange chromatography.[20,21] It is best to perform the next step of fractionation immediately since all the contaminating protease activities cannot be removed by this procedure alone. Table II lists some of the steps commonly used in the purification of polymerases.

Adsorption chromatography is a common feature for most polymerase purification procedures. Proteins bind to the exchanger resins by electrostatic forces. In practice, the conditions for adsorption will have to be found empirically. Even if the polymerase does not bind to any of the exchangers under the conditions used, a high degree of purification is possible due to

TABLE II

COMMON TECHNIQUES FOR PURIFICATION OF POLYMERASES

Technique	Property utilized	Characteristic	Comments
Precipitation Ammonium sulfate Polyethylene glycol Polyethyleneimine	Hydrophobicity	High capacity, very low resolution, fast	Useful with large volumes
Ion-exchange chromatography	Charge	High capacity, high resolution, fast	Low ionic strength and optimum pH critical for binding
Hydrophobic interaction chromatography	Hydrophobicity	Medium capacity and resolution, slow	High ionic strength for binding
Ligand affinity chromatography	Bioactivity	Medium-low capacity high resolution, slow	Ligand dependent
Immunoaffinity chromatography	Bioactivity	Medium capacity, high resolution, fast	Ligand dependent, elution under harsh conditions
Dye affinity chromatography	Mixture	Medium capacity, high resolution, slow	Low ionic strength
Gel permeation chromatography	Size	Low capacity, low resolution	Initial loading in small volume

the retention of other host-related proteins onto the column. For determining the optimal conditions, the three pH-related properties of the enzyme that should be considered are (a) the isoelectric point (pI), (b) the optimum pH for activity, and (c) the pH stability range. If the pI of an enzyme is 5 or below, that does not mean that it is stable at pH 5 or can be handled at pH 5. Table III gives a general list for choosing the appropriate ion exchanger, with the assumption that the polymerases are stable only in the pH 5.5 to 8.5 range. Information regarding the isoelectric point of the enzyme can be useful in deciding the choice of column and buffer conditions

TABLE III

CHOICE OF ION EXCHANGER FOR PURIFICATION
OF POLYMERASES

pI	Ion exchanger	Buffer pH
8.5	Cation	$</= 7.0$
7.0	Cation	$</= 6.0$
	Anion	$>/= 8.0$
5.5	Anion	$>/= 6.5$

TABLE IV
p*I* VALUES OF VIRAL POLYMERASES

Viral polymerase	Molecular weight	Theoretical p*I*
Coronavirus	309,218	7.27
Capripox virus	105,700	7.14
Epstein–Barr virus	113,417	7.38
Herpes simplex		
Type 1	136,602	7.34
Type 2	137,354	7.06
Type 6	115,819	7.11
HIV-I (RT)	64,385	9.14
HIV-II (RT)	64,753	7.69
HepC virus	65,173	8.76
Human retrovirus	98,935	9.84
Measles virus	247,645	7.92
Polio virus	52,482	6.52
Respiratory syncycial virus	26,978	4.35
Rhinovirus	52,246	6.44
T4 bacteriophage	103,608	6.20
Varicella zoster virus	134,046	7.80
VSV α-subunit	31,299	4.41
VSV β-subunit	241,581	8.75

to be used. For example, the p*I* values of HIV-1 and HIV-2 chimera reverse transcriptases (RT)[34] have been useful in their purification. Table IV lists theoretical p*I* values of some of the viral polymerases.

In general, with the polymerases, anion-exchange chromatography has been performed in the pH range of 6.2 to 7.5 and cation-exchange chromatography in the pH 7.2 to 8.0 range. Maintaining enzymes at pH <6.0 or >8.0 for a long duration can result in a decrease in enzyme activity. Column matrices such as fast-flow Sepharose Q (anion exchanger) and Sepharose S (cation exchanger, both from Pharmacia) are excellent exchange resins because they can handle faster flow rates. FPLC columns Mono S and Mono Q (Pharmacia) are very useful in final stages of purification for their high resolution capabilities. These columns have been used for purification of HSV polymerase,[28] adenovirus polymerase (Ad-Pol),[24] RNA-dependent RNA polymerase of HepC,[14] human DNA polymerase,[28,35] and HIV-1 reverse transcriptase.[32] Nucleic acids are often a major contaminant with polymerases. Nucleic acids bind to the anion exchangers. Under physiologi-

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cal conditions the charge on the nucleic acids is pH independent, and thus generally they elute in the salt concentration range of 0.5–1.0 M. If the polymerase of interest also elutes under similar salt concentrations, the chromatography should be performed at a lower pH so that the enzyme elutes at a much lower salt concentration than the contaminating nucleic acids.

Introduction of hydrophobic interaction chromatography (HIC) during purification can also provide some degree of resolution. The technique relies on the affinity between hydrophobic groups such as phenyl- or octyl-in the matrix and regions of hydrophobicity on the protein surface. Hydrophobic interactions become stronger with increasing salt concentrations. Thus, HIC can be run either after an ammonium sulfate step or ion-exchange chromatography. Phenyl agarose chromatography has been used in the purification of human DNA polymerase δ.[28,35]

Specific Chromatographic Methods

Phosphocellulose and Heparin Sepharose Chromatography

Table V summarizes the steps used in the purification of several polymerases from various sources. The most widely used steps in the purification of polymerases are phosphocellulose and heparin Sepharose chromatography. Proteins usually do not bind to cation exchangers at salt concentrations higher than 0.5 M at pH $\cong 7.5$, because at this pH, even proteins having a high isoelectric point do not have high net positive charge that would be required for a strong interaction with a cation-exchange resin. There are specific interactions between phosphocellulose and enzymes that interact with phosphorylated substrates. Phosphorylated nonreducing ends of the cellulose can bind to the dNTP or primer/template-binding sites on the polymerases and, in many cases, high salt concentrations are needed to break this pseudo-specific binding interaction. The column in fact works as a pseudo-affinity column rather than an ion exchanger. On the same principle, heparin, which is a sulfated polysaccharide and highly negatively charged, mimics the anionic charge of nucleic acids. Immobilized heparin has been used as a pseudo-affinity column in the pH range 7.2 to 7.5 with the elution of polymerase in high salt. One or both of these columns are used in most of the purification procedures described in this volume.[14,24,26,28,29,31,32]

DNA Affinity Chromatography

Another variation in the biospecific affinity chromatography for polymerase purification is the utilization of sequences in the polymerases that

TABLE V
SUMMARY OF PURIFICATION PROCEDURES OF SELECTED VIRAL POLYMERASES

Polymerase	Source (expression system)	Purification steps	Ref.
Adenovirus	Vaccinia virus	DEAE-Sephacel, Mono S, ssDNA cellulose, heparin Sepharose, glycerol gradient	24
	Baculovirus	High speed centrifugation, ssDNA Sepharose, hydroxyapatite	24
	Vaccinia and baculovirus (6× His tagged)	Nickel-nitrilotriacetic acid, metal-chelate chromatography	24
Poliovirus	E. coli	Ammonium sulfate precipitation, phosphocellulose, GTP agarose	31
HSV-1	Baculovirus	Salt precipitation, phosphocellulose, hydroxyapatite, heparin Sepharose, Sepharose Q	26
		High speed centrifugation, phosphocellulose, ssDNA agarose, glycerol gradient	
Hepatitis C NS5B	Baculovirus	DEAE-Sepharose, heparin Sepharose, poly(U) Sepharose, Mono Q	14
HIV-1 RT	E. coli (6× His tagged)	DE52, phosphocellulose, Mono S	32
		Nickel-nitrilotriacetic acid, metal-chelate chromatography	39, 40
Human DNA polymerase δ	Tissue culture cells	Phosphocellulose, Blue4-agarose, Sephacryl S-300, Mono Q, Mono S, glycerol gradient	28, 35
	Human tissue/tissue culture cells	DEAE-cellulose, phenyl agarose, anti-DNA polymerase immunoaffinity, ssDNA cellulose, protein A-Sepharose	

can bind to polynucleotides. Immobilized poly(U), poly(A), GTP, single-strand DNA, and double-strand DNA columns, when used under controlled ionic strength conditions to prevent nonspecific cation-exchange interactions, can retain DNA-binding proteins. Hepatitis C polymerase[14] has been purified using poly(U)–Sepharose columns while poliovirus polymerase has been purified with a GTP agarose column.[31] A high salt gradient is needed

for the elution of the former, and KCl together with ATP are needed for elution of the latter.[31]

Mechanism-Based Affinity Chromatography

This novel technique has been successfully applied to the purification of HSV polymerase by Reardon.[36] Acyclovir triphosphate is a specific inhibitor of HSV-1 polymerase. An affinity resin containing a DNA hook template-primer with an acyclovir monophosphate residue at the 3' primer behaves as a DNA agarose column in the absence of added nucleotides. However, in the presence of the next required nucleotide encoded by the template, in this case dGTP, the affinity of the polymerase is increased toward the immobilized acyclovir monophosphate template, and the enzyme is retained on the column, even in the presence of 1 M salt. The enzyme is then eluted with a salt gradient after removal of the nucleotide from the buffer.

Protein Interaction Chromatography

When two proteins form a stable complex, it is possible to purify them by immobilizing one of the components. Phage T7 DNA polymerase forms a strong 1 : 1 complex with reduced E. coli thioredoxin. Reduced thioredoxin that has been immobilized onto an agarose matrix has been used for the purification of T7 DNA polymerase.[37] Similarly, the DNA-binding domain of cellular transcription factor NF1, when immobilized on Sepharose, can retain adenovirus type 2 polymerase from insect cell extracts, resulting in a partial purification of the polymerase.[38]

Metal Chelate Affinity Chromatography

Immobilized metal affinity chromatography (IMAC) is now being widely used for the rapid purification of recombinant proteins. The introduction of six tandem histidine residues with molecular biology techniques at either terminus of the recombinant protein allows binding of the protein to transition metal ions, which are immobilized on a resin. Adenovirus polymerase and adenovirus preterminal protein were purified using nickel-nitrilotriacetic acid beads.[24] Histidine-tagged rhabdovirus polymerase from E. coli was successfully purified from the pellet, after guanidine HCl solubilization of the pellet, followed by IMAC chromatography,[25] using the competitive ligand imidazole for gradient elution. However, it is important to show

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that the addition of these histidine residues onto the polymerase sequence has no effect on the structure or function of the enzyme. It is possible to introduce the His tag and an additional protease cleavage site. Of course, it is important that the native enzyme not contain another cleavage site for the protease used to remove the His tag. Hexahistidine preceding a renin cleavage sequence has been engineered onto the N terminus of HIV-1 RT.[39] The chimeric protein is purified using IMAC.[39,40]

Immunoaffinity Chromatography

Immobilized antibody, raised against an enzyme, can provide a very tight binding adsorbent ($K_d \cong 10^{-8}$ to 10^{-12} M). Disruption of this binding under mild conditions and elution of the enzyme in its native state are major challenges of this procedure. DNA polymerase α, DNA polymerase δ, and DNA polymerase α–primase complex have been purified using an anti-human DNA polymerase α monoclonal antibody column and an anti-human DNA polymerase δ antibody column, respectively. Purification of the adenovirus preterminal protein has been achieved by taking advantage of its interactions with the Ad-Pol.[24] Ad-Pol is immobilized onto an Ad-Pol-specific antibody column and then extracts containing the preterminal protein are loaded onto the column. The preterminal protein forms a complex with the immobilized Ad-Pol and is eluted with buffer containing 2 M urea. DeVico and co-workers[41] have purified equine infectious anemia virus (EIAV) polymerase using an antibody against a synthetic peptide derived from a sequence within the C-terminal portion of HIV-1 RT. Since the C-terminal sequence is conserved in the predicted amino acid sequence of EIAV, the immunoaffinity chromatography yields EIAV-specific polymerase.

Methods for Purity Analysis

The techniques used for assessing the purity of the final product must be able to detect small levels of impurity. Table VI summarizes different techniques that can be employed. Electrophoretic methods such as SDS–PAGE, native gels, urea gels, gradient gels, isoelectric focusing gels, and two-dimensional gels are the simplest, least expensive, and the most widely used methods. Gel filtration chromatography and analytical reverse-phase HPLC are two other powerful tools used for checking homogeneity of the

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TABLE VI
ANALYTICAL METHODS FOR CHECKING PROTEIN PURITY

Method	Sensitivity	Property used
Electrophoresis	ng–μg	Charge, chain length
SDS–PAGE		
Native gels		
Gradient gels		
Two-dimensional gels		
Isoelectric focusing		
Chromatography	μg	Size, hydrophobicity, charge
Sedimentation	μg	Mass, shape, size
Amino acid analysis	μg	Composition
Sequence analysis	μg	Sequence
N-terminal		
C-terminal		
Mass spectroscopy	ng–μg	Mass, sequence
Electrospray		
MALDI-TOF		
Activity	ng–μg	Presence of active site

sample with respect to molecular weight and hydrophobicity, respectively.[20,21] N-terminal analysis of proteins can give some indication of homogeneity, provided the N-terminal amino acid is not blocked. If the sequence of the enzyme is known from the cDNA sequence, amino acid composition can also give an idea of purity. Immunological methods such as immunoblotting are a very sensitive and simple technique for detecting host cell proteins. Antibodies to *E. coli* or yeast proteins are commercially available. Enzyme-labeled (alkaline phosphatase or peroxidase) antibodies have been used to visualize very small amounts of antibody–antigen complexes.

In recent years, mass spectrometric methods have emerged as powerful tools for protein characterization.[42] One of these techniques employs electrospray ionization in which multiply charged ion species are produced, thus lowering the mass to charge ratio values for an accurate determination of molecular weights.[43,44] In the matrix-assisted laser desorption time-of-flight instrument, protein samples in the presence of certain matrices such as 2,5-dihydroxybenzoic acid are ionized with an ultraviolet laser beam and the mass of the molecules is analyzed.[45,46] Homogeneity of the HIV-1 RT

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subunits has been reported using laser desorption spectrometry.[47] Protein amino acid sequences can be determined by tandem mass spectrometry using a triple-quadruple mass spectrometer. In this process, protonated peptide ions are selected with the first mass spectrometer and are transmitted to a collision cell. Low energy collision with neutral inert gases such as argon results in fragment ions that are then detected in a second mass spectrometer. The fragmentation patterns of peptides obtained are indicative of their amino acid sequences and thereby allow for the determination of peptide sequences as well as determination of any posttranslational modifications occurring within the protein. Phosphorylation, sulfation, and glycosylation at specific amino acids in the purified proteins are now routinely identified by mass spectrometry. Since these techniques allow analysis of large molecules at the low femtomole level, they may prove to be very useful in purity determinations.

In summary, the purification protocol used for a particular polymerase will depend on the experiments to be performed with the pure enzyme. While a single affinity chromatography step may be sufficient for some experiments, for others several steps may be required to remove not only the minor contaminating proteins but also the nonspecific activities that can interfere with specific polymerase activities.

Acknowledgment

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[2] Expression, Purification, and Characterization of the Herpes Simplex Virus Type-1 DNA Polymerase

By PAUL E. BOEHMER

I. Introduction

Representatives of the *Herpesviridae* exist in virtually all vertebrate species that have been examined. To date, eight different human herpesviruses have been identified (Table I). In addition, humans are hosts for cercopithecine herpesvirus 1, a herpesvirus native to macaque monkeys.