JOURNAL OF VIROLOGY, Mar. 1996, p. 2008–2013 Vol. 70, No. 3
0022-538X/96/$04.00+0

NOTES

Purification and Characterization of Herpes Simplex Virus Type 1 Alkaline Exonuclease Expressed in *Escherichia coli*

JOEL C. BRONSTEIN AND PETER C. WEBER*

Infectious Diseases Section, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105

Received 25 September 1995/Accepted 11 December 1995

The alkaline exonuclease (AE) encoded by the herpes simplex virus type 1 (HSV-1) UL12 open reading frame was inducibly expressed in *Escherichia coli* and purified without the use of chromatographic separation. This recombinant AE was found to exhibit the same biochemical properties as the virus-encoded protein and was used to confirm the existence of a weak endonucleolytic activity in the enzyme. Antisera raised against the recombinant protein recognized several forms of the AE in HSV-1-infected cells. This expression and purification strategy will provide an economical and easily accessible alternative source of HSV-1 AE for future in vitro studies.

The process by which newly replicated copies of the herpes simplex virus type 1 (HSV-1) genome undergo maturation and packaging is poorly understood. One virus-encoded enzyme which appears to play a critical role in these events is a DNase encoded by the UL12 open reading frame (5, 6, 12) that is commonly referred to as alkaline exonuclease (AE) because of its high pH optimum (9). Studies with a recombinant HSV-1 carrying a deletion in the UL12 gene indicate that AE appears to play a role in the processing and packaging of viral DNA into infectious virions, probably by resolution of branched genomic structures (10, 13, 16). Additionally, this null mutant was able to replicate and encapsidate significant amounts of viral DNA but was impaired with respect to the egress of capsids from the nucleus into the cytoplasm, suggesting that AE may also play a structural role during viral replication. Despite these defects, the UL12 deletion mutant virus was still capable of replicating in noncomplementing cell lines but produced yields of infectious virions that were significantly lower than those of wild-type HSV-1 (13, 16).

Although AE appears to participate in the processes of genome maturation and encapsidation, further in vitro characterization is clearly needed in order to precisely define the functions of this enzyme. AE was originally purified from HSV-1-infected cells and analyzed in several laboratories (1, 7, 15). More recently, soluble forms of the Epstein-Barr virus homolog of the HSV-1 AE that were overexpressed and purified from *Escherichia coli* cells have been characterized (2, 4, 14). However, all of these sources of enzyme proved to be difficult to work with for several reasons, including reduced yields due to low levels of AE expression in virus-infected cells, the need for tedious and lengthy chromatographic separation methods, and enzyme instability and degradation, even in frozen stocks. To address these problems in this study, HSV-1 AE was synthesized in bacteria by using an inducible expression system. Following induction, high levels of recombinant AE (rAE) accumulated as an insoluble aggregate in bacterial cells, and this complex could be purified and reactivated to yield an active and stable protein without the need for any chromatographic separation. The bacterial and virus-encoded proteins were found to be indistinguishable with respect to their enzymatic, biochemical, and antigenic properties, suggesting that this alternative source of enzyme should greatly facilitate further in vitro studies of the HSV-1 AE.

Expression of functional HSV-1 AE in *E. coli*. The UL12 gene encoding HSV-1 AE was cloned into the prokaryotic expression vector pET-25b(+) (Novagen) to create the construct pET-AE. This process involved first isolating the UL12 gene which encodes the viral AE from HSV-1 (strain KOS) genomic DNA as a 4.3-kb *Hin*dIII-*Pst*I fragment. This segment was cloned into the *Hin*dIII and *Pst*I sites of pUC19 to create pHP-AE. Three subclonings were then performed to prepare the UL12 gene for insertion into pET-25b(+). First, the 2.1-kb *Nsp*I-*Dra*I fragment of pHP-AE was inserted into the *Sph*I and *Sma*I sites of pUC19 to generate pMC-AE. Second, the 2.1-kb *Hin*dIII-*Eco*RI fragment of pMC-AE was cloned into the *Hin*dIII and *Eco*RI sites of pGEM7 (Promega) to yield pGEM-AE. Finally, the 2.1-kb *Hin*dIII-*Xba*I fragment of pGEM-AE was inserted into the *Hin*dIII-*Nhe*I sites of pET25b(+) to create pET-AE. This final plasmid uses an inducible T7 RNA polymerase promoter to drive the expression of a rAE protein in which the first 7 amino acids of the viral enzyme were replaced by a 22-amino-acid pelB leader (which is cleaved after expression) and 17 amino acids derived from pET25b(+) DNA.

pET-AE was transformed into *E. coli* BL21 (DE3), whose λDE3 lysogen contains a T7 RNA polymerase gene under the control of an inducible *lacUV5* promoter for use in the expression of recombinant proteins (Novagen). Cells carrying the plasmid were grown in 400 ml of Luria-Bertani media supplemented with 200 μg of carbenicillin per ml until an optical density at 600 nm of between 0.6 and 1.0 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture at a concentration of 1 mM to induce expression of

* Corresponding author. Mailing address: Infectious Diseases Section, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Rd., Ann Arbor, MI 48105. Phone: (313) 998-3295. Fax: (313) 998-3318. Electronic mail address: WEBERP@AA.WL.COM.

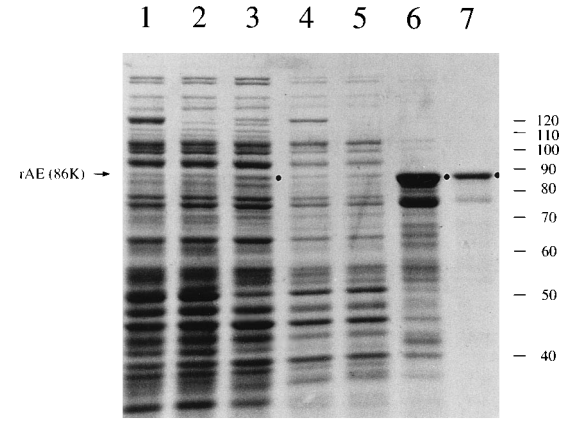

FIG. 1. IPTG-inducible expression of rAE protein in *E. coli* BL21 (DE3) cells. Fractionated bacterial extracts were prepared as described in the text. Various amounts of protein (60 μg of soluble fraction, 30 μg of insoluble fraction after solubilization, and 3 μg of insoluble fraction after solubilization and renaturation) were electrophoresed on an 8% polyacrylamide gel and stained with Coomassie brilliant blue. The mobility of molecular weight markers (in kilodaltons) is shown at the right, the mobility of the 86-kDa rAE protein is shown at the left, and bands corresponding to the rAE protein are indicated by black dots at the right of individual gel lanes. Lane 1 (soluble fraction), IPTG-induced plasmidless cells; lane 2 (soluble fraction), uninduced cells containing plasmid pET-AE; lane 3 (soluble fraction), induced cells containing pET-AE; lane 4 (insoluble fraction after solubilization), induced plasmidless cells; lane 5 (insoluble fraction after solubilization), uninduced cells containing pET-AE; lane 6 (insoluble fraction after solubilization), induced cells containing pET-AE; and lane 7 (insoluble fraction after solubilization and renaturation), induced cells containing pET-AE.

both the T7 RNA polymerase gene and the rAE gene. Cells were pelleted at 3 h postinduction, washed with phosphate-buffered saline, and stored at −70°C until fractionation. Once thawed, the cell pellets were resuspended in 40 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 100 mM NaCl) containing 20% (wt/vol) sucrose and 1 mg of lysozyme per ml. Following a 20-min incubation on ice, the cell suspension was quickly refrozen at −70°C and thawed in a water bath at 37°C. The freeze-thaw was repeated once, and the suspension was briefly sonicated for 30 s to reduce viscosity. Insoluble material was then pelleted, and the supernatant was stored on ice. The pelleted material was then resuspended by sonication in 40 ml of lysis buffer containing 1% (vol/vol) Triton X-100 and allowed to stand on ice for 15 min with occasional mixing. This suspension was pelleted again, and the resulting supernatant was combined with the previous one. This combined extract represented the soluble fraction, whereas the remaining pellet represented the insoluble fraction; both were stored at −70°C until needed.

Upon induction of pET-AE-transformed BL21 (DE3) cells, a protein whose molecular weight was consistent with that of the virus-encoded AE (1, 5, 15) could be detected in whole-cell lysates. Fractionation of these lysates as described above revealed a major protein band with an apparent molecular mass of 86 kDa in the insoluble fraction following induction (Fig. 1, lane 6). In contrast, a barely detectable amount of this protein was observed in the soluble fraction, which contained the cytosolic, periplasmic, and membrane-associated proteins of the bacterial cell lysates (Fig. 1, lane 3). The 86-kDa protein was not detected in the fractions of either untransformed bacterium cells following induction (Fig. 1, lanes 1 and 4) or transformed bacterium cells grown without induction (Fig. 1, lanes 2 and 5).

The existence of a functional nuclease activity in extracts containing the recombinant protein was assessed by a modification of an assay that quantified acid-soluble nucleotides released from degraded ^{3}H-labeled DNA (2). The assay mixture contained 50 mM Tris-HCl (pH 9.0), 3 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 μg of salmon sperm DNA, 25 nCi of ^{3}H-labeled *E. coli* DNA (DuPont NEN Research Products), and 0.3 μg of rAE in a total volume of 200 μl. All assay mixtures were incubated for 15 min at 37°C. The reaction was terminated by the addition of 1 ml of ice-cold 5% (wt/vol) trichloroacetic acid and 50 μl of 5-mg/ml bovine serum albumin, vortexed, chilled on ice for 10 min, and centrifuged at 15,000 × *g* for 5 min. Following centrifugation, 200 μl of the supernatant was transferred to scintillation vials containing 5 ml of Packard Ultima Gold XR scintillation fluid. Radioactivity was measured with a Beckman scintillation counter. One unit of exonuclease activity was defined as the amount (in micrograms) of acid-soluble nucleotides released after 1 min of incubation at 37°C. For specific activity measurements, protein concentrations were determined by the Bio-Rad protein assay or the Bio-Rad *DC* protein assay.

The identification of the 86-kDa protein as the rAE was indicated by the low but reproducible increase in exonucleolytic activity observed in the soluble fraction of IPTG-induced cells that carried pET-AE (Table 1). This exonucleolytic activity was consistently fourfold greater than the activity measured in comparable fractions lacking the 86-kDa protein, including those from induced plasmidless bacterial cells and those from uninduced bacterial cells that carried pET-AE (Table 1). In contrast, no activity was detected in the insoluble fraction of induced cells that carried pET-AE, even after it had been efficiently solubilized by the scheme outlined in the next section (Table 1). Thus, the insoluble fraction might contain the majority of the recombinant protein in induced cells (Fig. 1, lane 6), but the rAE appeared to exist in a denatured state in this preparation.

Purification and renaturation of insoluble rAE. Efforts to purity the soluble active form of rAE were met with the same difficulties encountered in earlier isolations of AE from HSV-1-infected cells (1, 7, 15), namely, low protein recovery and enzyme instability (3). As a result, purification efforts were redirected to the insoluble form of rAE. Although present in an inactive state, this species of rAE could be isolated in large amounts and appeared to be relatively free of contaminating

TABLE 1. Nuclease activities in fractionated bacterial cell extracts

Extract[a]	Sp act (U/μg of protein)[b]
Soluble fractions	
No plasmid, induced	1.5
pET-AE, uninduced	1.8
pET-AE, induced	6.5
Insoluble fractions after solubilization	
No plasmid, induced	0.5
pET-AE, uninduced	0.4
pET-AE, induced	0.4
Insoluble fractions after solubilization and renaturation	
pET-AE, uninduced	1.9
pET-AE, induced	257.6

[a] Fractions of lysates from bacterial cells with or without plasmid pET-AE and with or without induction by IPTG were prepared as described in the text.

[b] Specific activities were calculated for 0.3 μg of protein per reaction at 37°C by using [^{3}H]DNA degradation rates determined by the release of acid-soluble nucleotides and a unit definition of 1 μg of DNA degraded per min.

bacterial proteins (Fig. 1, lane 6). Moreover, aggregation of the insoluble enzyme into apparent inclusion bodies within the bacterial cell allowed for the development of a scheme whereby the rAE could be selectively solubilized and purified from these structures by a series of urea extractions.

To perform this purification, the pellet containing the insoluble fraction of rAE was first resuspended in 40 ml of solubilization buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 50 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 100 mM NaCl) containing 2 M urea. Following a 2-h incubation at room temperature with continuous mixing, the remaining insoluble material was pelleted. The supernatant, which contained a significant amount of contaminating protein but very little rAE, was discarded. At concentrations of urea greater than 2 M, the rAE in the pelleted material became increasingly soluble until a concentration of 8 M was reached, at which point no further solubilization was observed (3). On the basis of these findings, rAE could be routinely purified from the insoluble pellet by incubation in solubilization buffer containing 8 M urea for 2 h at room temperature with continuous mixing. The remaining insoluble material was pelleted and discarded. The final supernatant, which represented the solubilized insoluble fraction and contained purified rAE, was stored at −70°C until renaturation was performed.

Following this selective solubilization procedure, the rAE was completely denatured, reduced, and inactive (Table 1). In order to restore the native conformation and activity to this protein, the excess denaturing and reducing agents had to be slowly removed. This elimination of urea and dithiothreitol was accomplished by sequential dialysis of the solubilized rAE against renaturing buffer followed by extensive dialysis against storage buffer. Specifically, the frozen solubilized insoluble fraction was thawed and diluted to 100 μg of protein per ml of renaturing buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) containing 50 mM dithiothreitol, 0.5 M NaCl, and 6 M urea. The protein sample was then dialyzed overnight against 1 liter of renaturing buffer containing 10 mM dithiothreitol, 0.5 M NaCl, and 4 M urea. Half the dialysate was repeatedly removed following overnight dialyses and replaced sequentially with the following buffers: renaturing buffer containing 0.5 M NaCl, renaturing buffer containing 10% (vol/vol) glycerol, renaturing buffer containing 15% (vol/vol) glycerol but from which the EDTA is omitted, and renaturing buffer containing 30% (vol/vol) glycerol but from which the EDTA is again omitted. The purified rAE fraction was finally dialyzed twice overnight against 1 liter of storage buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM dithiothreitol, 20% [vol/vol] glycerol, 0.2% [vol/vol] Nonidet P-40) and centrifuged to remove any material that precipitated during dialysis. The renatured rAE fraction was stored at −70°C until further use. All steps in the purification of rAE were performed at 0 to 4°C, and all centrifugations were done at 20,000 × *g* for 15 min.

Examination of the final rAE preparation by the nuclease activity assay indicated that renaturation had restored functional activity to the enzyme (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of this active rAE revealed that the denaturation-renaturation process had also purified the enzyme to near homogeneity (Fig. 1, lane 7, and discussion below) without the use of any chromatographic separation procedures. The specific activity of the purified rAE was approximately 250 U/μg of protein, which represented a 40-fold increase over that observed in the original soluble fraction (Table 1). Negligible nuclease activity was detected in an identically prepared protein fraction from uninduced bacterial cells (Table 1), confirming that the activity that had been identified in the original soluble fraction of induced cells was due solely to the induced 86-kDa protein and not a nuclease that had been fortuitously isolated from *E. coli* cells. The recombinant protein was 5- to 20-fold less active than AE purified from HSV-1-infected cells (1, 7, 15); however, this difference was likely to be the result of variability in the ratio of labeled to unlabeled DNA substrates in the nuclease activity assays that were employed in the different studies.

While the specific activity of this rAE was comparable to that of the enzyme from other sources, its yield and long-term stability were clearly superior. Induced bacterial cell culture (400 ml) typically produced a total of 50 to 60 mg of solubilized rAE protein, of which approximately half (20 to 25 mg) was active enzyme. This yield represented a 270- to 10,400-fold increase over that of preparations of soluble forms of the Epstein-Barr virus homolog of the HSV-1 AE which were expressed in *E. coli* cells and which had to be purified by extensive chromatographic separation (2, 4, 14). Moreover, stocks of rAE stored for nearly a year at −70°C have been found to retain their original specific activity when thawed. Repeated thawing of these frozen enzyme stocks typically resulted in no detectable reduction of specific activity (3). These observations indicate that the AE expressed and purified from bacterial cells by this solubilization and renaturation scheme appears to be stable indefinitely.

Biochemical characterization of rAE. The rAE was characterized with respect to a variety of enzymatic parameters. Optimal enzyme activity was observed at a pH of between 9 and 10, and an alkaline pH value that was inhibitory for rAE could not be detected; in contrast, rAE retained only 25% of this optimal activity at physiological pH (7.5) and virtually no activity at pH 6 (Fig. 2A). The effects of ionic strength on rAE activity were also determined (Fig. 2B). Neither NaCl nor KCl had any effect on rAE activity up to a concentration of 50 mM; at concentrations greater than 50 mM, activity decreased linearly with increasing salt concentration, with a 50% inhibition concentration of 200 mM for both salts. Finally, the effect on rAE activity of varying the divalent cation cofactor was examined (Fig. 2C). rAE had an absolute requirement for a divalent cation, with a strong preference for Mg^{2+}. Of the other divalent cations tested, only Mn^{2+} gave significant enzyme activity, but this was just 30% of that obtained with Mg^{2+}. Peak enzyme activity could be achieved with Mg^{2+} or Mn^{2+} concentrations as low as 2 mM. Activity of rAE in the presence of Zn^{2+}, Ca^{2+}, and Cu^{2+} at any concentration was negligible (3). All of these biochemical properties of rAE were essentially indistinguishable from those described previously for enzyme purified from HSV-1-infected cells (1, 7, 15).

In addition to its well-characterized exonucleolytic properties, a weaker endonuclease activity has been described for some preparations of AE derived from HSV-1-infected cells (1, 8, 15). The availability of a bacterially expressed, purified AE allowed for a direct assessment of whether this endonuclease activity actually did reside in AE or was due to a copurifying cellular enzyme. This test was carried out by plasmid degradation assays in which supercoiled covalently closed circular pUC19 DNA served as an endonuclease substrate and *Eco*RI-linearized pUC19 DNA served as an exonuclease substrate. The standard reaction mixture contained 50 mM Tris-HCl (pH 7.5 or 9.0), 3 mM $MgCl_2$, 0.5 μg of plasmid DNA, and 0.3 μg of rAE in a final volume of 25 μl. The assays were initiated by the addition of enzyme, the mixtures were incubated at 37°C for various lengths of time, and the assays were terminated by the addition of 5 μl of gel loading buffer (0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol FF, 30% [vol/vol] glycerol, 150 mM EDTA, 6% [wt/vol] SDS). The

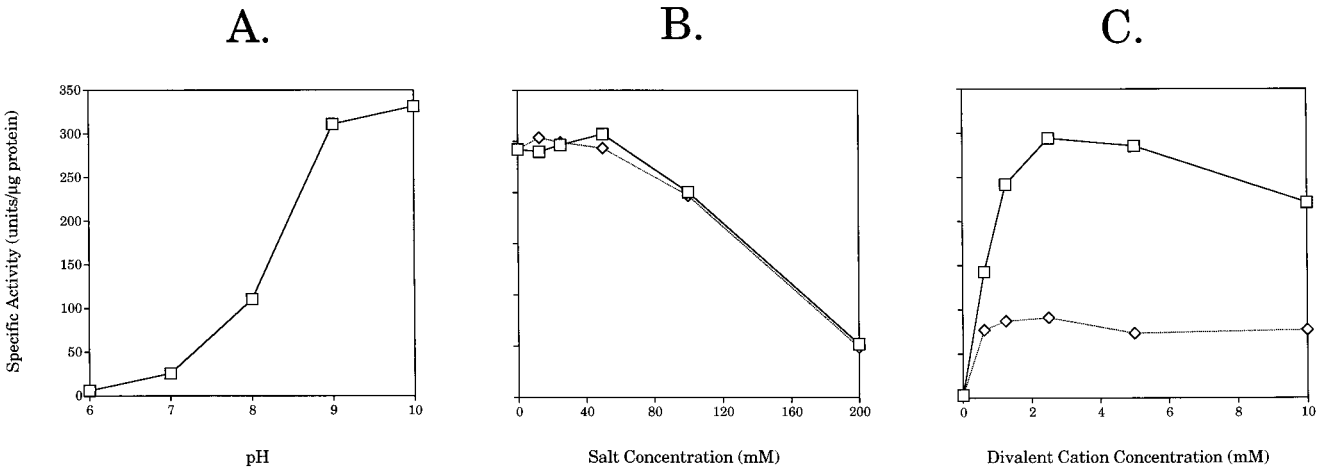

FIG. 2. Biochemical characterization of rAE. (A) Specific activity of rAE in the presence of Tris-HCl buffer at various pH values. (B) Specific activity of rAE in the presence of various concentrations of different salts (NaCl, boxes; KCl, diamonds). (C) Specific activity of rAE in the presence of various concentrations of different divalent cations ($MgCl_2$, boxes; $MnCl_2$, diamonds). All specific activities were determined by quantification of acid-soluble nucleotides released from rAE-degraded, ^{3}H-labeled DNA, with a unit definition of 1 μg of DNA degraded per min being used.

digested DNA products were loaded onto a 1% (wt/vol) agarose gel and detected following electrophoresis by staining with 5 μg of ethidium bromide per ml for 15 min and visualization with UV light. Representative results from these experiments are shown in Fig. 3.

Reaction rates in these experiments were determined by densitometric scanning of negatives of Polaroid photographs of the gels with a Hoefer GS 300 scanning densitometer to determine the levels of undegraded DNA after each reaction. In the case of the supercoiled template, this determination simply involved the quantification of the remaining uncut circular DNA, since nicking of a single nucleotide within the plasmid resulted in the creation of relaxed and linearized molecules; these slower-migrating forms were quickly degraded by the exonuclease activity of rAE. Quantification of degradation of the linear template was more complex, since removal of single nucleotides from the ends of this DNA could not be resolved by this assay. However, elimination of longer lengths of nucleotides was readily apparent by an increase in fragment electrophoretic mobility (Fig. 3). Therefore, a 100-bp integration window was created in the densitometry scan to allow for the quantification of degradation products that had lost fewer than 100 bp of nucleotides; as a result, any molecules that had lost more than this length were not detected by the densitometer and were therefore scored as degraded. These analyses were used to calculate reaction rates and specific activities for each pairing of DNA template and pH parameters.

The results obtained clearly show that rAE does have an associated endonuclease activity. Nevertheless, the exonuclease activity of the enzyme was considerably stronger: degradation of a linear template by rAE occurred 6 times faster at pH 9.0 (1,822 versus 303 U/μg of protein) and 12 times faster at pH 7.5 (304 versus 25 U/μg of protein) than did the degradation of a supercoiled template. Moreover, it is important to point out that these values could be underestimated by as much as 100-fold because of the differences in quantification strategies employed for the two templates: a linear template could suffer up to 100 nucleotide cleavage events and would still be included in the undegraded population of the 100-bp window of the densitometry scan, whereas a supercoiled template that underwent a single cleavage event was always removed from the undegraded population in the densitometry scan. Thus, it is likely that the exonuclease function in rAE is significantly (possibly several orders of magnitude) more active than its endonuclease function.

Finally, the results for exonuclease and endonuclease activities confirm and extend conclusions about the inhibitory effects of lower pH on rAE activity. The sixfold difference in exonuclease activity between pH 7.5 and 9.0 was comparable to the fourfold difference that was detected earlier by the ^{3}H-labeled DNA degradation assay (Fig. 2A). However, this effect was even more dramatic with the endonuclease function of rAE. In this case, the activity at pH 7.5 was just 8% of that at pH 9.0. Since it is thought that AE plays a crucial role in the HSV-1 life cycle by processing branched genomic intermediates (10), a reaction that would almost certainly involve an

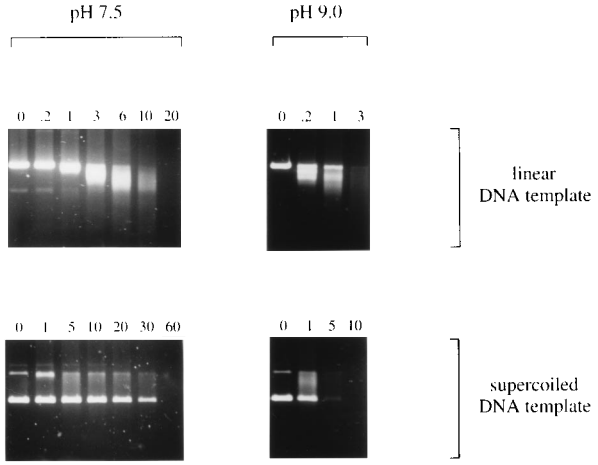

FIG. 3. Comparison of endonuclease and exonuclease activities associated with rAE. rAE was incubated with supercoiled and linearized pUC19 DNA for various lengths of time to assay its endonuclease and exonuclease activities, respectively. These digested samples were then electrophoresed on 1% agarose gels, stained with ethidium bromide, and quantified by densitometry to allow for the calculation of reaction rates. Assay times (in minutes) are given above each gel lane, the templates employed in the reactions (linear and supercoiled) are indicated at the right, and the pHs of the reactions (pH 7.5 and 9.0) are indicated at the top.

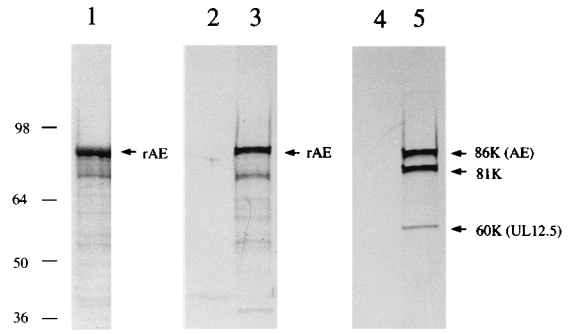

FIG. 4. Comparison of the antigenic properties of rAE and virus-encoded AE. Western blot analyses of bacterial and HSV-1-infected cell lysates were carried out with BWp12, an affinity-purified rabbit antiserum raised against a preparation of the 86-kDa rAE protein that had been purified by electroelution. The mobility of molecular weight markers (in kilodaltons) is shown at the left, and the mobilities of rAE in bacterial cell lysates or the three forms of AE present in HSV-1-infected cell lysates are indicated at the right of the appropriate panels. Lane 1, overloaded Coomassie brilliant blue-stained gel of purified rAE preparation; lanes 2 and 3, Western blot analysis of pET-AE-containing *E. coli* BL21 (DE3) lysates without and with IPTG induction, respectively; and lanes 4 and 5, Western blot analysis of mock- and HSV-1-infected Vero cell lysates, respectively.

endonuclease activity, the pH of the environment surrounding the catalytic site of the enzyme may act as a critical determinant in regulating this aspect of AE function.

Antigenic properties of rAE. As a final demonstration that the 86-kDa protein purified from bacterial cells was indeed the AE coded for by HSV-1, an affinity-purified rabbit polyclonal antiserum that was raised against electroeluted rAE protein (antiserum BWp12) (11) was used in Western blot (immunoblot) analysis of both bacterial and HSV-1-infected cell lysates. To prepare the latter, overnight cultures of African green monkey (Vero) cells cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum were infected with HSV-1 (strain *syn*17$^+$) at a multiplicity of infection of 10. The cells were harvested by scraping and low-speed centrifugation at 18 h postinfection. The resulting pellets were rinsed with phosphate-buffered saline prior to storage at −70°C. Cell pellets were lysed by the addition of loading buffer (125 mM Tris-HCl, 20% [vol/vol] glycerol, 2% [wt/vol] SDS, 25 μg of bromophenol blue per ml, 5% [vol/vol] β-mercaptoethanol) and heated to 100°C for 5 min. The lysates were cleared by centrifugation and diluted to enable 1.5 μg of protein to be loaded onto 8% polyacrylamide gels. Whole-cell lysates of induced and uninduced *E. coli* BL21 (DE3) cells carrying pET-AE were also prepared and diluted to enable 0.1 μg of protein to be loaded onto gels. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes with a NOVEX Western transfer apparatus. The resulting blots were incubated with a 1:20,000 dilution (80 ng/ml) of BWp12 antiserum. Protein reactivity was detected with a 1:5,000 dilution of goat anti-rabbit alkaline phosphatase-conjugated antibody (Promega) and 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium substrate (Kirkegaard & Perry Laboratories).

As expected, this antiserum specifically recognized the 86-kDa rAE protein in an extract from induced bacterial cells (Fig. 4, lane 3) but not from uninduced bacterial cells (Fig. 4, lane 2). More importantly, the antiserum also recognized a protein with an identical molecular weight in an extract from HSV-1-infected cells (Fig. 4, lane 5). The identity of this band as the viral AE was confirmed by its absence in extracts from uninfected cells (Fig. 4, lane 4) and from cells infected with a recombinant HSV-1 in which the UL12 gene that encodes AE has been deleted (11). The BWp12 antiserum was also able to detect two additional proteins with molecular masses of 81 and 60 kDa in HSV-1-infected cells (Fig. 4, lane 5). The 81-kDa protein is expressed at significant levels and is similar in size to a polypeptide that routinely copurified with AE from HSV-1-infected cell extracts (1, 15). However, this species is likely to represent a proteolytic degradation product of the full-length protein, since its appearance was influenced significantly by the method used to prepare the infected cell lysates (3). The 60-kDa species was expressed at a much lower level than the 86- or 81-kDa proteins. Recent studies have shown that this species represents a truncated form of AE coded for by the UL12.5 open reading frame of HSV-1 (11). This gene is identical to the UL12 open reading frame which encodes full-length AE, except that it utilizes an internal methionine codon of UL12 as a start codon, thereby creating an amino-terminal truncation in AE. The existence of this protein was consistent with earlier reports of an mRNA whose 5′ end mapped within the amino-terminal coding sequences of the UL12 gene and whose in vitro translation product was also a 60-kDa polypeptide (5, 6). Thus, an antiserum that was raised against the purified bacterial enzyme specifically recognized the virus-encoded AE and two of its derivatives in HSV-1-infected cells, thereby confirming the identity of rAE.

The BWp12 antiserum was also used to assess the purity of rAE, since a number of lower-molecular-weight proteins could be detected in the final protein preparation on Coomassie brilliant blue-stained gels containing overloaded lanes (Fig. 4, lane 1). These minor species could represent either contaminating bacterial proteins that copurify with rAE or degradation products of rAE. However, the BWp12 antiserum, which was raised specifically against the 86-kDa protein of rAE that had been electroeluted from polyacrylamide gels (11), recognized nearly all of these lower-molecular-weight proteins in Western blot analyses of the final rAE preparation (Fig. 4, lane 3). This result was confirmed by densitometric comparisons of bands present in both Coomassie brilliant blue-stained gels and Western blots, which gave nearly identical protein profiles (3). Since copurifying bacterial proteins would not be expected to be recognized by the BWp12 antiserum, these results indicate that most if not all of the minor species present in the purified rAE are derived from the 86-kDa rAE protein itself. Thus, it appears that the rAE had been purified to homogeneity and that the final preparation of renatured enzyme contained only low levels of rAE degradation products. The concentrations of the latter did not increase with extended storage at −70°C or with repeated thawing of enzyme stocks (3), indicating that they were probably generated during the denaturation and renaturation steps that were used to purify the rAE. This absence of further degradation over extended periods of time is likely to be responsible for the remarkable long-term stability in enzyme activity of the rAE protein.

Since the exact role of AE in the HSV-1 replication cycle is still unclear, further characterization of this enzyme in in vitro studies should help to elucidate its function. The expression scheme outlined in this work provides an economical and easily accessible alternative source of HSV-1 AE for these and other purposes. This enzyme could routinely be purified to homogeneity without the need for any chromatographic separation steps, and it was found to be stable indefinitely. The availability of large amounts of rAE should facilitate continued in vitro and in vivo characterization of HSV-1 AE.

We thank E. Nordby for the preparation of infected cell extracts and S. Spatz for technical advice.

REFERENCES

1. **Banks, L., D. J. Purifoy, P. F. Hurst, R. A. Killington, and K. L. Powell.** 1983. Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. J. Gen. Virol. **64:**2249–2260.
2. **Baylis, S. A., D. J. Purifoy, and E. Littler.** 1989. The characterization of the EBV alkaline deoxyribonuclease cloned and expressed in E. coli. Nucleic Acids Res. **17:**7609–7622.
3. **Bronstein, J. C., and P. C. Weber.** Unpublished data.
4. **Chen, J. Y., L. M. Y. Liu, T. Y. Hsu, S. M. Cho, and C. S. Yang.** 1993. Use of bacterially-expressed antigen for detection of antibodies to the EBV-specific deoxyribonuclease in sera from patients with nasopharyngeal carcinoma. J. Virol. Methods **45:**49–66.
5. **Costa, R. H., K. G. Draper, L. Banks, K. L. Powell, G. Cohen, R. Eisenberg, and E. K. Wagner.** 1983. High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-Dalton protein tentatively identified as a capsid protein. J. Virol. **48:**591–603.
6. **Draper, K. G., R. G. Devi, R. H. Costa, E. D. Blair, R. L. Thompson, and E. K. Wagner.** 1986. Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. **57:**1023–1036.
7. **Hoffmann, P. J., and Y. C. Cheng.** 1978. The deoxyribonuclease induced after infection of KB cells by herpes simplex virus type 1 or type 2. I. Purification and characterization of the enzyme. J. Biol. Chem. **253:**3557–3562.
8. **Hoffmann, P. J., and Y.-C. Cheng.** 1979. DNase induced after infection of KB cells by herpes simplex virus type 1 or type 2. II. Characterization of an associated endonuclease activity. J. Virol. **32:**449–457.
9. **Keir, H. M., and E. Gold.** 1963. Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. Biochim. Biophys. Acta **72:**263–276.
10. **Martinez, R., R. T. Sarisky, P. C. Weber, and S. K. Weller.** Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J. Virol., in press.
11. **Martinez, R., L. Shao, J. C. Bronstein, P. C. Weber, and S. K. Weller.** Virology, in press.
12. **McGeoch, D. J., A. Dolan, and M. C. Frame.** 1986. DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. Nucleic Acids Res. **14:**3435–3448.
13. **Shao, L., L. M. Rapp, and S. K. Weller.** 1993. Herpes simplex virus-1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology **196:**146–162.
14. **Stolzenberg, M. C., and T. Ooka.** 1990. Purification and properties of Epstein-Barr virus DNase expressed in *Escherichia coli*. J. Virol. **64:**96–104.
15. **Strobel-Fidler, M., and B. Francke.** 1980. Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. Virology **103:**493–501.
16. **Weller, S. K., M. R. Seghatoleslami, L. Shao, D. Rowse, and E. P. Carmichael.** 1990. The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. J. Gen. Virol. **71:**2941–2952.