Structural and Functional Properties of the 14-kDa Envelope Protein of Vaccinia Virus Synthesized in Escherichia coli*

Chingfeng Laït, Shiaoching Gong++, and Mariano Esteban§

From the Department of Biochemistry, State University of New York Health Science Center, Brooklyn, New York 11203

Vaccinia virus is a highly cytocidal virus, but the steps that lead to virus penetration into cells, the first event in virus pathogenesis, have not been elucidated. We have shown that a 14-kDa envelope protein of vaccinia virus might play a major role in virus-penetration acting at the level of cell fusion (Rodriguez, J. F., Paez, E., and Esteban, M. (1987) J. Virol. 61, 395–404; Gong, S., Lai, C., and Esteban, M. (1990) Virology 178, 81–91). To carry out structural and functional studies on the vaccinia 14-kDa protein, it would be desirable to have a high level expression system, since the amount of protein that can be obtained from purified virus or from infected cells is very limited. In this investigation we demonstrate that the 14-kDa envelope protein of vaccinia virus is expressed in Escherichia coli in soluble form and at high levels. We establish, by several criteria, that the 14-kDa vaccinia virus protein expressed in E. coli is similar to the protein found in the virus particle based on apparent molecular mass, occurrence of disulfide-linked oligomers, reactivity against specific monoclonal antibody, and identity in amino-terminal sequence with the predicted DNA sequence of the gene. We define several structural and functional properties concerning the 14-kDa envelope protein of vaccinia virus. 1) 14 kDa is a trimer of identical subunits. 2) A monomer binds to itself more strongly than to a dimer or a trimer. 3) Oligomerization does not require cellular factors. 4) Trimers induce high titer neutralizing antibodies in animals which correlate with overall immunogenicity. 5) 14-kDa binds with specificity to the cell surface of cultured cells.

Vaccinia virus is a highly cytocidal virus (1), but the mechanism of virus penetration into cells is not yet known (2). Through the use of monoclonal antibodies directed against structural proteins of vaccinia virus, we identified a 14-kDa protein as having a role in virus-penetration acting at the level of cell fusion. We have shown that the 14-kDa envelope protein of vaccinia virus might play a major role in virus-penetration acting at the level of cell fusion (Rodriguez, J. F., Paez, E., and Esteban, M. (1987) J. Virol. 61, 395–404; Gong, S., Lai, C., and Esteban, M. (1990) Virology 178, 81–91). To carry out structural and functional studies on the vaccinia 14-kDa protein, it would be desirable to have a high level expression system, since the amount of protein that can be obtained from purified virus or from infected cells is very limited. In this investigation we demonstrate that the 14-kDa envelope protein of vaccinia virus is expressed in Escherichia coli in soluble form and at high levels. We establish, by several criteria, that the 14-kDa vaccinia virus protein expressed in E. coli is similar to the protein found in the virus particle based on apparent molecular mass, occurrence of disulfide-linked oligomers, reactivity against specific monoclonal antibody, and identity in amino-terminal sequence with the predicted DNA sequence of the gene. We define several structural and functional properties concerning the 14-kDa envelope protein of vaccinia virus. 1) 14 kDa is a trimer of identical subunits. 2) A monomer binds to itself more strongly than to a dimer or a trimer. 3) Oligomerization does not require cellular factors. 4) Trimers induce high titer neutralizing antibodies in animals which correlate with overall immunogenicity. 5) 14-kDa binds with specificity to the cell surface of cultured cells.

Fusion of the virus envelope with the plasma membrane (3–6).

Further studies revealed the 14-kDa vaccinia virus protein to have functional properties analogous to those of the hemagglutinin (HA) of influenza virus. This is based on several experimental findings. First, the two proteins are known to form trimers in the virus particle (3, 6). Second, the two viruses had a similar optimum pH for acid-induced cell fusion (5, 7). Third, the two proteins have NH₂-terminal proximal regions involved in fusion (5, 8). Fourth, variants of influenza virus with charged amino acid substitutions in the amino-terminal region of HA can induce fusion at neutral pH (9), as do mutants of vaccinia virus with a charged amino acid point mutation in the NH₂ terminus of the 14-kDa protein (10). Thus, the fusion reaction catalyzed by the 14-kDa protein may be analogous to influenza HA protein. Because animal viruses enter eukaryotic cells either by direct fusion of the lipid membrane of the virus with the cell plasma membrane or by receptor-mediated endocytosis followed by a low pH-induced fusion of viral and endosomal membranes (11), understanding how the fusion reaction is catalyzed by a fusogenic protein is of major importance in viral pathogenesis. Although much is known about the structure of influenza virus HA (12), little is known about the structure of fusogenic proteins for other animal viruses. This is largely due to the difficulty in obtaining sufficient amounts of soluble proteins, either from purified virus envelopes or from virus-infected cells. Attempts to separately express proteins with fusogenic properties from animal viruses in E. coli had not been successful. This could possibly be due to the highly hydrophobic nature of most fusogenic proteins.

Vaccinia virus 14-kDa protein is highly hydrophilic. The protein has two large internal a-helical domains and a single potential glycosylation site (13). A priori, the above characteristics of the 14-kDa protein suggest that the protein may be produced in a soluble form in E. coli. In this investigation we report the production of the 14-kDa envelope protein of vaccinia virus in a high level prokaryotic expression system. We establish that the 14-kDa envelope protein of vaccinia virus is produced in a soluble form and in large amounts; and by several criteria, the E. coli-expressed 14-kDa protein is similar to the native protein found in the virus particle and in infected cells. We define several structural and functional properties concerning the 14-kDa virus envelope protein.

* This investigation was supported by Public Health Service Grant CA44262 from the National Institute of Health and by National Science Foundation Grant DMB-8609236. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ This work was done in partial fulfillment of the requirements for the Ph.D. degree in the Department of Biochemistry, State University of New York Graduate School, Health Science Center at Brooklyn.

§ To whom correspondence should be addressed.

1 The abbreviations used are: HA, hemagglutinin; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

22174
Vaccinia Virus 14-kDa Envelope Protein and Expression in E. coli

EXPERIMENTAL PROCEDURES

RESULTS

High Level Expression of the 14-kDa Envelope Protein of Vaccinia Virus in E. coli—To achieve high level expression of the 14-kDa vaccinia virus envelope protein in E. coli, we cloned a 0.42-kbp DraI-RsaI fragment from plasmid pE17 (16) into the Klenow-treated Ndel restriction site of the expression vector pT7-7 (14), so that the vaccinia 14-kDa-encoding gene was placed under the transcriptional control of the bacteriophage T7 promoter (Fig. 1). By digestion with the restriction enzyme Dral, we removed the single T upstream from the 14-kDa-encoding gene (13) and therefore removed the stop signal TAA which immediately preceded the start codon ATG, a characteristic sequence of vaccinia late genes (TAAAT). Unique restriction sites for creation of fusion proteins (after filling in the 5’ end) are indicated in Fig. 1. The cloning of the 14-kDa-encoding gene into the Klenow-treated Ndel site of pT7-7 vector enabled the 14-kDa-encoding gene of vaccinia virus to use its own start codon ATG and avoided the introduction of any extra amino acid residue at its amino terminus. The recombinant plasmid pT7Nd14K, containing the 14-kDa-encoding gene of vaccinia virus inserted in the sense orientation relative to the direction of transcription from the T7 promoter, was then used to transform E. coli lysogen BL21 (DE3) for expression via IPTG induction.

A typical result of 14-kDa protein expression in E. coli is shown in Fig. 1B. As revealed by staining with Coomassie Blue R-250 (panel A) and by immunoperoxidase staining with monoclonal antibody mAbC3 (panel B), the E. coli-expressed 14-kDa vaccinia protein is a major protein produced after induction and accumulated to high levels by 4 h. In addition, the 14-kDa protein is gradually processed to a 12-kDa form. Transformants carrying the pT7Nd14K in the antisense orientation relative to the T7 promoter did not produce the 14-kDa protein (data not shown). By measuring the recovery of the 14-kDa protein in supernatants after induced cells were freeze-thawed and sonicated in extraction buffer without detergent, we found high yields (80%) of the 14-kDa protein in soluble form.

The 14-kDa Envelope Protein of Vaccinia Virus Synthesized in E. coli Forms Disulfide-linked Oligomers—We have previously reported that the 14-kDa envelope protein of vaccinia virus apparently exists as a trimer both in the virus particle and in vaccinia virus-infected cells (3, 4, 10). Therefore, it was of interest to determine whether the 14-kDa protein synthesized in E. coli could form trimers. Because trimers are linked by disulfide bonds, lysates from induced cells were analyzed on 12% SDS-PAGE without treatment with β-mercaptoethanol. A typical result is shown in Fig. 2 (panels A and B). As indicated by Coomassie Blue staining (panel A), proteins of about 40, 28, and 14 kDa are clearly induced after IPTG treatment of E. coli BL21(DE3) cells harboring plasmid pT7Nd14K. These proteins reacted with mAbC3 (panel B). The size of the E. coli-expressed proteins corresponded with trimer, dimer, and monomer forms of the 14-kDa protein. Other mAbC3-reactive proteins of higher mass might represent protein aggregates.

When lysates of IPTG-induced E. coli cells expressing the 14-kDa vaccinia virus protein were treated with increasing concentrations of the chemical cross-linker disuccinimidyl suberate and analyzed by SDS-PAGE under reducing conditions, we observed an increase in the amount of mAbC3-reacting proteins with molecular masses about 28 kDa and about 40 kDa and a corresponding decrease of 14- and 12-kDa proteins (Fig. 2, panel C). The heterogeneity found in the 40 and 28 kDa regions is most likely due to mixing of 14- and 12-kDa subunits. These studies establish that 14 kDa forms trimers.

The 14-kDa Envelope Protein of Vaccinia Virus Synthesized in E. coli Has Similar Molecular Mass as the Protein in the Virus Particle and the Same Amino-terminal Sequence as That Predicted from the DNA Sequence—As determined by SDS-PAGE analysis, we found that the 14-kDa vaccinia protein expressed in E. coli (Fig. 3, lane E) has similar size, in both monomer, dimer, and trimer forms, to the protein present in virus-infected cells (lane I) and in the virus particle (lane V). Because with prolonged induction by IPTG, we observed...
processing of the 14-kDa protein into a form of lower molecular mass (12 kDa), we asked whether these two proteins had the same NH₂-terminal amino acid sequence. The two proteins were purified and NH₂-terminal sequence analysis was carried out as described under "Experimental Procedures." The E. coli-expressed 14-kDa vaccinia protein had the same amino terminus (30 amino acids) as that deduced from the DNA sequence (Met-Asp-Gly-Thr-Leu-Phe-Pro-Gly-Asp-Asp-Leu-Ala-Ile-Pro-Ala-Thr-Glu-Phe-Phe-Ser-Thr-Lys-Ala-Ala-Lys-Lys-PRO-Glu-Ala). However, the first two amino acids, Met and Asp, were deleted at the NH₂ terminus of the 12-kDa protein. The amino terminus of the 14-kDa vaccinia protein from purified virus was blocked, preventing determination of the NH₂-terminal amino acid sequence of the native protein.

Oligomerization of 14-kDa Protein Does Not Require Cellular

Factors and a Monomer Binds to Another Monomer More Strongly than to a Dimer or a Trimer—Since the 14-kDa protein contains two contiguous cysteine residues at amino acids 71-72 which are thought to be involved in trimerization by disulfide linkage (13), it was of interest to know if purified 14- and 12-kDa vaccinia proteins produced in E. coli could reassociate to oligomers in the absence of cellular factors. Indeed, when purified 14- and 12-kDa proteins are incubated at 4°C in 60 mM NH₄HCO₃ solution and then analyzed under non-reducing conditions, dimers and trimers are formed (Fig. 4, A and B). Further proof that the 14-kDa protein interacts with monomers and oligomers was provided by incubation of 125I-labeled 14-kDa protein with a blot containing lysates of purified virions (Fig. 4C, lane 1), virus-infected cells (Fig. 4C, lane 2) and E. coli-expressed 14-kDa protein (Fig. 4C, lane 3). To quantify the extent of interaction of the 125I-labeled 14-kDa protein to monomeric and oligomeric forms of this protein, binding experiments were carried out with blots containing different amounts of monomers, dimers, and trimers. Purified 40-kDa trimers were partially dissociated into dimers and monomers by increasing concentrations of β-mercaptoethanol. The proteins were fractionated by SDS-PAGE, and the relative abundance of each protein component was quantified by densitometric analysis of the Coomassie Blue-stained gel. A similar gel run in parallel was blotted, incubated with 125I-14-kDa protein and the extent of binding to each protein component was quantified by densitometric analysis of the autoradiogram. The results are shown in Fig. 5. It is clear that 125I-14-kDa protein binds to monomers more strongly than to dimers and trimers. For example, comparison of binding in lane 3 revealed that when the amount of each protein component in the stained gel (panel A) represents 33% (monomer), 22% (dimer), and 45% (trimer), the extent of binding of the labeled protein in the autoradiogram (panel B) accounts for 71% (monomer), 11% (dimer), and 18% (trimer).

Trimers Induce High Titer Neutralizing Antibodies Which Correlate with Overall Immunogenicity—Next, we studied an important biological property of the native 14-kDa protein, i.e. its ability to elicit humoral immune response and to induce neutralizing antibodies. Was this ability dependent on oligomerization of the protein?
BALB/c mice (three/group) were immunized with each of the three forms of purified E. coli-expressed vaccinia proteins (40, 14, and 12 kDa) or with a non-induced E. coli extract as control. Polyclonal antisera prepared from each group of mice were tested by Western blot (Fig. 6) with strips containing E. coli lysates from non-induced (lane 1), 4-h-induced cells (lane 2), and lysates of vaccinia virus-infected BSC-40 cells (lane 3). The origin of antisera was anti-E. coli extract (panel A), monoclonal antibody mAbC3 as ascites (panel B), anti-40-kDa trimer (panel C), anti-14-kDa monomer (panel D), and anti-12-kDa processed monomer (panel E). It is clear that both monomer and trimer forms of vaccinia 14-kDa proteins induced specific antibodies to the protein (panels C–E). The antibody levels induced by each protein were quantitated by enzyme-linked immunosorbent assay. Higher antibody levels were elicited by trimers than by monomers. The virus neutralization titers and the immunogenicity of the proteins (by enzyme-linked immunosorbent assay) are shown in Table I. Trimers elicited higher titer neutralizing antibodies than monomers and the differences in neutralization titers correlated well with the overall immunogenicity of these proteins. The same findings were obtained in individual serum from each group of mice.

Vaccinia Virus 14-kDa Protein Binds with Specificity to the Cell Surface of Cultured Cells—We have previously described the structure and function of a 32-kDa envelope protein of vaccinia virus with strong binding to the cell surface of cultured cells (24). In these studies, two other unknown virus envelope proteins of 21 and 14 kDa were found to bind to cells, but their intensity was lower when compared with binding of the 32-kDa protein (24). Because vaccinia 14-kDa protein might play a major role in virus penetration at the level of fusion of the virus envelope with the cell membrane (3–5), it was of interest to know if the 14-kDa protein was capable of binding to the surface of animal cells. Fig. 7A (upper panel) shows binding of $^{125}$I 14 kDa to HeLa cells in the absence or presence of competitors. It is clear that specific binding of $^{125}$I 14-kDa protein to cells occurs with time of incubation; binding was competed either by purified vaccinia virus or by purified 14-kDa trimer but not by bovine serum albumin. Poor binding to cells was observed with labeled protein A. Analysis of the bound products by SDS-PAGE and autoradiography confirm binding with time of either monomers or trimers to the cell surface (Fig. 7B, lower panel). Similar results were observed between Hela cells, BSC-40 cells, and mouse L cells (data not shown).

**DISCUSSION**

We have inserted the gene encoding the 14-kDa envelope protein of vaccinia virus into a high efficient bacterial expres-
sion vector, and we established that the E. coli-produced protein behaves as the native protein present in the virus particle. The expression plasmid was engineered to express only the entire coding sequence of the 14-kDa virus envelope protein.

The choice of the expression system was dictated by the difficulties encountered in purifying the milligram amounts of the 14-kDa protein either from purified virions or from virus-infected animal cells required for structural and functional studies of this fusogenic protein. As shown here, 15-20 mg of 14-kDa protein could be produced in soluble form from 1 liter of E. coli BL21(DE3) cells harboring the expression plasmid pT7Ndl4K. Of the total E. coli proteins produced by the bacteria about 5-10% represent the 14-kDa protein. The sequence of the first 30 NH₂-terminal amino acids of the purified E. coli-expressed 14-kDa protein was identical to the sequence predicted from the nucleotide sequence of the 14-kDa-encoding gene. Similar protein sequence analysis could not be performed with the 14-kDa protein from purified virus particles since the NH₂ terminus was found to be blocked. The prokaryotic expression system also generates another protein of about 12 kDa which increases in amount throughout the induction period, indicating cleavage by a cellular protease. The 12-kDa protein lacks the first 2 amino acid residues, Met-Asp, from the NH₂ terminus of the 14-kDa protein. Although we have not determined the COOH-terminal sequence of the 12-kDa protein, it is likely that the NH₂-terminal deletion accounts for the about 2 kDa reduction in apparent size on SDS-PAGE. In fact, we have previously reported that a single point mutation, Asp for Ala-25, alters the apparent size of the 14-kDa protein by about 1.5 kDa on SDS-PAGE (10). However, we cannot exclude proteolytic cleavage also occurring at the COOH terminus of the 12-kDa protein.

An important structural feature of the E. coli-expressed 14-kDa protein is its ability to form oligomers, dimers, and trimers. Oligomerization was demonstrated both by protein size analysis under non-reducing conditions and by chemical cross-linking with disuccinimidyl suberate (Fig. 2). Cellular factors are not required for oligomerization, since purified 14- and 12-kDa proteins reassociate in solution to form dimers and trimers (Fig. 4). The characteristic oligomerization of the 14-kDa protein was also observed after soluble monomers were incubated with 14-kDa protein immobilized on nitrocellulose paper. We found that nitrocellulose paper containing E. coli-expressed 14-kDa protein and 14-kDa protein from virus-infected cells readily binds to 125I-labeled 14-kDa protein (Fig. 4). Quantitation of the extent of binding demonstrates that 125I 14-kDa protein binds to monomers more strongly than to dimers and trimers, in ratios approximately of 2:1 (monomer), 1:2 (dimer), and 1:2.5 (trimer) (Fig. 5, for lane 3). These findings suggested that soon after synthesis, the 14-kDa protein assembles into oligomers in a host-independent manner. Oligomerization of 14-kDa protein must involve the 2 unique and contiguous cysteine residues, at positions 71-72. This is based on the observation that dissociation of
trimeres into monomers occurs in the presence of disulfide-reducing agents (Fig. 5). Because purified 14 kDa monomers spontaneously assemble into dimers and trimers (Fig. 4), it demonstrates that oligomers of 14 kDa are made of identical subunits.

The vaccinia virus 14-kDa protein is an integral membrane protein (3, 5), and many membrane proteins, like influenza HA, undergo extensive post-translational modification, such as trimming of sugars, terminal glycosylation, and fatty acid acylation, during transport from the endoplasmic reticulum to the cell membrane (21–23). Inspection of the primary structure of the 14-kDa protein reveals a single potential glycosylation site at amino acids 60–62 (13). Because E. coli cannot glycosylate proteins and since the 14-kDa protein has the same apparent molecular mass as the protein found in the virus particle (Fig. 5), the native vaccinia virus 14-kDa protein most likely exists as a non-glycosylated molecule. This is also supported by the results obtained after cell-free translation of the 14-kDa transcript in the rabbit reticulocyte lysate (10). Thus, lack of glycosylation might indicate that in contrast to other viral membrane proteins (21, 22), vaccinia 14-kDa protein does not undergo passage from the endoplasmic reticulum to the Golgi complex. Trimerization and transport to the viral membrane may then be sequential events with the hydrophobic COOH terminus providing the anchoring domain. In the case of influenza virus HA and vesicular stomatitis virus G protein, trimerization takes place in the endoplasmic reticulum, where monomers apparently acquire a compact and highly folded conformation before assembly, but trimerization alone is not sufficient for transport (21, 23). Although we have shown here that 14-kDa protein can assemble into oligomers in the absence of cellular factors, it remains to be established whether such factors are needed for intracellular transport of subunits and/or trimers to the viral membrane or if this is rather a conformation-dependent event. The availability of monomers, dimers, and trimers of 14-kDa protein provides the means to develop conformation-dependent monoclonal antibodies to study the biogenesis of the 14-kDa protein in animal cells.

The mechanism of fusion of vaccinia virus envelope with cell membrane components as a means to deliver the virus inside of the cell is not yet known. From experiments with deletion mutants of 14-kDa protein, we have provided evidence that the NH2 terminus of the 14-kDa protein is involved in cell fusion (5). Both phenomena, 14-kDa-mediated fusion-from-without and fusion-from-within were observed at the cell surface after acid-pH treatment of infected cells (5). We suggested that the fusion reaction requires either a conformational change of the protein to expose a hydrophobic peptide and bind to lipid bilayers or protonization of charged residues at the NH2 terminus (5). To initiate the fusion reaction a close interaction between 14-kDa protein and cell components must take place soon after virus attachment. As shown in this investigation 14-kDa protein binds to the cell surface with specificity. Whether 14 kDa can cause fusion directly or it needs to interact with other viral proteins remains to be determined.

A characteristic property of many envelope proteins of animal viruses is their ability to induce neutralizing antibodies in the infected host (11, 12). Like other viral envelope proteins, the purified vaccinia 14-kDa protein induces neutralizing antibodies in mice (Table I). Interestingly, trimers induced higher titer neutralizing antibodies than monomers and these titers correlated with antibody levels (Table I). Activation of a humoral immune response by 14-kDa protein might then play an important role in the control of virus infection in vivo.

Of the viral membrane proteins, the x-ray diffraction structure has only been elucidated for the trimer HA of influenza virus (8) and tetramer of neuraminidase (25). Crystallization of HA was only made possible after removal of the ectodomain of mature HA with bromelain to generate a soluble trimer (8). The difficulties encountered in resolving the three-dimensional structure of other viral membrane proteins are largely due to their hydrophobic nature and the occurrence of extensive post-translational modifications in the mature proteins. The apparently simple structure of vaccinia virus 14-kDa protein and our demonstration that large amounts of this protein can be obtained in soluble form in E. coli with apparently similar conformation to the native protein, suggest that elucidation of the structure of 14-kDa trimers by x-ray diffraction might be possible. Acknowledgments—We thank J. A. Lewis and T. C. Detwiler for critically reading the manuscript.

REFERENCES

Vaccinia Virus 14-kDa Envelope Protein and Expression in E. coli

Experimential Procedures

1. **Expression of Viral Proteins**

   a. The vaccinia virus 14-kDa envelope protein was expressed in E. coli. The protein was produced in large quantities and purified using a combination of affinity and size-exclusion chromatography.

2. **Expression in E. coli**

   a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

3. **Protein Analysis**

   a. The purified protein was analyzed using SDS-PAGE and Western blotting.

4. **Expression in Bacteria**

   a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

5. **Protein Analysis**

   a. The purified protein was analyzed using SDS-PAGE and Western blotting.

6. **Expression in E. coli**

   a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

7. **Protein Analysis**

   a. The purified protein was analyzed using SDS-PAGE and Western blotting.

8. **Expression in Bacteria**

   a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

9. **Protein Analysis**

   a. The purified protein was analyzed using SDS-PAGE and Western blotting.

10. **Expression in E. coli**

    a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

11. **Protein Analysis**

    a. The purified protein was analyzed using SDS-PAGE and Western blotting.

12. **Expression in Bacteria**

    a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

13. **Protein Analysis**

    a. The purified protein was analyzed using SDS-PAGE and Western blotting.

14. **Expression in E. coli**

    a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

15. **Protein Analysis**

    a. The purified protein was analyzed using SDS-PAGE and Western blotting.

16. **Expression in Bacteria**

    a. The expression vector used was pET28a. The protein was expressed in E. coli strain BL21 (DE3) and purified by Ni-NTA affinity chromatography.

17. **Protein Analysis**

    a. The purified protein was analyzed using SDS-PAGE and Western blotting.