ABSTRACT

African swine fever (ASF) soluble antigen, used for routine diagnostic tests, is produced from infected Vero cells. The residual infectivity of antigen after solubilization and ultracentrifugation ranges from $10^{6.5}$ to $10^{8.0}$ ID$_{50}$/ml. Antigen shipped from Plum Island Animal Disease Center high containment laboratory has to be completely inactivated while still retaining antigenicity. Since binary ethylenimine (BEI) inactivates nucleic acid with minimal effect on protein, it was used to inactivate the residual virus present in soluble ASF antigen. The effect of time, temperature and concentration of BEI was studied. Inactivation with 10 mM BEI at 37°C for 3 hours completely inactivates virus, but some loss of antigenic activity occurred. Treatment with 10mM BEI at 17°C inactivated 5 logs of virus after 5 hours of treatment, while 5.0 mM BEI inactivated 5 logs of virus in 7 hours. Inactivation with 10mM BEI for 20 hours at 17°C was used to assure complete loss of infectivity while still retaining most of the antigenic activity. Inactivated antigen was found to contain no residual infections ASF virus as demonstrated by inoculation of swine and tissue cultures.

INTRODUCTION

Soluble African swine fever (ASF) antigen is currently used for both the immunoosmoelectrophoresis (IEOP) (4), and enzyme-linked immunosorbent assay (2). Large scale production of antigen was initiated at the Plum Island Animal Disease Center (PIADC) as a result of the simultaneous report of outbreaks of ASF in Brazil and the Dominican Republic in 1978 (3). Over 10 liters of antigen have been prepared, inactivated, safety tested and stored at -70°C and are available for shipment. Antigen has been shipped both to the Dominican Republic and Haiti for laboratory use in order to monitor swine sera during the ASF eradication programs in those countries.

All biologicals shipped from PIADC must be free of infectious agents, thus, complete inactivation of antigen is mandatory before shipment. Acetylene (AEI), B-propiolactone, and glycidaldehyde have been shown to effectively inactivate ASFV (7), but the compounds are considered potentially carcinogenic for humans. Binary ethylenimine (BEI) may also be potentially carcinogenic, but is is generated from bromethylamine (BEA), a less toxic compound (1). Previous work had
shown that ASF antigen treated with 10 mM BEI for three hours at 37°C completely inactivates the residual virus in the antigen, but does result in some loss of antigenicity (6). Moreover, storage of antigen at temperatures above -70°C also reduces antigen titer. The kinetics of inactivation of ASF antigen was studied in order to establish optimal conditions of antigen inactivation and improve the quality of the antigen.

MATERIALS AND METHODS

Virus and cell culture.

The twelfth passage of ASFV/Brazil/78 virus adapted to Vero cell cultures was received from I. C. Pan of the PIADC. Serial passages of virus were made as described previously (6). A microtiter assay in Vero cells was used to titrate the virus infectivity. Serial ten-fold dilutions of virus were employed using four replicates per dilution. Titers were expressed in ID$_{50}$. Conditions of the assay will be described (manuscript in preparation).

Immunoosmoelectrophoresis and immunodiffusion assays.

Details of the assay were described previously (4, 6). All samples were tested with a hyperimmune serum obtained from a pig inoculated with ASFV/Dominican Republic/78 and ASFV/Lisbon 60. Serum was obtained from A. H. Dardiri of the PIADC. Samples for assay included the untreated virus, zero time and the final product after each treatment. Block titrations of antigen and antisera were done for the IEOP assay, while for the immunodiffusion (ID) assay serial two-fold dilutions of antigen were tested against a single concentration of antiserum.

Soluble ASF antigen.

The production of ASF antigen was described previously (4, 6). In brief, Vero cells grown in 110 x 285 mm glass roller bottles were inoculated with virus at a multiplicity of infection ranging from 0.5 to 1.0. After a 4 hour adsorption period at 37°C, infected cells were incubated at 33°C for 40 to 44 hours. ASF antigen was obtained from sonicated infected cells. After sonication, the supernatant fluid was clarified by low and high speed centrifugation. The final supernatant fluid was diluted with phosphate buffered saline, (PBS) aspH 7.4, and the antigen was stored at -70°C until it was activated. The antigen before inactivation has a residual virus titer ranging from 10$^{6.5}$ to 10$^{8.0}$ ID$_{50}$. The protein concentration was 5 mg/ml.

Determination of the optimal temperature of virus inactivation

The rate of inactivation of residual ASF virus present in antigen in the presence of 10 mM BEI was determined at 37°C, 27°C, 17°C and 7°C. The production of BEI from BEA was described previously (1). In brief, a solution of 100 mM BEA in 200 mM NaOH was heated at 37°C for 1 hour to produce 100 mM BEI. Two control tubes containing 200 mM NaOH and respectively, were also made. Three flasks of antigen and three tubes containing 100 mM BEI, 200 mM NaOH or PBS, respectively, were equilibrated for 30 minutes at the above temperatures. At zero time, 10
ml of BEI was added to 9 ml of antigen in a flask, followed by the addition of 1.0 ml of 200 mM NaOH or 1.0 ml of PBS to each of the respective flasks. Flasks were agitated by stirring. Inactivation of virus by BEI was stopped by the addition of an excess of Na₂S₂O₅. Accordingly, 0.9 ml samples from each flask were rapidly pipetted into 0.1 ml of 200 mM Na₂S₂O₅. Samples were taken at 2 minutes after initial inoculation, considered as zero time, and at appropriate intervals thereafter.

Determination of the optimal concentration of BEI.

The antigen was produced from the 23rd passage of ASFV/Brazil/78 in Vero cells. Four flasks containing 9 ml of antigen and 4 vials of BEI containing 100 mM, 50 mM, 25 mM and 10 mM BEI were equilibrated at 17°C. At 0 time, 1.0 ml of each concentration of BEI was added to a flask of antigen, which resulted in four flasks of antigen containing 10.0, 5.0, 2.5, and 1.0 mM BEI. Flasks were agitated by stirring. Samples of 0.9 ml were taken from each flask at 0, 2, 4, 5, and 24 hours post inoculation (p.i.) and diluted into 0.1 ml of 200 mM Na₂S₂O₅. Samples were stored at 5°C before assay.

RESULTS

Optimal temperature of inactivation.

The rate of inactivation of virus by 10 mM BEI is linear and decreases with the decrease in temperature at 37°C, 27°C and 17°C, with no viable virus detected at 1, 3 and approximately 10 hours, respectively. Reaction at 7°C was slower with some residual infectivity seen at 24 hours post inoculation. Antigen diluted in PBS showed no drop in titer when treated at 37°C for 2 hours, 27°C for 4 hours, 17°C and 7°C for 24 hours. In all cases, antigen treated with 200 mM NaOH at the above time and temperature was non-infectious.

IEOP assays on the original, zero time and final samples after treatment at different temperatures are shown in Figure 2. There was only a minimal effect on antigen treated with 10 mM BEI at 37°C and 27°C for 1.5 and 2 hours, respectively. Little or no reduction in antigenicity was seen in virus treated with 10 mM BEI at 17°C and 7°C. Figure 3 shows the results of titrations of antigen with 10 mM BEI at different temperatures. There appear to be at least 4 major precipitin lines detected in both unstained preparations of the IEOP and ID assays. The number of lines diminishes with antigen dilution. A principal antigen had a titer of 1/32 in the ID assay when virus was treated with BEI at 17°C and 7°C. However, the antigen had a titer of 1/8 when treated at 37°C for 1.5 hours with 10 mM BEI. The identity of these principal antigens is unknown at present.

Inactivation with 10 mM BEI at 37°C and 27°C is extremely rapid, whereas inactivation at 17°C was effective and readily controlled. This temperature was chosen as optimal for inactivation.

Optimal concentration of BEI.

The rate of inactivation at 17°C with varying concentrations of BEI is
seen in Figure 4. Inactivation with 1.0 mM BEI was slow with no inactivation occurring at 5 hours p.i. Some inactivation not indicated in the figure was seen at 24 hours p.i. Inactivation of virus with 2.5 mM BEI was first detected at 4 hours p.i. Treatment with 10 mM BEI showed inactivation of 5 logs of virus after 5 hours of treatment, while 5.0 mM BEI inactivated 5 logs of virus in 7 hours.

The residual virus titer in different lots of soluble ASF antigen can range from $10^{6.5}$ to $10^{6.0}$ ID$_{50}$/ml. Most lots of antigen prepared for inactivation represent a pool of approximately 360 ml. In order to assure complete inactivation of residual virus, antigen was inactivated with 10 mM BEI for 20 hours at 17°C.

DISCUSSION

Complete inactivation of residual virus present in soluble ASF antigen is necessary because the antigen is shipped both to Latin American countries and to the National Veterinary Services Laboratory at Ames, Iowa. Over 15 lots of ASF/Brazil/78 antigen, 400 ml of antigen per lot, have been treated by the above method. All of the lots have shown a complete loss of infectivity as indicated by inoculation of both animals and tissue cultures (unpublished data). Although 5 mM BEI treatment may also be effective, treatment with 10 mM BEI for 20 hours at 17°C results in minimal effect on antigenicity and assures inactivation of virus. Moreover, repeated tests on antigen stored at -70°C for as long as 2 years show but a small decrease in antigenicity as measured by the IEOP assay (unpublished data).

African swine fever virus is a extremely stable virus, which can withstand wide extremes of temperature and pH (5, 8). Our results show that ASFV is rapidly inactivated by 20 mM NaOH at a final pH in antigen of 9.0. This differs from previous reports which indicate that ASFV remains infectious after treatment for 6 hours at pH 13.0. Both the total amount of protein present in the preparation and, perhaps, differences among virus strains may account for the discrepancy in results.

The number of precipitin lines seen both by the IEOP and ID assays is an indication of the multiplicity of antigens present in the ASF antigen preparation. It is interesting to note that one of the antigens appears to be sensitive to treatment at 37°C. The identification and isolation of the principle viral proteins that react in the IEOP assay will result in an improved and more specific test.

Inactivation of residual ASF virus in antigen with 10 mM BEI at 17°C for 24 hours effectively inactivates infectivity of ASF virus with minimal effect on viral antigenicity.

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Figure 1. Comparison of the effect of different temperatures on the inactivation of ASF/Brazil/78 antigen treated with 10 mM BEI.
Figure 2. IEOP titration of ASF/Brazil/78 antigen using hyperimmune anti-ASFV swine serum. Antigen samples taken before treatment and after treatment with 10 mM BEI at different temperatures for 0, 1.5, 2.0, 5.0 and 24 hours. Numbers within the boxes refer to the number of precipitin lines found between each respective antigen and antiserum dilution.
Figure 3. Immunodiffusion titration of ASF antigen with hyperimmune anti-ASFV serum as described in Figure 2.

Figure 4. Comparison of the effect of different concentrations of BEI on the inactivation of ASFV/Brazil/78 at 17°C.
REFERENCES


