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Thermal Inactivation of High Pathogenicity Avian Influenza Virus in Dried Egg White

Colleen Thomas and David E. Swayne

Southeast Poultry Research Laboratory, Exotic and Emerging Viral Disease Research Unit, ARS-USDA, 934 College Station Road, Athens, GA 30605

SUMMARY. This study presents thermal inactivation data for the high pathogenicity avian influenza virus (HPAIV) strain A/chicken/PA/1370/83 (H5N2) (PA/83) in dried egg white with an average moisture content of 7.5%. The 95% upper confidence limits for D-values calculated from linear regression of the survival curves at 54.4, 60.0, 65.5, and 71.1ºC were 475.4, 192.2, 141.0, and 50.1 minutes, respectively. The line equation y = [(0.05494)(ºC)] + 5.5693 (RMSE = 0.0711) was obtained by linear regression of experimental D-value versus temperature. Conservative predictions based on the thermal inactivation data suggest that standard industry pasteurization protocols would be very effective for PA/83 HPAIV inactivation. For example, these calculations predict that after approximately 2.6 days at 54.4ºC there is a 1:100 probability of 1 EID_{50}/g PA/83 HPAIV remaining when the starting titer is 5-log EID_{50}/g.

INTRODUCTION

High pathogenicity avian influenza virus (HPAIV) strains cause severe disease with high mortality in chickens and related gallinaceous poultry. In chickens, the initial replication site for HPAIV is the respiratory or intestinal tract which is followed by systemic spread of the virus. During the 1983-1984 outbreak of HPAIV in the northeastern U.S., HPAIV was isolated from albumen, yolk, and the shell surface of eggs obtained from affected flocks in Pennsylvania (*3*). In one experimental study, HPAIV titers as high as $10^{4.9}$ EID_{50}/ml of egg product were found in eggs laid by infected hens (M. Brugh, unpublished data).

To prevent transmission of HPAIV to susceptible commercial poultry flocks via the movement of infected or contaminated poultry products, the World Organization for Animal Health (OIE) recommends that poultry products from HPAIV-infected countries, zones, or compartments be treated to inactivate HPAIV prior to export (*6*). The demonstration of heat inactivation of avian influenza viruses in poultry products suggests that thermal processing could be an effective treatment (*5*, *7*, *8*, *10*, *11*).

A previous study performed in our laboratory reported D-values for the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) in various egg products (*8*). Using this data, calculations were done to determine whether U.S. industry standards for egg product pasteurization, developed to inactivate contaminating *Salmonella*, are also sufficient for HPAIV inactivation. The calculations predicted that 15 days would be required to completely inactivate $10^{4.9}$

EID_{50}/ml HPAIV in dried egg white at 54.4ºC, rather than the 7-10 days specified by the industry standard. However, the moisture content of the freeze-dried egg white prepared for the previous study was not controlled, and was probably much lower than that found in commercially available spray-dried egg white products.

For the current study, HPAIV-contaminated freeze-dried egg white with an average moisture content of 7.5% was prepared and used for thermal inactivation experiments. PA/83 HPAIV was inactivated much more rapidly in this product than in that prepared for the previous study, indicating that moisture content does affect HPAIV inactivation in dried egg white. Calculations done for the current study predict that standard industry pasteurization protocols would effectively inactivate PA/83 HPAIV with a large margin of safety.

MATERIALS AND METHODS

Virus strain and preparation of virus-infected material. Working stocks of the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) were prepared by propagation in embryonating chicken eggs following standard procedures (*9*). All work with PA/83 and with PA/83-infected materials was performed in USDA-certified biosafety level 3 agriculture (BSL-3Ag) facilities.

Preparation of samples for freeze-drying. Liquid egg white was prepared by mixing 0.2 g dried egg white per ml of sterile deionized and distilled water, and then filtering the suspension through sterile cheesecloth. One ml of PA/83 stock was added to 100 ml of the filtrate, and no virus was added to the remainder. Sterile 5 ml serum vials were arranged in semicircular drying trays, and 1.5 ml of liquid egg white was dispensed into each vial according to the pattern shown in Fig. 1A. Stoppers were placed in the vials in the “up” position used for freeze-drying. The vial trays were placed in sealed containers and frozen at -80ºC for a maximum of 3 days before freeze-drying.

Freeze-drying procedure. The drying time and sample configuration required for producing dried egg white with 6.5-8.0% moisture was determined empirically, then repeated to confirm reproducibility. This section describes the final protocol used to prepare freeze-dried egg white containing PA/83 HPAIV. Sample vials were freeze-dried in a Micromodulyo 1.5 L freeze dryer, with two vial trays (Fig. 1A) placed on each of the middle shelves of the drying chamber as shown in Fig. 1B. Vacuum was supplied by an Edwards E2M8 pump. Samples were dried for 7 hours and 45-50 minutes, and then the vials were sealed under vacuum. Vials were treated with 70% ethanol to remove any surface contamination. The vials from the inner and middle rows of each tray were discarded. The vials from the outer rows were capped and then stored at -80ºC.

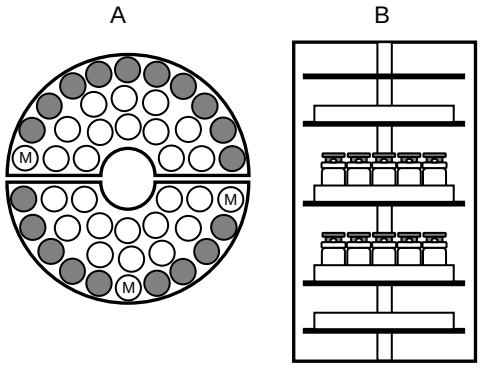

Figure 1. (A) Serum vials containing liquid egg white with PA/83 AIV (gray circles) or no virus (white circles) placed in trays for freeze drying. "M" indicates samples for moisture analysis. (B) Schematic diagram showing placement of the vial trays on vial stoppering shelves in the freeze dryer chamber.

Moisture analysis. The percent moisture was estimated from sample vials from the tray positions shown in Fig. 1A. Triplicate samples for moisture analysis were prepared by combining the contents of two sample vials. To reduce error from combining samples, the sample vials were ranked by sample weight and combined accordingly (i.e. the two heaviest samples were combined). The flat end of a plunger taken from a syringe was used to grind the samples in the vials. Moisture determination was done by the AOAC vacuum oven method for dried eggs (*1*), except that 0.5 g of sample was used for analysis.

Thermal inactivation procedure. Sample vials were completely submerged in a Precision water bath (Thermo Scientific 280 series) set to the target temperature. The time required for sample in the vial to reach each target temperature was determined by simultaneously monitoring water and negative control sample temperatures with a data logger thermometer. Samples were immediately chilled on ice after removal from the water bath. Unless otherwise noted, triplicate samples were treated for each data point.

Virus isolation and titration. 1.5 ml of 4ºC sterile phosphate-buffered saline was added to each sample vial. The samples were placed on ice for 30-60 minutes and allowed to dissolve, then were gently mixed by swirling and pipetting. Virus isolation and titration were performed following standard procedures (*9*). The 50% endpoints were calculated by the method of Reed and Muench (*12*), and virus titer measurements were converted to log EID_{50}/g dried egg white. The detection limit of the assay was 0.8 log EID_{50}/g.

Statistics and graphs. Statistical operations were performed with Sigma Stat version 2.03 (1992-1997, SPSS, Chicago, IL). Graphs were prepared with Sigma Plot version 6.00 (2000, SPSS). Experimental D-values were calculated from linear regression of virus titer versus time at the given temperature (D-value = -1/slope). The upper limit of the 95% confidence interval for each experimental D-value was calculated from the following equation:

$$b_1 + t^*(s_\varepsilon)$$

where b_1 is the slope coefficient, t^* is obtained from a t-test critical values table (two-tailed test, a = 0.05), and s_ε is the standard error of the slope coefficient. The z-value was calculated from linear regression of log D-values (minutes) versus the temperature in ºC (z-value = -1/slope). The upper limit of the 95% confidence interval for the z-value was calculated as described for the D-values, except that the following generic equation was used to calculate the upper limit of the 95% confidence interval for the slope coefficient:

$$b_1 + 2(s_\varepsilon)$$

For each D-value calculated from the z-value graph regression line equation, the upper limit of the 95% prediction interval for the D-value was calculated as follows:

$$y + 2(\text{RMSE})$$

where y is the predicted log D-value (minutes) and RMSE is the root mean square error, or the standard error of the y estimate.

RESULTS

Freeze-drying method development and moisture analysis of dried egg white samples. The position of the sample vials relative to the center of the drying chamber and to the condenser unit affected the drying rate. As shown in Fig. 1A, only the sample vials in the outer rows of the drying trays were used for thermal inactivation experiments or for moisture analysis. Drying of samples in the interior vials was slower and less uniform, but their presence in the drying chamber improved the drying uniformity of the samples in the outer rows. Samples placed on the top or bottom shelves of the chamber dried more quickly or more slowly (respectively) than samples placed on the middle shelves, so only the middle shelves were used for freeze-drying (Fig. 1B). As shown in the table below, samples analyzed from each of the four batches of freeze-dried egg white used in this study had an estimated moisture content of 7.4 to 7.6%, with a maximum standard deviation of 0.5%.

Batch number	% Moisture[a]	
	Range	Average (SD)[b]
1	6.8 - 7.7	7.4 (0.5)
2	7.5 - 7.8	7.6 (0.2)
3	7.2 - 8.1	7.6 (0.5)
4[c]	7.8 - 9.2	8.4 (0.7)
5	7.1 - 7.9	7.5 (0.4)

[a] Moisture content estimated by loss of mass after drying for 5 hours at 100ºC. Data is from triplicate samples
[b] SD, standard deviation
[c] Not used in this study

Survival curves and D-values for PA/83 HPAIV in dried egg white. Figure 2 shows survival curves for PA/83 HPAIV in dried egg white at 54.4ºC, 60.0ºC, 65.5ºC, and 71.1ºC. The coefficients of determination (R^2) and the D-values calculated from each survival curve are shown in the table below the graphs. A linear model provided a fair-to-good fit for the survival curves, with R^2 values of 0.90 or higher for all curves except for the 65.5ºC curve. As shown in the graph, the 4 hour time point for 65.5ºC had an unusually large standard deviation. Similar results were obtained when a second set of triplicate samples was analyzed for this time point. This variability probably accounts for the relatively low R^2 value for this curve (0.83), as the R^2 values were obtained from graphs that included all of the data points rather than just the average values. For each survival curve, the final data point includes at least one sample in which PA/83 was not detected: 1/3 samples for 54.4ºC, 2/3 samples for 60ºC, 3/3 samples for 65.5ºC, and 2/3 samples for 71.1ºC. Negative samples were graphed as 0.7 log EID_{50}/g, which is just below the detection limit of the assay (0.8 log EID_{50}/g).

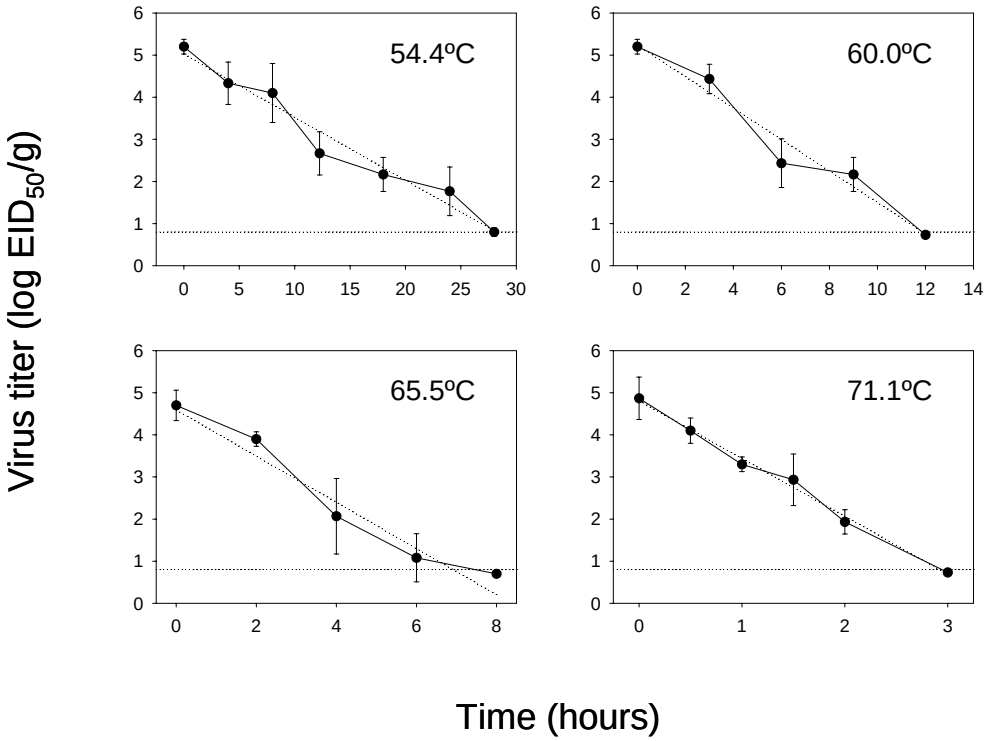

Figure 2. Survival curves for PA/83 virus in dried egg white. Each data point represents the average titer of at least three samples, and the error bars indicate standard deviations. The detection limit of the assay is 0.8 log EID_{50}/g.

Temp (ºC)	D-value (min)[a]	D-value 95% UCL[b]	R^{2c}
54.4	400.6	475.4	0.90
60.0	160.7	192.2	0.93
65.5	109.4	141.0	0.83
71.1	43.7	50.1	0.95

[a] Calculated from the inactivation curves shown in Figure 2
[b] 95% upper confidence limit for the D-value
[c] Coefficient of determination

Regression line equation and the z-value. Figure 3 shows a linear regression plot of log D-value versus temperature for PA/83 in dried egg white. The line equation and RMSE for the linear model are shown in the figure legend. A z-value of 18.2ºC was calculated from the graph, with a 95% upper confidence limit of 23.0ºC.

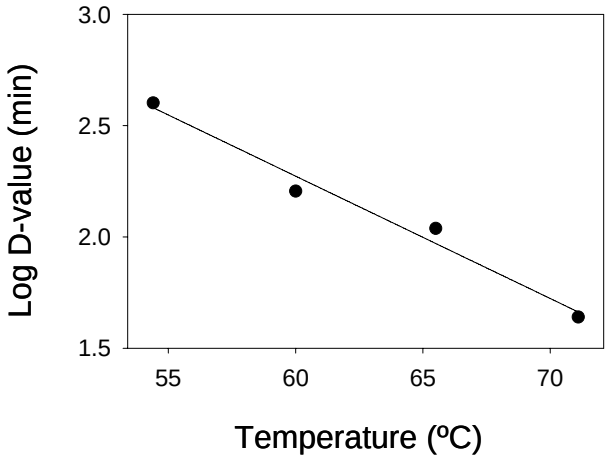

Figure 3. Linear regression plot of log D-value (in minutes) versus temperature (in ºC) for PA/83 virus in dried egg white. Line equation: y = [(-0.05494)(ºC)] + 5.5693. RMSE (root mean square error) = 0.0711. $R^2 = 0.98$.

Reduction of PA/83 HPAIV titer during pasteurization of dried egg white. The table below shows the predicted reduction in virus titer expected in dried egg white pasteurized following standard industry protocols (*2*, *4*). These estimates are based on the upper limits of the 95% prediction intervals for the D-values, calculated from the data shown in Figure 3. As shown in the table below, both pasteurization methods are predicted to be very effective for PA/83 HPAIV inactivation with a very large margin of safety.

Pasteurization standard		95% PI upper limit for D-value (hours)[a]	Predicted time (hours) for 7-log reduction[b]	Predicted number of log reductions after pasteurization[c]
Temp (ºC)	Time (days)			
54.4	7 - 10	8.80	61.6	19.1 - 27.3
67	15	1.79	12.5	201

[a] Upper limit of the 95% prediction interval for the D-value, calculated from the regression line equation + 2RMSE (Fig. 3). All of the predictions in the table are conservative estimates based on this number. RMSE, root mean square error
[b] 1:100 probability of 1 EID_{50}/g remaining when the starting titer is 5-log EID_{50}/g
[c] Assuming that the required pasteurization temperature is maintained for the length of time specified in the pasteurization standard

CONCLUSIONS

In the current study, liquid egg white containing the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) was freeze-dried to an average moisture content of 7.5%, and this product was used for thermal inactivation studies. Calculations based on this thermal inactivation data predict that standard industry pasteurization protocols would be very effective for PA/83 HPAIV inactivation. For example, calculations predict that after approximately 2.6 days at 54.4ºC, there is a 1:100 probability of 1 EID_{50}/g PA/83 HPAIV remaining when the starting titer is 5-log EID_{50}/g.

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