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Co-operative thermal inactivation of herpes simplex virus and influenza virus by arginine and NaCl

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ABSTRACT

Elevated temperatures have been used to inactivate viruses for plasma-derived biopharmaceuticals. This paper describes the effects of arginine and NaCl in conjunction with elevated temperature for inactivation of two enveloped viruses, i.e., herpes simplex virus type 1 (HSV-1) and influenza virus type A at neutral pH. In phosphate-buffered saline, a significant inactivation of HSV-1 occurred above 40 °C, resulting in less than 10% surviving virus (over 90% virus inactivation) at 50 °C. Arginine concentration dependently decreased the temperature required for virus inactivation, leading to temperature shift by almost 17 °C at 1.2 M. NaCl also decreased the inactivation temperature, but to a considerably lesser extent, indicating that virus inactivation effect of arginine is not simply due to ionic strength. Influenza virus was also inactivated by high temperature, but its responses to arginine and NaCl were different from those on HSV-1, suggesting that virus inactivation mechanism is different between these two viruses, i.e., the effects of these reagents are virus specific.

1. Introduction

Incubation of plasma-derived products or related materials at elevated temperatures is a conventional practice to inactivate viruses (Evengard et al., 1989; Norwak et al., 1992; Smales et al., 2000; Swayne and Beck, 2004; Tomasula et al., 2007; Graham and Staples, 2007). Although higher temperature and prolonged incubation inactivate viruses more effectively, they can cause damages on proteins (Smales et al., 2000; Ross et al., 1984; Yu and Finlayson, 1984). Knowledge of temperature dependence of virus inactivation should open a way for rational approach of the inactivation process. We have undertaken a study on virus inactivation by various solvents and shown that arginine has a synergistic effect with low pH on virus inactivation (Yamasaki et al., 2008; Katsuyama et al., 2008).

Arginine has been widely used for protein refolding (Arora and Khanna, 1996; Suenaga et al., 1998; Liu et al., 2007a, b), solubility enhancement (Ho et al., 2003; Tsumoto et al., 2003; Umetsu et al., 2005; Arakawa et al., 2008; Hirano et al., 2008) and column chromatography (Arakawa et al., 2005; Ejima et al., 2005; Tsumoto et al., 2007). In the previous studies, we have shown that arginine is effective in virus inactivation under mildly acidic pH at low temperature (Yamasaki et al., 2008; Katsuyama et al., 2008). In this paper, we investigated the temperature dependence of inactivation of two enveloped viruses, i.e., herpes simplex virus and influenza virus at neutral pH and compare the effects of arginine with that of NaCl on the temperature dependence.

2. Materials and methods

2.1. Materials

L-Arginine hydrochloride (simply described as arginine) was obtained from Ajinomoto Co. Inc. Aqueous solutions containing arginine was prepared in 20 mM acetic acid. The pH was adjusted with HCl; 20 mM acetic acid was insufficient to titrate arginine. The pH meter was routinely calibrated using pH calibration standards.

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2.2. Cells and viruses

Vero and MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum. Herpes simplex virus type 1/strain F (HSV-1) and influenza virus A/Aichi/68 (H_3N_2) were used throughout the experiments. The viruses were propagated in Vero (for HSV-1) or MDCK (influenza virus) cells in MEM supplemented with 0.5% fetal bovine serum (for HSV-1) or 0.1% bovine serum albumin (BSA) (for influenza virus). For influenza virus, acetylated trypsin (4 μg/ml) was supplemented to the culture medium for the activation of viral infectivity. The viruses were stored at −80 °C until use. The amount of virus in the stock preparation was measured by a plaque assay on Vero cells (for HSV-1) or MDCK cells (for influenza virus) as described previously (Koyama and Uchida, 1989; Kurokawa et al., 1999).

2.3. Assay for virucidal activity

All the starting materials were stored on ice prior to the virus inactivation experiments. A large excess volume of solvents was mixed with the virus stock so that the concentration and pH would not be affected: i.e., a 5 ml of the solvents containing different concentrations of arginine or NaCl to be tested received 100 μl of virus preparations (approximately 10^7 or 10^8 plaque-forming units (PFU)/ml). This virus preparation was divided into 200 μl aliquot in glass test tube on ice and the sample mixture was incubated at the indicated temperature for 5 min. After incubation, aliquots of these virus samples as well as those stored on ice for the same period were 100-fold diluted with Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing 1% calf serum (for HSV-1) or 0.1% BSA (for influenza virus). The viruses were further diluted with ice-cold PBS containing 1% calf serum or 0.1% BSA and the number of infectious virus in the treated preparation was measured by a plaque assay. There was a considerable variation in the plaque assay in the samples with higher concentrations of arginine. To reduce this variation to the extent that the observed variation does not affect the conclusion, we determined (1) the number of the infectious virus in the ice-stored samples simultaneously with the incubated samples at each time point to avoid the effect of virus-inactivation on ice and (2) samples at each time point were prepared in tetraplicate. There was little virus inactivation in PBS and hence the amount of infectious virus (also expressed as relative infectivity) in PBS was close to constant.

Virus inactivation data are normally plotted as a logarithmic fashion, i.e., log (relative infectivity) vs. variables (e.g., pH or concentration of antiviral agents). Such plot emphasizes the log-scale degree of virus inactivation, but does not depict the actual extent of surviving virus population. Although a difference in the degree of virus inactivation between 10^{-2} and 10^{-3} has a biological significance, for example, the amount of surviving virus is only 1 and 0.1% of the total and hence a majority of viruses are dead. In this paper, a normal plot was used to emphasize the population (concentration) of surviving viruses in the total virus population.

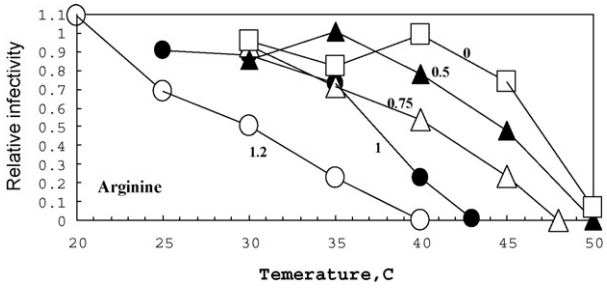

Fig. 1. Temperature dependence of virus inactivation for HSV-1 in the absence and presence of arginine at indicated concentration. Experimental errors are within the size of the symbols in this and the following figures.

3. Results and discussion

3.1. Inactivation of HSV-1

Temperature dependence of HSV-1 inactivation in PBS was examined for a varying incubation period of 2–20 min over a temperature span of 4–50 °C. Independent of incubation time, there was no significant virus inactivation below 25 °C, suggesting a critical temperature of virus inactivation for HSV-1. In the following experiments, the incubation period was fixed at 5 min. A precise control of incubation time is critical in the presence of arginine, as the rate of virus inactivation increases with arginine concentration. Fig. 1 shows a representative data of HSV-1 inactivation observed in 5 °C interval between 30 and 50 °C. No significant virus inactivation was observed up to 40 °C in PBS (open square). There is a sharp drop in relative infectivity above this temperature, i.e., 75% surviving virus at 45 °C, 50% at 47 °C and 10% at ∼50 °C (see also Table 1).

Thermal inactivation of HSV-1 was compared for PBS and arginine under the similar experimental conditions. It is evident in Fig. 1 that temperature-dependent inactivation curve shifted toward lower temperature with increasing arginine concentration. Virus inactivation was already apparent around 35 °C in the presence of 0.5 M arginine, i.e., about 5 °C decrease in the threshold temperature of virus inactivation. The temperature of virus inactivation was ∼2, 7, 10 and 17 °C lower in 0.5, 0.75, 1.0 and 1.2 M arginine solution. Table 1 summarizes the temperature, at which 50 and 90% viruses are inactivated, as a function of arginine concentration, demonstrating the trend seen in Fig. 1. At the highest arginine concentration tested, i.e., 1.2 M, the virus inactivation was already significant at 25 °C (open circle), leading to a nearly linear progression of virus inactivation with temperature.

Table 1
Temperature of 50 and 90% virus inactivation.

Solvent	Temperature at 50% inactivation (°C)		Temperature at 90% inactivation (°C)	
0 (PBS)	47		50	
Solvent	Temperature at 50% inactivation (°C)		Temperature at 90% inactivation (°C)	
	Arginine	NaCl	Arginine	NaCl
0.5 M arginine	45	47	47	
0.75 M arginine	40	43	45	48
1.0 M arginine	37	41	40	
1.2 M arginine	30	40	34	46

On average, each temperature has an experimental error of ±0.5 °C.

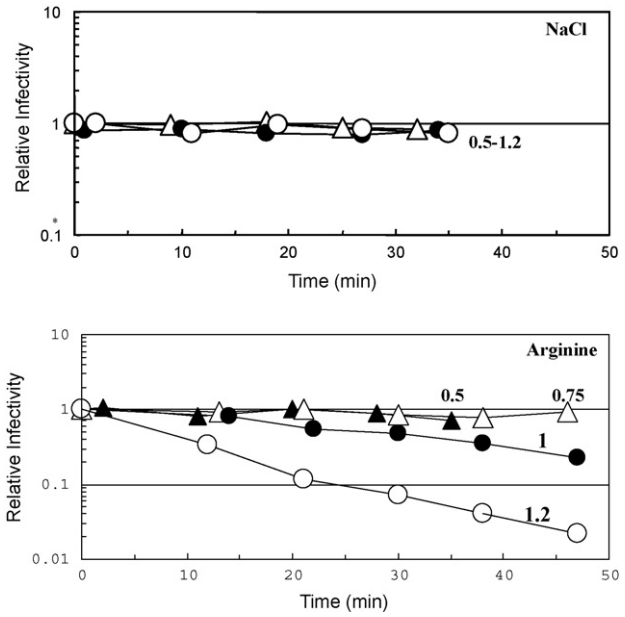

Fig. 2. Time course of virus inactivation for HSV-1 on ice.

The above results clearly demonstrate that arginine facilitates thermal inactivation of HSV-1. As arginine is ionic at pH 7, the effects may be simply due to the ionic strength. The effects of NaCl at 0.5–1.2 M on inactivation temperature are therefore examined and summarized in Table 1. A significant change in inactivation temperature was observed in the presence of NaCl. For example, the temperature of 50% virus inactivation was 7 °C lower in 1.2 M NaCl solution than in PBS. However, the virus inactivation was consistently greater in arginine solution than in NaCl solution at identical concentrations, indicating that the observed decrease in virus inactivation temperature is not simply due to ionic strength, although the ionic strength does contribute.

As shown above, HSV-1 showed inactivation in 1.2 M arginine solution even slightly above 20 °C within 5 min incubation, suggesting a possibility that inactivation may occur on ice, in which viruses have always been maintained for a prolonged period before and after heat treatment. As shown in Fig. 2, no significant virus inactivation was observed up to 0.75 M arginine over 60 min incubation on ice. When arginine concentration was increased to 1 M (solid circle), inactivation slowly occurred even on ice, leading to ~80% inactivation after 48 min incubation. Inactivation was further enhanced in 1.2 M arginine (open circle). Conversely, NaCl at 0.5–1.2 M showed no effect on virus inactivation on ice, although 1.2 M NaCl significantly decreased the inactivation temperature (from 47 to 40 °C for 50% inactivation). This suggests that the virus inactivation mechanism may be fundamentally different between arginine and NaCl. Virus inactivation is linear when plotted as log (relative infectivity) vs. time, indicating the first order reaction. This is expected from the most likely reaction mechanism that inactivation is a single molecule (virus particle) reaction.

3.2. Inactivation of influenza virus

A similar experiment was carried out with influenza virus, except for incubation time; i.e., 20 min for influenza virus. As shown in Fig. 3, there is little inactivation up to 45 °C in PBS, above which a sharp temperature-dependent inactivation was observed, leading to 50 and 90% reduction in relative infectivity at 51 and 54 °C. It thus appears that influenza virus is significantly more stable (about

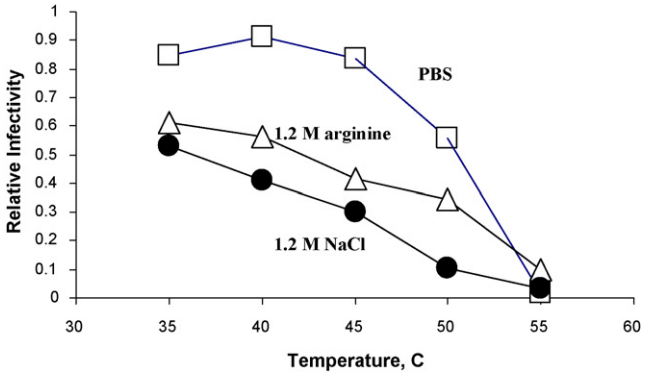

Fig. 3. Temperature dependence of virus inactivation for influenza virus in PBS and 1.2 M arginine and NaCl.

4 °C more stable) than HSV-1 against heat treatment, as HSV-1 showed 50 and 90% inactivation at 47 and 50 °C. It should be noted that even longer incubation period (20 min) was used for influenza virus than for HSV-1 (5 min). As observed for HSV-1, inactivation of influenza virus in 1.2 M arginine occurred gradually with temperature, surviving virus already being ~60% at 35 °C. The effects of 1.2 M arginine were much weaker on influenza virus than on HSV-1; temperature of 50% inactivation was ~43 °C, which was 8 °C lower than the value in PBS. The change for HSV-1 by 1.2 M arginine was 17 °C. A major difference in the presence of 1.2 M arginine between HSV-1 and influenza virus was virus inactivation at high temperature. Influenza virus was much more stable against higher temperature, as the temperature of 90% virus inactivation was 55 °C, 1 °C higher than in PBS. It thus appears that 1.2 M arginine does not enhance virus inactivation at higher temperature for influenza virus, different from the synergistic HSV-1 inactivation by arginine and temperature. Another difference between influenza virus and HSV-1 was the effect of NaCl. As shown in Fig. 2, 1.2 M NaCl was more effective in inactivation of influenza virus than was arginine. For HSV-1, arginine was more effective than NaCl. This clearly indicates that influenza virus and HSV-1 respond differently to these reagents, suggesting that these viruses are inactivated by different mechanisms: in other words, the effect of inactivation agent is virus-specific.

3.3. Comparison with protein thermal unfolding

The above temperature dependence indicates presence of a critical (threshold) temperature of virus inactivation, below which no significant inactivation occurs and above which co-operative transition of virus inactivation occurs. Such co-operative transition is reminiscent of phase transition, e.g., thermal melting of proteins and nucleic acids. We have previously characterized the effects of arginine on thermal transition of lysozyme and ribonuclease (Arakawa and Tsumoto, 2003). As summarized in Table 2, the effects are small. Arginine decreased the melting temperature of both proteins at most by 1–3 °C even in 1–2 M arginine.

Table 2
Change in melting temperature (°C).

Arginine concentration (M)	Lysozyme	RNase
0.1	0	0
0.2		−1
0.5	−1	−1
1.0	−1	−3
2.0	0	−3

However, arginine is highly effective in suppressing aggregation of proteins (Arakawa and Tsumoto, 2003), a major reason why this amino acid is used as a formulation excipient. It prevents aggregation from various stresses during storage, shipment and handling. NaCl is a protein-stabilizing agent and hence increases the melting temperature of proteins (Arakawa and Timasheff, 1982; Gouda et al., 2003; Saboury et al., 2005). Thus, these results indicate that arginine-induced inactivation of virus cannot be simply ascribed to destabilization of protein structure. Based on the results with two enveloped viruses, the effects of arginine may be due to its effects on protein–protein or protein–lipid interaction. The effects of NaCl also indicate that the inactivation is not simply due to protein conformation, as NaCl increases the melting temperature of proteins (Arakawa and Timasheff, 1982; Gouda et al., 2003; Saboury et al., 2005). As arginine or NaCl does not reduce protein stability, they may be used to enhance virus inactivation by elevated temperatures for pharmaceutical products.

Whatever the specific mechanisms involved, the present experimental results have immediate practical value in the fields of plasma fractionation and recombinant pharmaceuticals, potentially including vaccines and viral vectors for gene therapy, as well as therapeutic proteins. In addition to the protein aggregation-reducing and stability-conserving abilities of arginine and NaCl, their ability to support effective virus inactivation at reduced temperatures suggests the possibility of achieving viral safety with less stress to labile protein structures. The ability of arginine to achieve significant inactivation of HSV-1 at 0 °C provides valuable supporting data for the use of arginine in conjunction with other non-thermal inactivation methods, such as exposure to low pH, as has been demonstrated (Yamasaki et al., 2008; Katsuyama et al., 2008). It is also possible that the changes in viral structure these agents mediate may make viruses more susceptible to the effects of intercalating agents and/or treatment with UV. The differences in the relative response of HSV-1 and influenza virus to arginine and NaCl further suggest the possibility of their use to selectively inactivate contaminating virus species while perhaps leaving a viral vector product relatively unaffected. Additional studies will help to define the full scope of their potential.

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