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Butyroyl-arginine as a potent virus inactivation agent

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ABSTRACT

Virus inactivation is a critical step in the manufacturing of recombinant therapeutic proteins, in particular antibodies, using mammalian expression systems. We have shown in the previous paper that arginine is effective in inactivation of herpes simplex virus type 1 (HSV-1) and influenza virus at low temperature under mildly acidic pH, i.e., above pH 4.0; above this pH, conformational changes of most antibodies are negligible. We have here extended virus inactivation study of arginine to other enveloped viruses, such as Sendai virus and Newcastle Disease Virus (NDV), and observed that arginine was ineffective against both viruses under the similar conditions, i.e., on ice and above pH 4.0. However, an arginine derivative, butyroyl-arginine, showed a strong virucidal potency against Sendai virus, leading to a 4 log reduction in virus yield at pH 4.0, but not against NDV. In addition, although arginine and butyroyl-arginine were equally effective against influenza virus having a cleaved form of hemagglutinin spike proteins, only butyroyl-arginine was significantly effective against the same virus, but having an uncleaved hemagglutinin spike proteins. Furthermore, butyroyl-arginine was more effective than arginine against HSV-1 at pH 4.5; i.e., it has a broader pH spectrum than does arginine.

1. Introduction

Virus inactivation is one of the critical steps in the manufacturing of therapeutic proteins, including monoclonal antibodies (mAbs), using mammalian host cells. Conventionally low pH, e.g. pH ~3.5, is used to inactivate viruses (Preuss et al., 1997; Brorson et al., 2003, 2004; Shi et al., 2004; Farshid et al., 2005; Buchacher and Iberer, 2006), which can cause conformational changes in proteins, in particular antibodies (Martsef et al., 1994, 1995; Vermeer and Norde, 2000; Ejima et al., 2007). It is safer to use pH range above 4.0 for antibody stability during virus inactivation and processing. We have shown before that arginine is highly effective above this pH, even at low temperatures, in inactivating enveloped viruses, i.e., herpes simplex virus type 1 (HSV-1) and influenza viruses, but not a non-enveloped poliovirus (Yamasaki et al., 2008). Thus, virus inactivation above pH 4.0 allows the entire process of mAb production under such mildly acidic conditions, as arginine is also effective in eluting antibodies from Protein-A columns above pH 4.0 (Arakawa et al., 2004; Ejima et al., 2005). Here we have examined the effects of arginine on other enveloped viruses, i.e., Sendai virus and Newcastle Disease Virus (NDV) and two forms of influenza virus. We have used in the previous study (Yamasaki et al., 2008) only one form of influenza virus, in which hemagglutinin (HA) spike proteins have been proteolytically cleaved. Another form of influenza virus used here has uncleaved HA spike proteins and requires correct processing to become infectious. Arginine displayed marginal inactivation at or above pH 4.0 on ice against these viruses, as reported in this paper. Therefore, we sought a more effective virus inactivation agent, which may also be safe in human and animal use. Butyroyl-arginine is an arginine derivative having butyroyl-group at the amino group and hence is a zwitter ion at and above pH 4 with a positively charged guanidinium group and a negatively charged carboxyl group. This reagent displayed a much stronger virucidal potency than arginine against Sendai virus, HSV-1 and influenza virus, but not NDV. Strong virus inactivation effects of butyroyl-arginine may be due to the addition of hydrophobicity to the structure of arginine, which may have caused an enhanced binding to the viral components, e.g., spike proteins.

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2. Materials and methods

2.1. Materials

L-Arginine hydrochloride (simply described as arginine) and L-butyroyl-arginine (butyroyl-arginine) were obtained from Ajinomoto Co. Inc. Butyroyl-arginine has a modification at the amino group of arginine and hence has little net charge above pH 4.0. Aqueous solutions containing arginine and butyroyl-arginine were prepared in 20 mM acetic acid, although high concentration of these provided enough buffer action at the pH tested. The pH was adjusted to the indicated values with HCl; 20 mM acetic acid is insufficient to titrate arginine or butyroyl-arginine solutions. Aqueous citrate solution was adjusted to the indicated pH with NaOH. The pH meter was routinely calibrated using pH calibration standards. To mimic the antibody purification process, a mAb at 5 mg/ml was included in these solvents. Comparison was initially made between 0.7 M arginine, 0.7 M butyroyl-arginine and 0.1 M citrate, as these are the concentrations effective for Protein-A purification (Arakawa et al., 2004; Ejima et al., 2005). A strong salting-out property of citrate makes it difficult to use at high concentrations for mAb purification due to decreased solubility of mAb (Kaushik and Bhat, 1999).

2.2. Cells and viruses

Vero and MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 10% newborn calf serum. Herpes simplex virus type 1/strain F (HSV-1), poliovirus type 1/Sabin vaccine strain, Sendai virus/strain Z, Newcastle Disease virus/strain Miyadera (NDV) and influenza virus A/Aichi/68 (H_3N_2) were used throughout the experiments. Two forms of influenza virus were tested. While the infectious form of virus has proteolytically cleaved hemagglutinin (HA) spike proteins (here referred to as CHA-influenza virus), another form has uncleaved HA spike proteins (referred to as UCHA-influenza virus). UCHA-influenza virus requires prior proteolytic cleavage of HA proteins for the infection to cells. All the viruses except influenza virus were propagated in Vero cells in MEM supplemented with 0.5% fetal bovine serum (for poliovirus and HSV-1) or 0.1% bovine serum albumin (BSA) (for Sendai virus and NDV). For Sendai virus, acetylated trypsin (4 μg/ml) was supplemented to the culture medium. Both CHA- and UCHA-influenza viruses were propagated in MDCK cells in the medium supplemented with 0.1% BSA; acetylated trypsin (4 μg/ml) was also supplemented for the preparation of CHA-influenza virus, but not UCHA-influenza virus. 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, poliovirus, Sendai virus and NDV) or MDCK cells (for influenza virus) as described previously (Koyama and Uchida, 1989; Kurokawa et al., 1999; Koyama et al., 2001, 2003; Uozaki et al., 2007; Yamasaki et al., 2007).

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 950 μl aliquot of the solvents to be tested was placed in 1.5 ml-plastic tube on ice and received 50 μl of virus preparations (approximately 10^7 or 10^8 plaque-forming units [PFU]/ml). This was immediately followed by vigorous mixing and the sample mixture was incubated on ice for 60 min. After incubation, aliquots of these virus samples were 100-fold diluted with Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing 1% calf serum (for HSV-1 and poliovirus) or 0.1% BSA (for influenza virus, Sendai virus and NDV). 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 as described in the previous section. For the plaque assay of the UCHA-influenza virus, the virus was incubated with acetylated trypsin at 37 °C for 10 min prior to plaque assay to cleave HA spike proteins and hence activates the virus infectivity. The activated virus preparation was chilled immediately on ice and further diluted with PBS containing 0.1% BSA and subjected to plaque assay. All the experiments were done in duplicate or triplicate, as shown in the tables for some data. Others were omitted for brevity. There was little variation in the plaque assay, to the extent that the observed variation does not affect the conclusion. Virus yield was determined from the number of plaques. Log Reduction Value (LRV) was calculated by

$$\mathrm{LRV} = \log\left\{\frac{(\text{virus yield in PBS})}{(\text{virus yield in the test solvent})}\right\}$$

There was little virus inactivation in PBS and hence the virus yield in PBS was close to constant. Variation of virus yield between assays was checked with PBS and such virucidal compounds as octylgallate and coffee extracts.

Table 1
Inactivation of Sendai virus by arginine and butyroyl-arginine

Solvent	Relative virus yield	LRV
PBS control	1	
Isotonic pH 3.0 inactivation buffer[a]	0.0004	3.4
0.1 M citrate, pH 3.5	0.49/0.71	0.31/0.14
0.1 M citrate, pH 4.0	0.72	0.14
0.7 M arginine, pH 3.5	0.25	0.60
0.7 M NaCl, pH4.0	0.88/1.04	0.06/−0.02
0.35 M arginine, pH 4.0	0.85	0.07
0.7 M arginine, pH 4.0	0.73	0.14
0.7 M butyroyl-arginine, pH 4.0	0.0002/0.00018 (below detection)	3.7/3.7

[a] 40 mM citric acid, 5.0 mM KCl, 125 mM NaCl, pH 3.0 (Koyama and Uchida, 1987).

2.4. Protein-A chromatography

Cell culture media containing humanized IgG1 were loaded onto HiTrap Protein-A column. After extensive washing, the bound IgG1 was eluted with a strong acid of 0.1 M citrate, pH 2.9 and mildly acidic 0.7 M arginine and butyroyl-arginine at pH 4.0. The eluted IgG1 was analyzed by size exclusion chromatography (SEC) on a G3000SWXL column in 0.1 M Na phosphate, 0.2 M arginine, pH 6.8 at a flow rate of 0.8 ml/min.

3. Results and discussion

3.1. Sendai virus

Previously we have observed that arginine is effective in inactivating two enveloped viruses, HSV-1 (a member of herpesvirus family) and influenza virus (a member of orthomyxovirus family), but is incapable of inactivating non-enveloped poliovirus (a member of picornavirus family). Here we have extended to other viruses, Sendai virus and NDV (members of paramyxovirus family). Table 1 summarizes the results on Sendai virus; the results for the test solvents are given as a relative value to the virus yield in PBS.

A conventional buffer at pH 3.5 containing 0.1 M citrate showed marginal inactivation, the virus yield being only ∼50–70% of PBS control. As expected from the above result, 0.1 M citrate at pH 4.0 was totally ineffective. However, the isotonic pH 3.0 inactivation

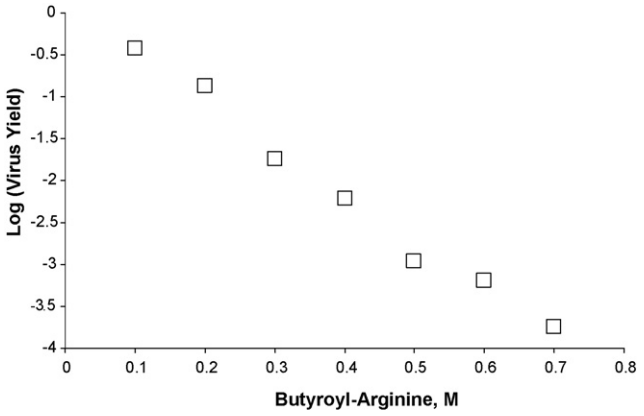

Fig. 1. Inactivation of Sendai virus at pH 4.0 as a function of butyroyl-arginine concentration.
The data at 0 M corresponds to the value in PBS, not pH 4 solution.

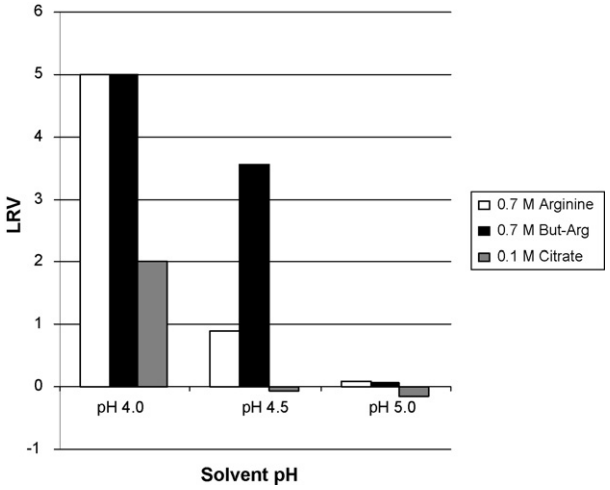

Fig. 2. Inactivation of HSV-1 by 0.1 M citrate (gray bar), 0.7 M arginine (white bar) and 0.7 M butyroyl-arginine (But-Arg, black bar) at different pH.

buffer resulted in 3 log reduction of virus yield (Table 1). Arginine was also ineffective against this virus at pH 4.0; both 0.35 and 0.7 M arginine showed virus yield close to the PBS control and that of 0.7 M NaCl (pH 4.0); thus, even high salt concentration was ineffective at this pH. At pH 3.5, 0.7 M arginine showed a slight, though marginal, inactivation effect (0.25 relative to the PBS control). On the contrary, butyroyl-arginine showed a strong virucidal potency at pH 4.0; this reagent at 0.7 M resulted in almost 4 log reduction (Table 1); in certain cases, the level of virus was undetectable. Thus, only butyroyl-arginine appears to be effective against Sendai virus among the solvents tested.

3.2. Concentration dependence

Inactivation of Sendai virus by butyroyl-arginine was examined at pH 4.0 as a function of concentration. As shown in Fig. 1, the virus inactivation potency of butyroyl-arginine was concentration dependent, leading to only 0.5 log reduction at 0.1 M. While arginine was marginally effective against Sendai virus even at 0.7 M, 0.1 M butyroyl-arginine showed a significant virus inactivation potency against this virus, indicating a potent virucidal activity of butyroyl-arginine.

3.3. pH dependence

The virus inactivation at pH 4.0 revealed superior ability of butyroyl-arginine over arginine or citrate to inactivate Sendai viruse as described above. Is this butyroyl-arginine effect universal against any viruses? We have shown before that the ability of arginine and citrate to inactivate HSV-1 rapidly diminishes as the pH of the solvent was increased (Yamasaki et al., 2008). Fig. 2 shows this trend. At pH 4.0, arginine (white bar) and butyroyl-arginine (black bar) both at 0.7 M were equally effective against HSV-1, both leading to 5 log reduction: note that they could be more potent, as the virus inactivation reached upper limit of the assay used here. At this pH, 0.1 M citrate (gray bar) only resulted in 2 log reduction and hence 1000-fold less effective than the above two solvents. At pH 4.0, increasing citrate concentration had marginal effects on virus inactivation of HSV-1 as described later. When the solvent pH was raised to 4.5, the virus inactivation potency of 0.7 M arginine decreased to less than 1 log reduction, over 10,000-fold decrease from the effect at pH 4.0. On the contrary, 0.7 M butyroyl-arginine maintained high virucidal activity, retaining the potency of ~3.5 log reduction (com-

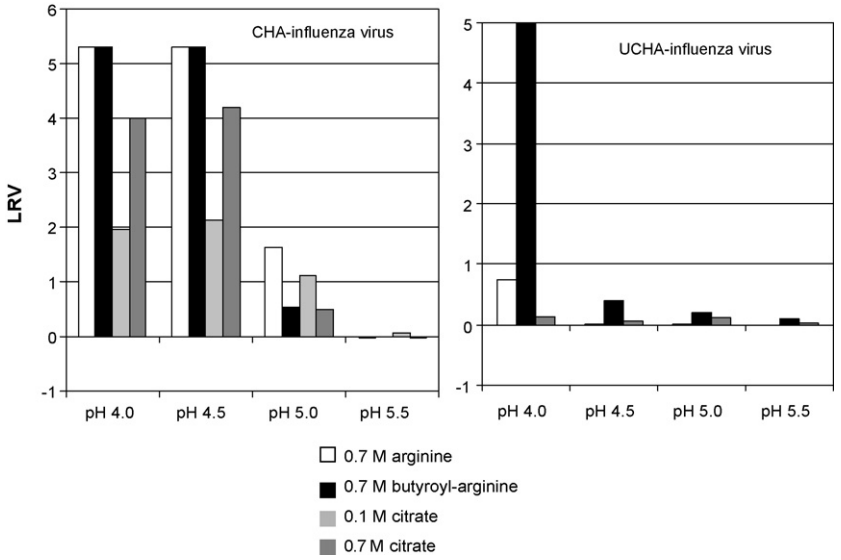

Fig. 3. Inactivation of influenza virus by 0.1 M citrate (light gray), 0.7 M citrate (dark gray), 0.7 M arginine (white) and 0.7 M butyroyl-arginine (black) at different pH. Left panel, CHA-influenza virus (cleaved HA spike proteins). Right panel, UCHA-influenza virus (uncleaved HA spike proteins).

pare with ~5 log reduction at pH 4.0). At pH 4.5, 0.1 M citrate was totally ineffective. Increasing citrate concentration to 0.7 M at pH 4.5 resulted in little change in virus inactivation effectiveness (data not shown), similar to the effect of increasing citrate concentration at pH 4.0. At pH 5.0, even butyroyl-arginine lost effectiveness. These results again demonstrate a stronger ability of butyroyl-arginine to inactivate viruses, when arginine or citrate becomes less effective at increasing pH.

The dependence on pH was also tested on influenza virus. As shown in Fig. 3 (CHA-influenza virus), both arginine (white bar) and butyroyl-arginine (black bar) at 0.7 M were highly effective at pH 4.0 and 4.5: in fact, the numbers of surviving viruses in both solvents were below the detection level. There was no apparent decrease in inactivation at pH 4.5 compared to pH 4.0. Virus inactivation was significantly weaker, i.e. >100-fold weaker, with 0.1 M citrate (gray bar) at pH 4.0 and 4.5; i.e., 0.1 M citrate also showed little pH dependence at these two pHs. However, increasing citrate concentration to 0.7 M greatly enhanced virus inactivation at both pHs (compare light and dark gray bars) to ~4 log reduction level, although still below the level of 0.7 M arginine and butyroyl-arginine. Conversely, there was little concentration dependence for arginine and butyroyl-arginine; i.e., their effects at 0.1 M were similar to those at 0.7 M as described later; i.e., these two reagents were highly effective even at 0.1 M, different from the observed lower potency of butyroyl-arginine at 0.1 M than 0.7 M against Sendai virus. As the pH was further raised to 5.0, the potency of both arginine and butyroyl-arginine at 0.7 M significantly decreased, more significantly for butyroyl-arginine (see black bar at pH 5.0). Arginine (white bar) and citrate (gray bar) showed a similar trend, namely, partial loss of potency. At pH 5.0, 0.7 M citrate was weaker than 0.1 M citrate. Thus, the order of CHA-influenza virus inactivation at pH 5.0 is 0.7 M arginine > 0.1 M citrate > 0.7 M butyroyl-arginine = 0.7 M citrate, which is different from the order at pH 4.0 and 4.5. The reason for this peculiar order at pH 5.0 is not clear, although the observed differences at pH 5.0 between different solvents are only 1 log reduction. There was little inactivation at pH 5.5. It is clear that at pH 5.0 CHA-influenza virus is more susceptible to inactivation by low pH than is HSV-1, as there is still significant inactivation for influenza virus, different from no inactivation for HSV-1 at pH 5.0 (see Fig. 2). This is consistent with the conformational changes known to occur on cleaved HA spike proteins at endosomal pH of ~5.0. Thus, a similar conformational change may occur in the test solvents at pH 5.0, leading to the observed partial inactivation of influenza virus, while the spike proteins on HSV-1 surface may be too stable to be altered at pH 5.0. The observed sensitivity of CHA-influenza virus to low pH is consistent with the flexibility of proteolytically cleaved HA proteins.

Influenza virus is known to require a proteolytic cleavage of HA spike proteins on virus particles for the infectivity, while the virus is produced from the infected cells in vitro as an uncleaved HA-form (UCHA-influenza virus). While the difference in the virucidal activity between arginine and butyroyl-arginine was small on the CHA-influenza virus as described above (Fig. 3A), a large difference was observed on UCHA-influenza virus. As shown in Fig. 3B, this virus showed strong resistance to low pH with both arginine and citrate at pH 4.0, different from the observed sensitivity of the CHA-influenza virus. Thus, these acid stable HA proteins, that have been exposed to 0.7 M arginine or 0.1 M citrate, can be correctly activated by acetylated trypsin added prior to the plaque assay (see Methods section). At pH 4.0, there was small inactivation with 0.7 M arginine (below 1 log reduction), but not much with 0.1 M citrate. Conversely, 0.7 M butyroyl-arginine exhibited 5 log reduction of the infectivity of this form of the virus, indicating a strong inactivation potency of butyroyl-arginine. This suggests that conformational changes of HA spike proteins, that may occur upon exposure to

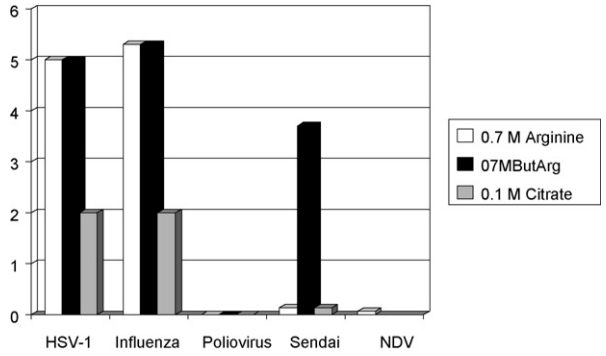

Fig. 4. Effects of 0.1 M citrate (gray), 0.7 M arginine (white) and 0.7 M butyroyl-arginine (But-Arg, black) at pH 4.0 on the inactivation of different viruses.

0.7 M butyroyl-arginine at pH 4.0, lead to either correct proteolytic cleavage but with altered conformation, or incorrect proteolytic cleavage of the HA spike proteins by acetylated trypsin added prior to plaque assay, resulting in loss of infectivity (i.e., virus inactivation). Above pH 4.5, virus inactivation by 0.7 M arginine diminished to zero, but 0.7 M butyroyl-arginine was still effective, although slightly, at pH 4.5. As expected from the marginal virucidal activities of arginine, butyroyl-arginine and citrate at pH 4.5, virus inactivation was barely detectable at pH 5.0 and 5.5, while significant virus inactivation was observed at pH 5.0 for the CHA-influenza virus, again indicating the stability of uncleaved HA spike proteins against low pH.

3.4. Virus dependence

Having established that butyroyl-arginine is superior to arginine in virus inactivation, it is important to examine its efficacy against different viruses. Fig. 4 compares the effects of citrate (0.1 M), arginine (0.7 M) and butyroyl-arginine (0.7 M) at pH 4.0 on five different viruses. Arginine and butyroyl-arginine were equally effective against HSV-1 (5 log reduction) and influenza virus (>5 log reduction). These solvents were more effective than 0.1 M citrate (~2 log reduction) on these two viruses; i.e., these reagents are about 1000-fold more active against influenza virus and HSV-1, respectively, than is 0.1 M citrate, pH 4.0. As described above, only butyroyl-arginine was active against Sendai virus. None of these solvents were effective against NDV and poliovirus. However, the isotonic pH 3.0 inactivation buffer was also ineffective against NDV (data not shown), indicating its strong resistance to low pH.

Although limited to influenza virus and HSV-1 at pH 4.0, these two viruses responded differently to the different concentration of the reagents. While Influenza virus (CHA-influenza virus) was concentration dependently inactivated by citrate (Fig. 5A), little citrate concentration dependence was observed for HSV-1, ranging from ~1.5 to 2 log reduction (Fig. 5B). Although there is no clear explanation for this difference, it may be due to different aggregation tendency between the two viruses, assuming that virus aggregation occurs at pH 4.0. Citrate is a strong salting-out salt (Kaushik and Bhat, 1999) and hence may enhance virus aggregation. The observed increased virus inactivation at higher citrate concentration for influenza virus may be due to its aggregation, not the actual virus killing, provided that HSV-1 does not aggregate even at 0.7 M citrate. Alternatively, it may be due to further destabilization of HA spike proteis of infectious influenza virus at higher citrate concentration. Conversely, the concentration effects of arginine and butyroyl-arginine were opposite between these two viruses. There was no concentration dependence of arginine and butyroyl-arginine for influenza virus; i.e., both reagents were

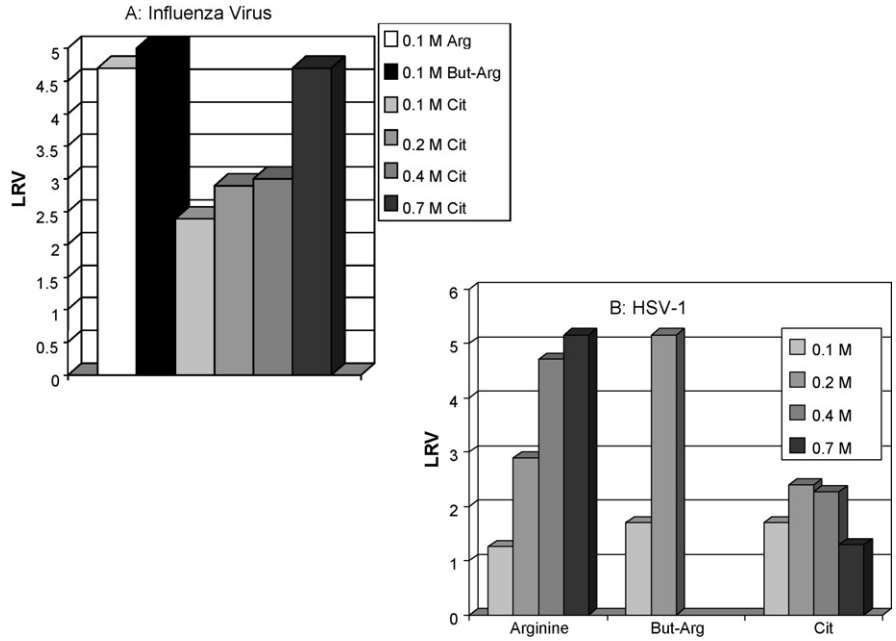

Fig. 5. Inactivation of influenza virus (CHA-influenza virus (A)) and HSV-1 (B) at pH 4.0 in the presence of arginine, butyroyl-arginine (But-Arg) and citrate (Cit). Concentrations are indicated in the figure.

nearly maximally effective at 0.1 M. It suggests that these reagents are strong enough to alter conformation of HA spike proteins even at low concentration. The HSV-1 inactivation increased concentration dependently for arginine and butyroyl-arginine; although the effect of butyroyl-arginine appeared to saturate already at 0.2 M. Thus, more stable HSV-1 requires higher concentration of arginine and butyroyl-arginine. These results suggest different charged state and the stability of the viral components, making the virus respond differently to the solvent conditions.

4. Antibody purification with butyroyl-arginine

Having established that butyroyl-arginine is highly effective in virus inactivation, we have tested its ability to elute IgG1 from Protein-A columns. The recovery of IgG1 by different solvents is shown in Fig. 6 as percentage to the recovery with 0.1 M citrate, pH 2.9. Due to the strong acidity, 0.1 M citrate (panel A) was more effective than 0.7 M arginine, pH 4.0 (B). Conversely, 0.7 M butyroyl-arginine at pH 4.0 (C) showed greater recovery than 0.1 M citrate,

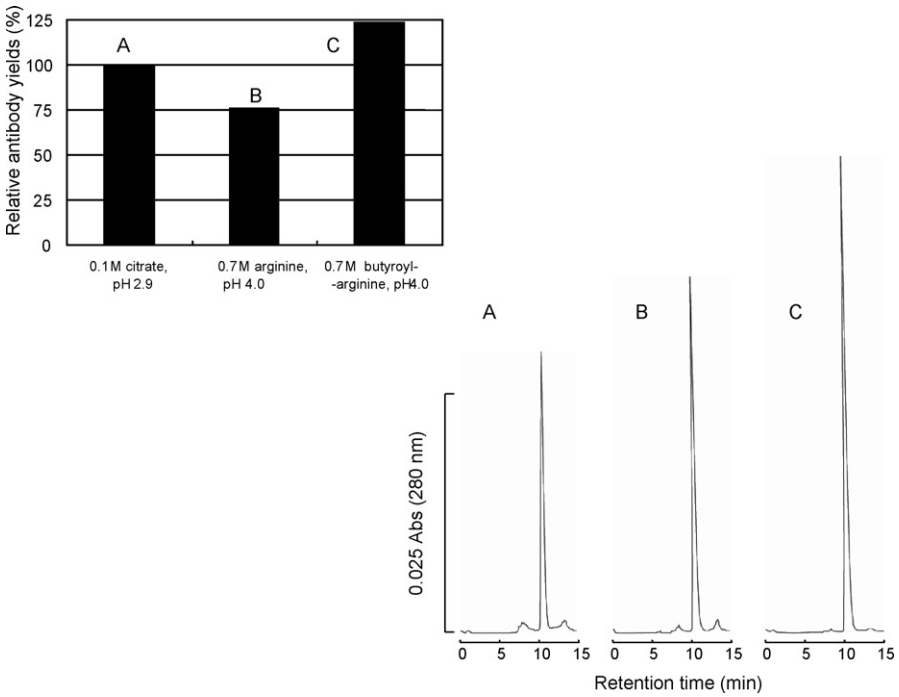

Fig. 6. Protein-A chromatography and SEC analysis of IgG1.
Recovery of IgG1 from Protein-A column is shown as percentage to the recovery with 0.1 M citrate, pH 2.9.
SEC analysis of IgG1 eluted with 0.1 M citrate, pH 2.9 (A), 0.7 M arginine, pH 4.0 (B) and 0.7 M butyroyl-arginine (C).

pH 2.9, indicating its strong dissociating capability on IgG1 binding to the Protein-A. SEC analysis showed presence of aggregates in the 0.1 M citrate eluted material (panel A), while aggregation was much less in 0.7 M butyroyl-arginine (C). There was also no apparent difference in low molecular weight species in these eluted materials, suggesting that there is no adverse effect of butyroyl-arginine on IgG1. Thus, it appears that elution with butyroyl-arginine reduces aggregation of IgG1 and is unlikely that incubation of antibodies with this solvent on ice for 60 min would alter the integrity of the antibodies.

The observed virus inactivation by arginine and in particular butyroyl-arginine at mildly acidic pH (i.e., above, 4.0) means that antibodies will never be exposed to a pH of ~3.5 or below, a normally used pH for virus inactivation, since with these reagents antibodies can be eluted from Protein-A columns as shown above. This should minimize acid induced conformational changes, which often result in aggregation, of pharmaceutical antibodies.

5. Mechanism of arginine and butyroyl-arginine effects

Virus inactivation is one of the most critical processes for production of therapeutic proteins using mammalian host cell expressions. Low pH is generally used to inactivate viruses for mAB processing, indicating the contribution of charges on the virus to the virus stability. Virus inactivation was almost identical at pH 4.0 and 4.5 and still significant at pH 5.0 for CHA-influenza virus (Fig. 3), while inactivation is enhanced at pH 4.0 for HSV-1 (Fig. 2). This suggests a critical contribution of titration group(s) between pH 4.5 and 5.0 to the stability of influenza virus, while titration involved in HSV-1 inactivation continues to pH 4.0. Such difference in pH dependence of virus stability should be due to different virus structure between HSV-1 and influenza virus, most likely due to the instability of cleaved HA spike proteins of influenza virus as described above. Response to ionic strength is also different between these two viruses; i.e., increase in citrate concentration made no difference in HSV-1 inactivation, but strongly enhanced influenza virus inactivation, perhaps consistent with their different charged state or stability of spike proteins involved in virus inactivation. Independent of the virus structure, both arginine and butyroyl-arginine are more effective in virus inactivation. Recently, protein denaturants have been shown effective in virus inactivation (Roberts and Lloyd, 2007). However, protein denaturants are useless as they damage proteins. We have shown that arginine, which is not a protein denaturant, is highly effective in virus inactivation even at mildly acidic pH (>pH 4.0) (Yamasaki et al., 2008). Here we have shown that butyrol-arginine is superior to arginine, as it has a broader spectrum of virus inactivation activities, i.e., broader effective pH and less selectivity on different viruses. Why is butyroly-arginine stronger? Unlike arginine, whose solution properties have been relatively well characterized (Kita et al., 1994; Lin and Timasheff, 1996; Shiraki et al., 2002; Ho et al., 2003; Arakawa and Tsumoto, 2003; Tsumoto et al., 2003, 2004, 2005; Ishibashi et al., 2005; Reddy et al., 2005; Umetsu et al., 2005; Arakawa et al., 2006; Tsumoto et al., 2007), little information is available on butyroyl-arginine. As described in the introduction, butyroyl-arginine is a zwitter ion and has almost no net charges at pH 4.0. This should make butyroyl-arginine less hydrophilic than arginine, which has at least one net positive charge at pH 4.0. Such hydrophobic nature may add to the ability of arginine to inactivate viruses.

Before analyzing the mechanism of butyroyl-arginine effect, it may be informative to summarize the information on the ability of arginine to inactivate viruses. Arginine has been shown to suppress aggregation of proteins and protein-surface interactions (Shiraki et al., 2002; Ho et al., 2003; Arakawa and Tsumoto, 2003; Tsumoto et al., 2003, 2004, 2005, 2007; Ishibashi et al., 2005; Reddy et al., 2005; Umetsu et al., 2005; Arakawa et al., 2006, 2007). More relevant data may be the ability of arginine to solubilize insoluble proteins trapped in loosely formed protein aggregates, as soft inclusion bodies (Tsumoto et al., 2003; Umetsu et al., 2005). These effects of arginine are due to weak ability of arginine to bind to the proteins (Kita et al., 1994; Lin and Timasheff, 1996). Arginine is also known to bind to membranes (Futaki, 2005). Such binding of arginine may be responsible for inactivation of viruses, although the precise mechanism of arginine binding and resultant virus inactivation is to be elucidated. Enhanced hydrophobic property of butyroyl-arginine may add additional binding strength to the ability of arginine to bind the viruses. This enhanced hydrophobic property appears to play a key role in inactivation of UCHA-influenza virus, as butyroyl-arginine was the only effective reagent among the conditions tested. Resultant enhanced binding of butyroyl-arginine to the virus surface may have caused HA spike proteins to assume non-native conformation or non-native interactions at pH 4.0 (not at pH 4.5, as seen Fig. 3, right panel), leading to incorrect conformation or processing of HA by trypsin and hence loss of the UCHA-influenza virus to infect.

As described above, butyroyl-arginine has no net change near neutral pH, while arginine is a mono-valent cation. When the protein solution after virus inactivation needs to be further processed, e.g., by ion exchange chromatography, butyroyl-arginine has an advantage over arginine, as there is little increase in ionic strength with butyroyl-arginine, which renders protein binding to the resin more straightforward. It is evident that more studies are required for butyroyl-arginine with regard to its interaction with proteins and the effects on protein stability and viruses or cells. As described in the previous section, we have limited data on the effects of butyroyl-arginine on antibodies and hence more studies are underway.

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