

Virus inactivation by protein denaturants used in affinity chromatography

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Abstract

Virus inactivation by a number of protein denaturants commonly used in gel affinity chromatography for protein elution and gel recycling has been investigated. The enveloped viruses Sindbis, herpes simplex-1 and vaccinia, and the non-enveloped virus polio-1 were effectively inactivated by 0.5 M sodium hydroxide, 6 M guanidinium thiocyanate, 8 M urea and 70% ethanol. However, pH 2.6, 3 M sodium thiocyanate, 6 M guanidinium chloride and 20% ethanol, while effectively inactivating the enveloped viruses, did not inactivate polio-1. These studies demonstrate that protein denaturants are generally effective for virus inactivation but with the limitation that only some may inactivate non-enveloped viruses. The use of protein denaturants, together with virus reduction steps in the manufacturing process should ensure that viral cross contamination between manufacturing batches of therapeutic biological products is prevented and the safety of the product ensured.

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1. Introduction

Of potential concern in therapeutic products derived from biological materials is the possible presence of pathogenic viruses. For instance, in the case of cell-derived products such as monoclonal antibodies [1–4], viruses such as endogenous retroviruses or minute virus of mice may be present. In order to prevent this, testing is carried out on the start material and/or cell substrate as well as process intermediates and final products as appropriate. In addition, proven virus inactivation/removal steps [5], such as those used for plasma products [6], are increasingly being incorporated into the manufacturing processes of cell-derived products [3]. Cross contamination between different manufacturing batches is one route by which virus contamination can potentially occur and which can be prevented by the use of effective segregation and decontamination methods.

A number of protein denaturants and chaotropic agents are used for the elution of purified proteins from chromatographic

gels or for cleaning prior to reuse or for storage. While many of these agents are able to remove protein contaminants and thus to contribute to decontamination by this method, they also have the ability to inactivate viruses.

In the present study, the virus inactivation capabilities of a number of protein denaturants, particularly those used with affinity chromatography media such as Protein-A or -G have been evaluated. They have been tested under similar conditions to those commonly used at laboratory or industrial scale. Sodium hydroxide, a particularly severe denaturing agent and also known for its virus inactivating properties has also been included in the study for comparative purposes.

2. Materials and methods

2.1. Viruses

Vaccinia, Sindbis and herpes simplex virus type 1 (HSV-1), were propagated in BHK-21 cells grown in minimal essential medium (MEM) with 5% newborn-calf serum. Polio-1 virus was propagated in Vero cells maintained in medium 199 with 4% newborn-calf serum and 1% foetal calf serum.

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Virus-containing cell supernatants or cell extracts were harvested from infected cultures and, after clarification by centrifugation and/or filtration, stored at -70°C before use.

2.2. Protein denaturants

The acidic pH 2.6 buffer was prepared as either 0.2 M acetate or 0.2 M phosphate/citrate buffer. All the chemicals were prepared in water except 6 M guanidinium chloride, which used 20 mM phosphate buffer pH 5.0 and 20% ethanol, which was prepared in 50 mM acetate buffer pH 4.5.

2.3. Effects on the virus assays

Control experiments were carried out in order to test the effect of the test chemicals on the cell-culture and infectivity assay for each virus. Initially tests were carried out by using a dilution series of 10^{-1} to 10^{-4} for each test chemical in phosphate buffered saline (PBS) and adding this to cell monolayers of each cell type used in the virus assays. From these initial experiments, appropriate dilutions for testing the effect on the virus infectivity assay were selected (Table 1). Serial dilutions of virus were made in the test chemical dilutions or a PBS control. These were then assayed using the standard procedures described below. Any effect of the test chemical on the cells or cell monolayer, plaque morphology or virus titre compared to the controls were noted.

2.4. Inactivation studies

Virus, at a dilution of 1/10 or greater, was added to the test chemical and incubated at 22°C . Samples were removed at

various time points and diluted to an appropriate non-cytotoxic dilution i.e. 10^{-1} to 10^{-4} in cell-culture medium depending on the virus and chemical involved (as determined in Section 2.3 above). All samples were assayed, together with a control consisting of virus in cell-culture medium, as described below.

2.5. Virus assays

Virus infectivity was determined by plaque assay using monolayers of Vero (polio-1) or BHK-21 (Sindbis, Vaccinia and HSV-1) cells. Where necessary, samples were adjusted to neutral pH before assay. Serial log dilutions of virus were prepared in phosphate buffered saline and a volume of 0.5 ml added to the cells. However, in order to increase the sensitivity of virus detection in treated samples, larger volumes i.e. 7 ml, of the least diluted samples were assayed where appropriate. After a 45 min incubation period an overlay of 0.4% high-viscosity CMC was added. The minimum dilution of each chemical shown to have no cytotoxic effect on the assay was used as the starting dilution for the virus assay.

Virus titres were calculated from sample dilution, assay volume and plaque number. The variation between individual assays was about 0.5 log/ml. Where virus was undetectable, the titre was calculated assuming one plaque was present in the total volume assayed and expressed as a 'less than' value. Virus reduction values were calculated by subtracting log virus titres after treatment from that determined for the untreated control.

3. Results

3.1. Control studies

The effect of the various protein denaturants on the assays used for determining virus infectivity was first tested (Table 1). As might be expected, several of the chemicals proved to be extremely toxic to the cells used for the virus assays and/or to the assay detection system itself. In particular the 6 M guanidinium chloride and 6 M guanidinium thiocyanate were very toxic. The magnitude of the effect was also dependent on the virus involved. For instance guanidinium chloride inhibited the polio-1 virus assay while guanidinium thiocyanate inhibited the Sindbis virus assay. These experiments allowed the minimum dilution for each chemical that had no significant toxic or inhibitory effect on the virus assay to be determined. For each chemical/virus combination the minimum dilution that had no significant effect was used as the start dilution when assaying each particular virus.

3.2. Low pH

The inactivation of a range of viruses by low pH conditions of pH 2.6 is given in Table 2. The enveloped viruses Sindbis and HSV-1 were effectively inactivated i.e. by >4 log. However, there was almost no i.e. <1 log inactivation of the non-enveloped virus polio-1.

Table 1
Control studies on the effect of protein denaturants on virus assays

Chemical	Dilution ^b	Change in virus titre (log) ^a			
		Sindbis	HSV-1	Vaccinia	Polio-1
NaSCN	10^{-1}	T	T	T	T
	10^{-2}	-0.1	-0.2	-0.1	+0.1
0.5 M NaOH	10^{-1}	+0.1	+0.2	+0.3	+0.1
6 M GdCl	10^{-1}	T	T	T	T
	10^{-2}	-0.1	0.0	-0.2	T or -0.5
	10^{-3}	+0.1	0.0	-0.2	+0.1
6 M GdSCN	10^{-2}	T	T	T	T
	10^{-3}	-4.8	-0.1	-0.3	-0.1
	10^{-4}	+0.1	-0.4	+0.2	-0.4
8 M Urea	10^{-1}	+0.4	-0.5	-0.3	-0.8
	10^{-2}	+0.1	0.0	-0.1	+0.1
20% EtOH	10^{-1}	+0.1	-0.2	+0.1	-0.2
70% EtOH	10^{-1}	+0.2	-0.3	+0.1	+0.3

Abbreviations used: NaSCN, sodium thiocyanate; NaOH, sodium hydroxide; GdCl, guanidinium chloride; GdSCN, guanidinium thiocyanate; EtOH, ethanol. T, cell toxicity was seen.

^a Positive values indicate a virus titre increase and negative values a decrease.

^b Samples were adjusted to neutral pH where necessary before assay.

Table 2
Virus inactivation by pH 2.6

Virus	Virus inactivation (log)		
	5 min	30 min	40 min
Sindbis	3.3	nd	4.4
HSV-1	5.4	>6.0	nd
Polio-1	0.5	0.3	0.7

nd, not determined.

3.3. Sodium thiocyanate

Virus inactivation by 3 M sodium thiocyanate is shown in Table 3. Both vaccinia and HSV-1 were effectively inactivated and this occurred within 1 min in the case of HSV-1. However, Sindbis and polio-1 proved to be relatively resistant.

3.4. Sodium hydroxide

All four viruses tested proved to be susceptible to inactivation by 0.5 M sodium hydroxide (Table 4). Inactivation was extremely rapid in all cases with high levels of inactivation occurring within 1–2 min.

3.5. Guanidinium chloride

Sindbis, HSV-1 and vaccinia virus proved susceptible to inactivation by 6 M guanidinium chloride (Table 5), however, polio-1 virus was completely resistant. Inactivation was particularly rapid in the case of HSV-1.

3.6. Guanidinium thiocyanate

All the viruses tested, including polio-1, were inactivated by 6 M guanidinium thiocyanate (Table 6). Virus inactivation was very rapid and occurred within 1 min in all cases.

3.7. Urea

Sindbis, HSV-1, vaccinia and also polio-1 virus, were all inactivated by 8 M urea (Table 7). Inactivation occurred most rapidly with HSV-1 and vaccinia virus. In the case of polio-1, the inactivation kinetics were particularly distinctive with no inactivation at all occurring during the first 10 min of the incubation period, but the virus suddenly became undetectable after 30 min.

Table 3
Virus inactivation by 3 M sodium thiocyanate

Virus	Virus inactivation (log)						
	1 min	2 min	5 min	10 min	30 min	1 h	2 h
Sindbis	0.0	0.3	0.2	1.3	1.4	1.6	2.4
HSV-1	>6.0	>6.0	>6.0	nd	nd	nd	nd
Vaccinia	1.2	1.5	2.3	3.1	5.0	5.4	6.1
Polio-1	0.0	0.0	0.2	0.0	0.3	1.4	3.2

nd, not determined.

Table 4
Virus inactivation by 0.5 M sodium hydroxide

Virus	Virus inactivation (log)						
	1 min	2 min	5 min	10 min	30 min	1 h	2 h
Sindbis	4.8	4.9	nd	5.0	5.1	5.6	nd
HSV-1	>6.5	>5.8	>6.5	>6.4	>6.5	>6.5	nd
Vaccinia	2.6	6.0	nd	6.3	6.4	>6.8	nd
Polio-1	4.9	nd	nd	4.5	4.1	4.1	4.5

nd, not determined.

Table 5
Virus inactivation by 6 M guanidinium chloride

Virus	Virus inactivation (log)						
	1 min	2 min	5 min	10 min	30 min	1 h	2 h
Sindbis	3.4	nd	3.3	4.2	4.3	4.4	>5.4
HSV-1	5.0	5.7	nd	>6.3	>6.3	nd	nd
Vaccinia	nd	1.5	nd	4.9	4.6	5.1	>5.5
Polio-1	nd	nd	nd	-0.1	-0.1	0.0	0.0

nd, not determined.

Table 6
Virus inactivation by 6 M guanidinium thiocyanate

Virus	Virus inactivation (log)						
	1 min	2 min	5 min	10 min	30 min	1 h	2 h
Sindbis	4.9	5.0	5.2	5.4	5.9	>6.2	nd
HSV-1	>4.8	>4.8	>4.8	>4.8	>4.8	>4.8	>4.8
Vaccinia	>5.4	>5.4	>5.4	nd	nd	nd	nd
Polio-1	4.1	>4.1	5.1	>4.1	>4.1	>4.1	nd

nd, not determined.

Table 7
Virus inactivation by 8 M urea

Virus	Virus inactivation (log)							
	1 min	2 min	5 min	10 min	30 min	1 h	2 h	5 h
Sindbis	nd	2.0	2.7	4.8	>4.8	>5.5	nd	nd
HSV-1	nd	>6.4	>6.4	>6.4	>7.1	nd	nd	nd
Vaccinia	3.5	4.9	>5.6	>5.6	>5.6	nd	nd	nd
Polio-1	0.1	0.0	0.0	0.0	4.3	4.3	>4.7	>4.7

nd, not determined.

Table 8
Virus inactivation by 20% ethanol

Virus	Virus inactivation (log)					
	2 min	10 min	30 min	1 h	2 h	5 h
Sindbis	2.8	4.7	5.5	6.4	nd	nd
HSV-1	3.2	>7.0	>7.0	>7.0	nd	nd
Vaccinia	1.7	3.1	6.6	>7.9	nd	nd
Polio-1	nd	0.1	0.1	0.2	0.2	0.2 ^a

nd, not determined.

^a No inactivation seen after 2 days.

Table 9
Virus inactivation by 70% ethanol

Virus	Virus inactivation (log)						
	1 min	2 min	5 min	10 min	30 min	1 h	2 h
Sindbis	>8.1	>7.4	>7.4	nd	>5.8	nd	>7.4
HSV-1	>7.8	>7.1	>7.3	nd	nd	nd	nd
Vaccinia	>8.6	>5.6	>7.6	>7.6	>5.6	>5.6	>5.6
Polio-1	1.6	2.9	3.3	3.2	3.7	4.1	3.6

nd, not determined.

3.8. Ethanol

Virus inactivation by 20% and 70% ethanol is shown in Tables 8 and 9, respectively. The three enveloped viruses were effectively inactivated by 20% ethanol. However, polio-1 virus was completely resistant even when very long incubation periods of up to 5 h were used. The use of a higher ethanol concentration i.e. 70% increased the rate of virus inactivation so that all three of the enveloped viruses became undetectable after 1 min. Polio-1 was inactivated at this higher ethanol concentration although inactivation was slower and only partially effective. About 3–4 log of virus was inactivated after about 5 min but this did not significantly increase on further incubation.

4. Conclusion

The results of virus inactivation studies using the various chemical denaturants, based on a 30 min incubation period, are summarised in Table 10. Sodium thiocyanate at a concentration of 3 M and low pH are two methods that are commonly used for eluting proteins from affinity gels such as those based on Protein-A or -G. However, whilst these conditions inactivated the tested enveloped viruses, they had no effect on the non-enveloped polio-1 virus.

Sodium hydroxide at a concentration of 0.5 M proved to be more effective at inactivating viruses including the non-enveloped polio-1 virus. This agent is widely used for cleaning and removing microbial contamination from chromatographic media [7] and is thus something of a benchmark against which to compare other methods. However, this denaturant is generally considered too severe for repeated use with Protein-A or -G resins. For this reason other milder agents such as 6 M guanidinium chloride, 6 M guanidinium thiocyanate or 8 M urea are commonly used. These three agents proved to be essentially as effective as sodium hydroxide for inactivating all the viruses tested although inactivation by 8 M urea or 6 M guanidinium chloride was slower. In addition, 6 M guanidinium chloride was not effective against the non-enveloped polio-1 virus.

Ethanol is very widely used as a general antimicrobial agent. It is generally used at a concentration of 70–80% in water to ensure that it is fully effective. In the current study it was found that ethanol, even at the relatively low concentration of 20%, commonly used for the storage of gel media, was

able to inactivate the enveloped viruses tested but not polio-1. However, increasing the concentration to 70% did ensure that polio-1 was inactivated.

The results of the current study support the contribution of commonly used protein denaturants for the viral decontamination of chromatographic gels. Using the optimum elution, cleaning/sanitisation and storage regimes, virus inactivation of at least 15 log is possible for the less resistant enveloped viruses. However, the figure is likely to be much lower for highly resistant non-enveloped viruses where inactivation of ca. 5 log is more likely to occur. These figures are conservative estimates of the total reduction value as many of these chemicals are also powerful cleaning agents and thus will, in addition, remove viruses by this mechanism. Further inactivation studies with any specific viruses e.g. murine retroviruses, minute virus of mice may be needed where these are specific concerns for a particular product. In addition, testing of a wider panel of viruses will further confirm the effectiveness of any particular agent. While the present study has concentrated on single chemicals, a combination of two or more denaturants at a lower concentration may be beneficial. There is some evidence that such an approach may have a less detrimental effect on the quality of the gel [8,9]. Other issues such as changes in the performance of the chromatographic gel, both with regard to product characteristics and contribution to virus removal upon repeated treatment with the denaturants, also need to be considered [10–12]. Critically, the chromatographic medium and equipment must be completely exposed to the denaturant if the benefits of cleaning/virus inactivation are to be fully realised.

Sodium hydroxide at 0.1–1 M is generally considered an effective sanitisation method and is widely used. While, in general, affinity gels such as Protein-A and -G cannot withstand such conditions, a new gel with an engineered Protein-A has recently been described which can withstand treatment with 0.5 M NaOH [11,13]. Other new variants of these ligands are being developed [14,15]. A number of the denaturants tested here appear to be equally effective as NaOH for virus inactivation. Also the inclusion of dedicated virus inactivation or removal steps in the manufacturing process, will further reduce the viral risks. With the continuing development of new ligands it is likely that even more severe decontamination methods for chromatography gels will be possible in the future.

Table 10
Summary of virus inactivation by chemical denaturants

Virus	Virus inactivation (log) at 30 min							
	Chemical							
	pH 2.6	3 M NaSCN	0.5 M NaOH	6 M GdCl	6 M GnSCN	8 M Urea	20% EtOH	70% EtOH
Sindbis	3.3–4.4	1.4	5.1	4.3	5.7	>4.8	5.5	>5.8
HSV-1	>6.0	>6.0	>6.5	>6.3	>4.8	>7.1	>7.0	>7.3
Vaccinia	nd	5.0	6.4	4.6	>5.4	>5.6	6.6	>5.6
Polio-1	0.3	0.3	4.1	0.0	>4.1	4.3	0.1	3.7

nd, not determined.

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