Japanese Journal of Veterinary Research 58(3&4): 179–183, 2010

NOTE

Inactivation strategy for pseudorabies virus in milk for production of biopharmaceuticals

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Received for publication, May 7, 2010; accepted, July 6, 2010

Abstract

By selecting pseudorabies virus (PrV) as a model virus, this study assessed the feasibility of applying viral inactivation strategies to manufacturing medicinal products from the milk of transgenic sows. The efficacy of heat, acidic/alkaline and detergent treatments was also evaluated with respect to their ability to inactivate PrV in milk samples. Experimental results indicate that PrV was inactivated obviously at least 7.125 $\log_{10}$ for 30 min at 60°C. At alkaline values of pH 10 and acidic value of pH 4, PrV infectivity was reduced to 3.625 $\log_{10}$ and exceeded 5 $\log_{10}$, respectively. Moreover, PrV virus was inactivated efficiently ($>$ 3.875 $\log_{10}$) by using 0.25 ～ 1% of Triton X-100 treatment and without a loss of biological activity of the recombinant human coagulation factor IX (rhFIX). Results of this study demonstrate the effectiveness of the proposed detergent inactivation method for PrV inactivation of rhFIX production from transgenic products, especially in milk materials.

Keywords: Biopharmaceuticals, milk, virus inactivation

Given the extensive use of biotech products derived from transgenic livestock in clinical and nonclinical research[17], the potential introduction of viral contaminants has become a major parameter for determining the safety of biological products produced from cell cultures or animal tissues[2]. Although the efficacy of virus inactivation strategies in the production of dairy products[3,14] and plasma-derived medicinal products[11,16] has been investigated, milk-derived therapeutic protein has seldom been addressed.

Although capable of eliminating infectious agents for bioactive compounds, extreme chemical and physical conditions may destroy protein structures[10]. An effective means of achieving viral safety for protein pharmaceuticals involves removing or inactivating a model virus during the downstream purification process[2,7]. Pseudorabies virus (PrV) is the aetiological agent of Aujeszky's disease in swine[9]. Infection of non-natural hosts causesneurological symptoms, often proving fatal[12]. PrV has also been applied as a surrogate for the hepatitis B virus (HBV), human herpes virus (HSV) and Epstein-Barr

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virus (EBV) in many viral inactivation studies[1,4]. Despite the extensive attention paid to the inactivation of PrV, the inactivation kinetics of PrV in milk samples has not been examined.

Human coagulation factor IX has been prescribed to treat type B hemophiliacs for three decades[1]. The Animal Technology Institute Taiwan (ATIT) has developed transgenic pigs to produce the recombinant human coagulation factor IX (rhFIX), which carries dual transgenes that combine porcine lactoferrin with human coagulation factor IX. In addition, rhFIX was expressed and secreted in the milk of lactating transgenic pigs. Skim milk stocks were further administered for rhFIX purification following downstream manufacturing processes such as Q-Sepharose FF chromatography, Heparin-Sepharose chromatography, IgA affinity column, and nanofiltration. To minimize the risk of rhFIX from transgenic pigs, PrV was selected as a model virus; its inactivation kinetics and the stability of rhFIX activity were also examined in transgenic sow's milk.

Pseudorabies virus was obtained from the Division of Animal Medicine, Animal Technology Institute, Taiwan (ATIT-PrV). The ATIT-PrV virus was inoculated into LLC-RK1 cells (CCRC 60329, rabbit kidney cells) and maintained in a M199 medium with 10% horse serum. Following 72 hr of cultivation, the viral harvest was clarified by centrifugation at 2,000 rpm for 10 min. Finally, the ATIT-PrV master stocks and working stocks were stored in a liquid nitrogen tank and in a freezer set at −80°C, respectively.

The efficacy of heat, acidic/alkaline and detergent treatments was evaluated for their ability to inactivate PrV in milk samples. Thermal inactivation was performed at 4, 22, 37 and 60°C, followed by analysis of the kinetics of virus inactivation at 60°C. The ATIT-PrV viral stock was spiked into various media with pH values of 4.0, 6.0, 8.0 and 10.0 to assess the effect of virus inactivation. Various detergent solutions (1% Tween 20, 1% Tween 80 and 1% Triton X-100) were prepared in 50 mM Tris-HCl, pH 7.2. A 10^8 $TCID_{50}$/mL quantity of ATIT-PrV was added to different sterile-filtrated formulations. Virus infectivity was quantified by estimating 50% of tissue culture infectious dose ($TCID_{50}$) based on use of standard cell culture conditions[6].

Next, an attempt was made to select the most appropriate strategy for viral inactivation of rhFIX derived from transgenic sow's milk by assessing the feasibility of the inactivation treatment and the stability of rhFIX activity simultaneously. The starting material (skim milk) was then adjusted to acid condition (pH 4.0), alkaline condition (pH 10.0) or neutral condition (pH 7.2) containing 0.25% ～ 1% of Triton X-100. The ATIT-PrV viral stock (one-tenths of total volume) was spiked into various treatments and incubated at 4°C for 1 ～ 4 hr. Samples with and without virus were removed periodically for virus titration and rhFIX activity assay. Finally, the procoagulation activity of the recombinant hFIX was tested by the activated partial thromboplastin time (aPTT) using the fibrometer method[13].

Table 1 summarizes the effect of temperature, pH values and various detergent concentration on the inactivation kinetic of ATIT-PrV. Inactivation of ATIT-PrV at 60°C over 30 min revealed a mean reduction of 7 $\log_{10}$ in virus infectivity. These experimental results correspond to those of other studies, indicating that ATIT-PrV is less resistant to heat inactivation than other porcine viruses[8]. Since there is no inactivation of porcine parvovirus[5] or porcine circovirus[18] was seen after 15 minutes at 70°C. The ATIT-PrV stabilized in a mildly acidic and neutral condition. At alkaline value of pH 10 and acidic value of pH 4, ATIT-PrV infectivity was reduced to 3.625 $\log_{10}$ and exceeded 5 $\log_{10}$ respectively after 4 hr of treatment. According to our results, viral inactivation using 1% Tween 20 and 1% Tween 80 treatment was ineffective whereas a significant reduction ($>$ 3.833 $\log_{10}$) was achieved after 4 hr of treatment with 1% Triton X-100.

Table 1. The Effects of the temperature, pH values and various detergent concentrations on the inactivation kinetics of PrV

Treatment	Virus titer (log_{10} $TCID_{50}$/mL)	LRV
4°C / 48 hr	8.000 ± 0.000	0.125
22°C / 48 hr	7.750 ± 0.000	0.375
37°C / 48 hr	7.813 ± 0.088	0.312
60°C / 30 min	ND	> 7.125
pH 4.0 / 4 hr	ND	> 5.000
pH 6.0 / 4 hr	5.563 ± 0.265	0.437
pH 8.0 / 4 hr	5.563 ± 0.265	0.437
pH 10.0 / 4 hr	2.375 ± 0.000	3.625
Buffer only / 4 hr	6.917 ± 0.144	− 0.084
1% Tween 20 / 4 hr	6.583 ± 0.191	0.250
1% Tween 80 / 4 hr	6.500 ± 0.331	0.333
1% Triton X-100 / 4 hr	ND	> 3.833

LRV: log_{10} reduction value.
ND: no detectable viruses were found in the solution.
Buffer: 50 mM Tris-HCl, pH 7.2.

Table 2. ATIT-PrV inactivation for the raw milk harvest containing rhFIX

Treatment	Factor IX activity		Virus titer	
	IU/mL	Reduction rate (%)	log_{10} $TCID_{50}$/mL	LRV
Initial titer	92.91	--	6.511 ± 0.088	--
1% Triton X-100 / 4 hr	91.63	1.38	ND	> 3.511
acid (pH 4.0) / 4 hr	4.61	95.04	4.136 ± 0.442	2.375
alkaline (pH 10.0) / 4 hr	36.60	60.61	4.636 ± 0.088	1.875
60°C / 0 min	23.39	--	7.917 ± 0.144	--
60°C / 5 min	0.57	97.56	3.208 ± 0.473	4.708
60°C / 10 min	0.16	99.32	ND	> 6.917

Reduction rate (%) = (Starting − Ending) ÷ Starting × 100%.
LRV: log_{10} reduction value.
ND: no detectable viruses were found in the solution.

Table 2 shows the results of ATIT-PrV inactivation for the raw milk harvest containing rhFIX. ATIT-PrV is inactivated by a pH 4.0 condition (LRV = 2.375), a pH 10 condition (LRV = 1.875) and 10 min treatment at 60°C (LRV > 6.917) with a significant loss of rhFIX activity. In terms of the requirements of bioprocessing conditions, virus inactivation and the maintenance of protein activity must be balanced carefully[2). Thus, thermal and acid/alkaline treatments are infeasible for milk harvesting owing to its time consuming and destructive features. The PrV infectivity was significantly reduced (> 3.511 log_{10}), but without a significant loss of biological activity of the rhFIX in milk by using detergent treatment (1% Triton X-100).

Table 3 summarizes the results from various concentrations of Triton X-100 treatment. ATIT-PrV spiked sow's milk with 0.25%, 0.5% and 1% of Triton X-100 for 1 ∼ 4 hr resulted in

Table 3. Viral reduction values and rhFIX activity after treated with Triton X-100 for various time periods

Treatment	Factor IX activity (IU/mL)	Virus titer ($\log_{10}$ $TCID_{50}$/mL)	LRV
Initial titer	76.70	6.875 ± 0.177	--
0.25% Triton X-100 / 1.0 hr	81.39	ND	$> 3.875 \pm 0.177$
0.25% Triton X-100 / 2.0 hr	77.55	ND	$> 3.875 \pm 0.177$
0.25% Triton X-100 / 4.0 hr	76.27	ND	$> 3.875 \pm 0.177$
0.5% Triton X-100 / 1.0 hr	73.71	ND	$> 3.875 \pm 0.177$
0.5% Triton X-100 / 2.0 hr	89.07	ND	$> 3.875 \pm 0.177$
0.5% Triton X-100 / 4.0 hr	90.35	ND	$> 3.875 \pm 0.177$
1% Triton X-100 / 1.0 hr	92.91	ND	$> 3.875 \pm 0.177$
1% Triton X-100 / 2.0 hr	82.67	ND	$> 3.875 \pm 0.177$
1% Triton X-100 / 4.0 hr	91.63	ND	$> 3.875 \pm 0.177$

LRV: $\log_{10}$ reduction value.
ND: no detectable viruses were found in the solution. Limit of detection 3.0 $\log_{10}$ $TCID_{50}$/mL.

significant inactivation (LRV > 3.511) without reducing rhFIX activity.

Among the various methods adopted in the biopharmaceutical industry to inactivate viruses include heating, ultraviolet irradiation and solvent/detergent treatment[7,15]. However, these studies were limited in applications to biological products from a cell line-based bioreactor[1,4] and plasma derived biologicals[11,16]. This study is, to our knowledge, the first to describe an inactivation strategy using a model virus (ATIT-PrV) for manufacturing rhFIX from the milk source of transgenic pigs. In conclusion, results of this study demonstrate that 1 hr of treatment with 0.25% of Triton X-100 is appropriate for ATIT-PrV inactivation in unprocessed milk before the rhFIX purification downstream process. Additionally, the biological activity of rhFIX in raw milk without any changed following detergent treatment.

Acknowledgments

The authors would like to thank the Ministry of Economic Affairs of the Republic of China, Taiwan, for financially supporting this research. Ted Knoy is appreciated for his editorial assistance.

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