Inactivation of viruses by pasteurization at 60°C for 10 hours with and without 40% glucose as stabilizer during a new manufacturing process of α 2-Macroglobulin from Cohn fraction IV

Chaoji Huangfu ^{a, #}, Yuyuan Ma ^{a, #}, Junting Jia ^a, Maomin Lv ^a, Fengxuan Zhu ^a, Xiaowei Ma ^b, Xiong Zhao ^{a, *}, Jingang Zhang ^{a, *}

Abstract

Pasteurization is regularly used to inactivate viruses for the safety of plasma derivatives. Influence of pasteurization at 60°C for 10 h on α 2-Macroglobulin activity and virus inactivation were studied. With 40% sugar as stabilizers more than 70% α 2-Macroglobulin activity was reserved after pasteurization compared with 20% in control. Glucose presented a better activity protection effect than sucrose and maltose. By pasteurization without stabilizer the virus titers of pseudorabies virus, Sindbis virus, porcine parvovirus and encephalomyocarditis virus were reduced more than 5.88 log₁₀, 7.50 log₁₀, 4.88 log₁₀, and 5.63 log₁₀ respectively within 2 h. By pasteurization with 40% glucose vesicular stomatitis virus was inactivated more than 5.88 log₁₀ within 1 h. Only 2.71 log₁₀ reduction was achieved for encephalomyocarditis virus after 10 h. 40% glucose protected α 2-M activity and viruses simultaneously from pasteurization. Other viral inactivation methods need to be incorporated to ensure viral safety of this manufacturing process of α 2-Macroglobulin.

Keywords: α 2-Macroglobulin; Pasteurization; Virus inactivation; Cohn Fraction IV

1. Introduction

α 2-M (α 2-Macroglobulin) is a 720 kDa plasma protein which is abundant in the fluids of vertebrates and several invertebrates [1]. α 2-M presents a potential therapeutic effect on radiation injury [2-4] and scald [5]. Highly purified α 2-M has

1 been prepared from Cohn Fraction IV (precipitation of Fraction IV-1 and IV-4 in one
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3 step) which is usually discarded as a waste fraction in the industrial preparation of
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5 albumin in China. The new manufacturing process of α 2-M using Cohn Fraction IV as
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7 starting material may enhance the utilization of plasma.
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11 The use of purified plasma derivatives still has a risk to transmit blood-borne
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13 infectious agents, though the development of virus inactivation and/or removal
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15 technologies has greatly improved the safety. Pasteurization at 60°C for 10 h is
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17 effective enough to inactivate a wide range of viruses. It was applied in some
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19 manufacturing process of plasma derivatives [6, 7, 8]. However, studies about
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21 influence of pasteurization on α 2-M activity and virus inactivation by pasteurization
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23 in the new manufacturing process of α 2-M have not been reported.
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31 In this report, liquid α 2-M was exposed to 60°C for 10h. Virus inactivation
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33 without stabilizer was investigated for pseudorabies virus (PRV), Sindbis virus,
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35 porcine parvovirus (PPV) and porcine encephalomyocarditis virus (EMCV). Virus
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37 inactivation with 40% glucose as stabilizer was investigated for vesicular stomatitis
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39 virus (VSV) and EMCV. Three stabilizers were optimized to evaluate their protection
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41 for α 2-M activity during pasteurization, and abnormal toxicity of α 2-M treated by
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43 pasteurization was also studied.
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53 **2. Materials and methods**

54 **2.1. Preparation of α 2-M**

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56 The α 2-M used in this study was isolated as described [1]. 4% (w/v) α 2-M was
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1 treated with pasteurization before filtrating, and lyophilization (Figure 1 A). The
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3 abnormal toxicity of $\alpha 2$ -M was performed by the abdominal injection of $\alpha 2$ -M
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5 solution (0.5ml) into 19-21 g Kunming mice (Obtained from Weitong Lihua Co. Ltd,
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7 Beijing, China), with the subsequent monitoring of toxicity and skin reactivity for 7
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9 days post treatment. Negative controls were conducted using same batch of mice
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11 without treatment.
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16 2.2. Influence of pasteurization on $\alpha 2$ -M activity

17 $\alpha 2$ -M activity was detected by inhibition of trypsin with a chromogenic assay [1].
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19 The influence of pasteurization at 60°C for 10 h on $\alpha 2$ -M activity was evaluated either
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21 stabilized by glucose (40%, w/v), sucrose (40%, w/v), and maltose (40%, w/v) based
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23 on previous report [9] or not. Using $\alpha 2$ -M solution stored at 4°C without
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25 pasteurization as control.
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33 2.3. Viruses and cells

34 Viruses with a range of physicochemical properties (Table 1 and Table 2) were
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36 chosen to test the ability of system to eliminate viruses as described [10]. All cell lines
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38 and viruses were obtained from American Type Culture Collection. Both PRV and
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40 PPV were propagated in PK-15 cells. EMCV was propagated in BHK-21 cells. VSV
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42 and Sindbis virus were propagated in Vero-E6 cells. Cells were grown in a Dulbecco's
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44 modified Eagle's medium (DMEM, Gibco, Carlsbad, USA) containing 5% fetal
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46 bovine serum (Gibco, Carlsbad, USA). Virus-containing cell supernatants or extracts
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48 were harvested from infected cultures and after clarification by centrifugation (2000 g,
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50 10 minutes), stored at -70 °C before use.
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2.4. Virus inactivation studies

In each virus-spiked run, 9 mL of α 2-M solution was spiked with 1 mL of virus, and the samples (pH 6.7) with 40% glucose and without stabilizer were subjected to pasteurization at $60\pm 1^\circ\text{C}$ (temperature sensors were calibrated regularly by a special calibration laboratory) for 10 h. Duplicate assays on the condition of $58\pm 1^\circ\text{C}$ for 10 h without stabilizers were conducted to assess worst-case inactivation conditions (data not shown). The samples were heated within 15 to 20 minutes to target temperature, and stirred slowly during pasteurization. A heated water bath with recirculating water was used, and the temperature was continuously monitored and recorded. Samples were removed at 0.5h, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h respectively, and were immediately cooled by immersion into a cold water bath. Then the titers of the viruses were measured.

2.5. Virus assays

Virus infectivity was determined using monolayers cells (Same cell lines as described in Section 2.3). An aliquot of each sample from the virus inactivation studies was titrated after being collected in 8-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose assay. Indicator monolayers cells in 96-well culture plates were infected using at least eight replicates of 0.1 ml of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected using DMEM medium. Cytopathic effect was examined after incubation (37°C , 5% CO_2 concentration, 95% relative humidity) for 3 days (PRV, Sindbis virus, VSV, and EMCV) or 6 days (PPV). Virus reduction values were

calculated by subtracting log virus titres after treatment from that determined in the spiked starting materials.

3. Results

3.1. Influence of pasteurization on α 2-M activity

Sugars are able to prevent or reduce protein denaturation during pasteurization. Glucose, sucrose, and maltose were chosen as stabilizers during pasteurization. Stabilizers during pasteurization improved α 2-M activity by more than 300 percent points compared with the unstabilized control (Figure 1 B). More than 70% activity recovery was reserved in the presence of sugars, of which glucose showed a better protection effect for α 2-M activity than others. The weight of mice injected with pasteurized glucose-stabilized α 2-M increased normally and no abnormal toxicity was observed

3.2. Virus inactivation during pasteurization treatment

The virucidal efficacy of the pasteurization was studied using viruses of different resistance characteristics. Both both PRV and Sindbis virus are very sensitive to pasteurization process without any stabilizer. PRV and Sindbis virus were inactivated with 2.29 $\log_{10}$ and 4.79 $\log_{10}$ reduction respectively after pasteurization for 0.5 h (Table 1) and both were completely inactivated to undetectable levels within 1 h. Enveloped properties of these two viruses may account for the rapid inactivation. PPV and EMCV were less sensitive to pasteurization process. PPV was inactivated by 1.42 $\log_{10}$ and 2.79 $\log_{10}$ within 0.5 h and 1 h, respectively and the remaining infectivity

was inactivated below the limit of detection within 2 h. An average reduction of 2.00 $\log_{10}$ and 3.30 $\log_{10}$ was achieved after 0.5 h and 1 h for EMCV and no infectious EMCV was detected after 2 h pasteurization. Robustness studies with reduced temperature (58°C) indicated that no impact of temperature reduction on the virus reduction factors for these viruses was observed (data not shown). The presence of 40% glucose greatly increased the resistance of EMCV with only 2.71 $\log_{10}$ reduction after 10 h, while more than 5.88 $\log_{10}$ was inactivated for VSV within 1 h in the presence of 40% glucose (Table 2).

4. Discussion

Sugar is able to protect proteins against heat denaturation because of its effect on the water structure and the strength of hydrophobic interactions [11]. Wang et al [9] reported that no activity loss of α 2-M stabilized by xylitol (40 g/dl) and no immunological characteristic changes were observed after pasteurization. However, Wang et al didn't investigate virus inactivation. Glucose presented a significant better stabilizing effect for α 2-M activity than other sugars. Only 20% α 2-M activity was reserved without stabilizer indicates that liquid α 2-M is quite susceptible to pasteurization, and sugars could protect α 2-M activity from pasteurization. The injection of α 2-M solution treated by pasteurization into mice did not cause abnormal toxicity against mice. These results are encouraging for the clinical use of pasteurized α 2-M but more structural and functional analysis need to be investigated for further application.

To evaluate the effectiveness of pasteurization for the inactivation of viruses, spiking studies were carried out using five viruses, which are models of HAV, HBV, HCV, and B19V respectively. Pasteurization has been successfully incorporated into several manufacturing processes of plasma derivatives [6, 7, 8]. In this study, viral inactivation by pasteurization with and without stabilizer was investigated during the new manufacturing process of α 2-M. Significant difference was observed for the viral inactivation of EMCV depending on the presence of 40% glucose or not. 5.63 Log₁₀ EMCV was inactivated within 2 h without stabilizer, while only 2.71 Log₁₀ reduction was achieved after 10 h with 40% glucose as stabilizer. Stabilizer was considered to protect viruses from heat denaturation [12], which may account for this difference. Sindbis virus and VSV share some same characteristics (Table 1), which were both inactivated rapidly by pasteurization. These results suggest that sugar play a significant influence on α 2-M activity protection and viral inactivation treated by pasteurization. Other stabilizers such as amino acids or acetate need to be investigated on α 2-M activity reservation and viral inactivation after pasteurization. It is desirable to incorporate at least two distinct effective virus inactivation steps which complement each other for plasma-derived medicines [13], so another viral inactivation method such as dry-heat or filtration need to be incorporated for the viral safety of this process. This study may lay a foundation for the selection of virus inactivation treatment in the industrial preparation of α 2-M.

Acknowledgements

The study is funded by National High-tech Research and Development Program of China (Grant No. 2012AA021902) and Beijing Natural Science Foundation (Grant No. 7162140).

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Author Contributions

C.J.H.F. and Y.Y.M. searched data, performed the experiments, wrote the manuscript,
and contributed equally to this article; X.Z. and J.G.Z. designed the study and gave a
critical view of manuscript writing; M.M.L., J.T.J., X.W.M., and F.X.Z. collected and
analyzed the data. All the authors read and approved the final manuscript.

Figure 1. Process of α 2-M purification and recovery of α 2-M activity after pasteurization. A: Process flow for α 2-M purification from Cohn Fraction IV. B: Recovery of α 2-M activity after pasteurization. Data is expressed as means $\pm$ standard deviation from three experiments, and t-test was employed. Glucose presented significantly better protection on recovery of α 2-M activity than other groups. * $P < 0.05$ vs Glucose; $\times$ $P < 0.01$ vs Glucose.

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Table 1 Virus inactivation by pasteurization (60°C for 10 h) without stabilizer

Virus	Envelope	Corresponding virus of concern for human plasma	Initial titer of spike virus	Virus titers reduction (Log)*			
				0.5h	1 h	2 h	3-10 h
PRV	+	HBV	5.88	2.29±0.08	≥5.88	≥5.88	≥5.88
Sindbis virus	+	HCV	7.50	4.79±0.19	≥7.50	≥7.50	≥7.50
PPV	—	B19V	4.88	1.42±0.07	2.79±0.08	≥4.88	≥4.88
EMCV	—	HAV	5.63	2.00±0.13	3.30±0.14	≥5.63	≥5.63

* Three independent experiments were conducted for each virus. Data are expressed as means ± standard deviation.

Table 2 Virus inactivation by pasteurization (60°C for 10 h) with 40% glucose

Virus	Envelope	Corresponding virus of concern for human plasma	Initial titer of spike virus	Virus titers reduction (Log)*				
				0.5h	1 h	4 h	8 h	10 h
VS	+	HCV	5.88	4.50±0.12	≥5.88	≥5.88	≥5.88	≥5.88
EMCV	—	HAV	6.00	0.21±0.08	0.79±0.07	1.70±0.14	2.62±0.13	2.71±0.08

* Three independent experiments were conducted for each virus. Data are expressed as means ± standard deviation.

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PRV	+	HBV	5.88	2.29±0.08	≥5.88	≥5.88	≥5.88
Sindbis virus	+	HCV	7.50	4.79±0.19	≥7.50	≥7.50	≥7.50
PPV	—	B19V	4.88	1.42±0.07	2.79±0.08	≥4.88	≥4.88
EMCV	—	HAV	5.63	2.00±0.13	3.30±0.14	≥5.63	≥5.63

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Virus	Envelope	Corresponding virus of concern for human plasma	Initial titer of spike virus	Virus titers reduction (Log)*				
				0.5h	1 h	4 h	8 h	10 h
VSV	+	HCV	5.88	4.50±0.12	≥5.88	≥5.88	≥5.88	≥5.88
EMCV	—	HAV	6.00	0.21±0.08	0.79±0.07	1.70±0.14	2.62±0.13	2.71±0.08

* Three independent experiments were conducted for each virus. Data are expressed as means ± standard deviation.

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