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Comparison of Methods for The Purification of Goat Lactoferrin and Antiviral Activity to Human Papillomavirus

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Abstract Lactoferrin (Lf) has been reported for its multifunctional properties such as antifungal, antibacterial, antiviral, antioxidant and anticancer activities. This protein is an 80 kDa iron-binding protein of the transferrin family. It can be isolated from milk and has been identified from various mammalian secretions. The aims of this study focused on the isolation and purification of Lf from etawa goat colostrum using several methods of chromatography and observing its activities as antiviral human papillomavirus (HPV). Purification was performed by gel filtration chromatography using Sephadex G-75 and G-100, for cation exchange chromatography with carboxymethyl Sephadex C-50, and two-steps chromatography of cation exchange resin carboxymethyl Sephadex C-50 and gel filtration resin Sephadex G-75. All columns were prepared by manual packaging. The native isolated Lf was characterized by SDS-PAGE electrophoresis. Our result showed that the best purified goat lactoferrin (gLf) was by two-steps chromatography with yield of 364 µg/mL and molecular weight 82 kDa. The pure gLf showed anti-HPV effect at concentration of 100 µg/mL after 72 hours incubation by the increased in cycle threshold (Ct) value, from 26 Ct to 36 Ct.

Keywords: *goat lactoferrin, purification, ion exchange chromatography, gel filtration chromatography, anti-human papillomavirus*

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1. Introduction

Cervical cancer is the second most common cancer in women worldwide. Evidence from WHO [43], every year there are mortality of more than 270000 women from cervical cancer, more than 85% of these death are in low and middle income countries. Infection of *human papillomavirus* (HPV) can cause cervical cancer in women reproduction system. At least 70 % of cervical cancers worldwide were caused by HPV-16 and 18. Efforts to find cure or prevention continues including the use of natural products. Reference [11] reported that milk product can protect against cancer. One of the compound proteins in milk is lactoferrin (Lf). The highest concentration of lactoferrin can be found in colostrum [13,24,27,36,44,45]. The concentration of Lf in colostrum and milk varies widely from one species to another [32].

Lf is an iron-binding protein that has an important role in the non-specific immune system such as in the defense mechanisms against virus [8,9,15,16,21,29,35,38,40,41,47], bacteria [10,30] and fungi [25,28,39]. The antiviral activity of Lf act in the early phase of the viral infection thus preventing entry of virus into the host cell, either by blocking cellular receptors or by direct binding to virus particles. The most probable mechanism preventing virus entry by interacting with the viral attachment receptor heparan sulfate [3,4,7,18,33,34,37].

The molecular weight of Lf is about 80 kDa and have positive charge. Lf can be purified from colostrum and milk by several methods such as ion exchange chromatography, gel filtration and affinity chromatography. By ion exchange chromatography, recent study used the column S-Sepharose [23], carboxymethyl-cellex [14], cationic surfactant cetyldimethylammonium bromide [2], sulphopropyl Sepharose [10], carboxymethyl Sepharose FF [43], carboxymethyl Sephadex C-50 [11,30]. Reference [30] showed that purified Lf with one-step chromatography yielded purity efficiency of 90%. For Lf purification by gel filtration used Sephadex G-100 [17]. Beside ionic exchange chromatography and gel filtration, another step to purify Lf was by affinity chromatography. Reference [16] combined 2 steps of cation exchange chromatography using Mono S 5/50 GL and gel filtration using Superdex 200 5/150 column to purified human (hLf), camel (cLf),

bovine (bLf) and sheep (sLf) lactoferrin. Recent study used the affinity chromatography with matrix heparin Sepharose 6 fast flow [22] and heparin Sepharose [26]. Several study showed that Lf can be detected by immunosensor [6], enzyme-linked immunosorbent assay (ELISA) [22] and HPLC [12,17].

The aims of this study were to focus on the isolation and purification of Lf from Indonesian local goat colostrum. In this study we used different methods to purify goat lactoferrin (gLf). First we used the gel filtration chromatography (column Sephadex G-75 and Sephadex G-100), second we used the cationic exchange chromatography by carboxymethyl Sephadex C-50 and the last we used the combination two type chromatography ion exchange (carboxymethyl Sephadex C-50) and gel filtration (Sephadex G-75). All columns were prepared by manual packaging. The best fraction of purified isolated of gLf was further analyzed for its anti-HPV activity

2. Materials and Methods

2.1. Lactoferrin Isolation

Colostrum of etawa goat (*Capra aegagrus hircus*) were purchased from goat farm (Bogor, Indonesia). At first colostrum was skimmed by centrifugation in 10000 xg, 20 minutes at 4 °C. Casein removed from skim milk in acidic condition using 3N HCl incubated at 37 °C for 30 min, neutralized to 6.8 with NaOH 1N. Proteins were concentrated using dialysis membrane (Spectra/por MWCO 12-14000) on polyethylene glycol (PEG). Skimmed milk was used for Lf purification and diluted with phosphate buffer pH 7.4. Lf was purified by gel filtration on Sephadex G-75 and G-100 elute using phosphate buffer saline (PBS) pH 7.4. By cation exchange chromatography on carboxymethyl Sephadex C-50 resin elute at by increasing stepwise the molarity of NaCl 0-0.5 M in the PBS and by two steps with cation exchange chromatography (CM Sephadex C-50) - gel filtration chromatography (Sephadex G-75). Purity was checked by sodium deodecyl sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) and compared with commercial bLf. The concentration of gLf determined by bicinchoninic acid assay (BCA) kit.

2.2. Inhibition Effect of gLf

HeLa cells (1.0 x 10^5 cells/mL) was incubated with DMEM media containing 10% fetal bovine serum (FBS) and 1% (100 Units of penicillin and 100 µg streptomycin) in T-25 flask. The cells were incubated in 48-well plate for 24 hours at 37 °C, supplied with 5% CO_2 and 85% humidity, and then purified gLf was added at concentrations of 50, 100 and 200 µg/mL. HeLa cells (1.0 x 10^5 cells/mL) without treatment were used as negative control and HeLa cells with bLf of the same concentrations were used as comparison with gLf. The cells were incubated at 24, 48 and 72 hours at 37 °C, 5% CO_2 and 85% humidity.

2.3. Real Time PCR to Evaluate Antiviral Activity of the gLf Againts HPV

We used real time PCR to examine the ability of gLf to prevent the replication of HPV at concentration 25, 50, 100 µg/mL. After the incubation, HeLa cells were washed three times from debris and dead cells using PBS, then HPV DNA was isolated and extracted from HeLa cells by QiaAmp DNA mini blood kit (Qiagen). Amplification of HPV DNA in samples and controls was measured by real time PCR (iQ5 Real Time PCR, BioRad), and to determine the pattern of inhibition of HPV DNA replication, we used SYBR Green (BioRad). Quantitative analysis by shifting of Ct (Cycle threshold) using iQ5 Real Time PCR (BioRad).

3. Results and Discussion

Gel filtration is a process of separation of proteins and other biological molecules based on size exclusion. Sephadex G-75 have the ability to separate molecules with molecular weight range of 3-80 kDa and Sephadex G-100 separates molecules in the weight range of 4-150 kDa. Both of this resin should separate the Lf with molecular weight of about 80 kDa. Electrophoretic profile of gLf purification using Sephadex G-75 and Sephadex G-100 showed in Figure 1.

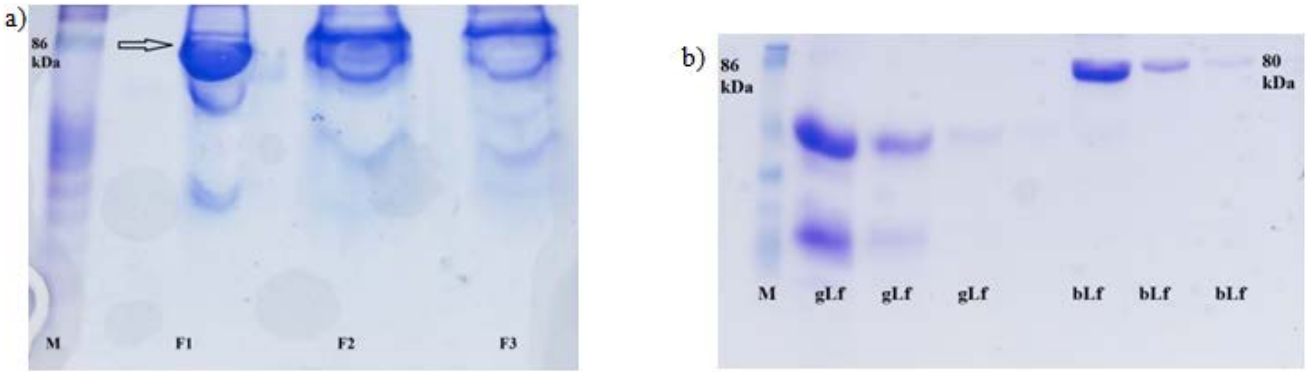

Figure 1. Electrophoretic profile SDS-PAGE purification of gLf by (a) Sephadex G-75 (arrow indicated Lf in the range of 80 kDa) and (b) Sephadex G-100 (M: marker broad range; F1, F2 and F3 are fractionated proteins; bLf : bovine lactoferrin)

The results of purification by using resins Sephadex g-75 showed better separation compared to Sephadex G-100 (Figure 1a). This was apparent from the electrophoregram profile that the fraction of purification by using Sephadex G-75 capable of protein separation with molecular weight in the range of 80 kDa. However, it is not completely

purified yet due to the separation was only based on the molecular weight, not on the charge of protein. Electrophoregram on Figure 1b showed there was no band in the range of 80 kDa. This may be due to the weight range molecules of Sephadex G-100 too large. The disadvantage of using manual packaging can be also affected the purification process since it was difficult to determine the release of Lf from the resin. Our result was different with reference [17], they were successful in obtaining purified camel lactoferrin (cLf) with Sephadex G-200 elution using HPLC equipment.

The separation principle on ion exchange chromatography is the opposite functional group charged with the charge of protein. Cation exchange chromatography is the most efficient method for the separation of positively charge protein. Carboxymethyl Sephadex C-50 that has a negative charged group will bind Lf. Elution process with 0.5 M NaCl is the best ionic strength to elute the gLf from carboxymethyl group based on the resin. The Na^+ from NaCl solution exhanged with gLf whereas bind to the carboxymethyl. Figure 2 showed that separation of proteins but not yet purified lactoferrin, there still many bands over the fractination. Reference [30] were succesful in purification the bovine lactoferrin (bLf) by one step ion exchange chromatography using CM Sephadex C-50, they were used ultraviolet absorption at 280 nm to monitor the eluent of protein.

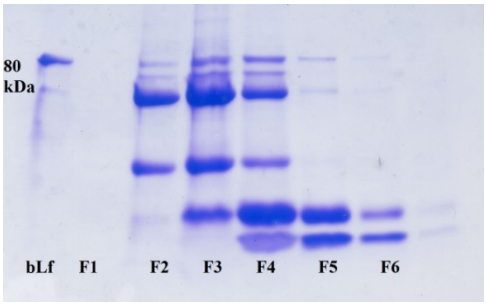

Figure 2. Electrophoretic profile SDS-PAGE og gLf seperated by CM Sephadex C-50 (bLf: bovine lactoferrin; F1 to F6: fractionated gLf)

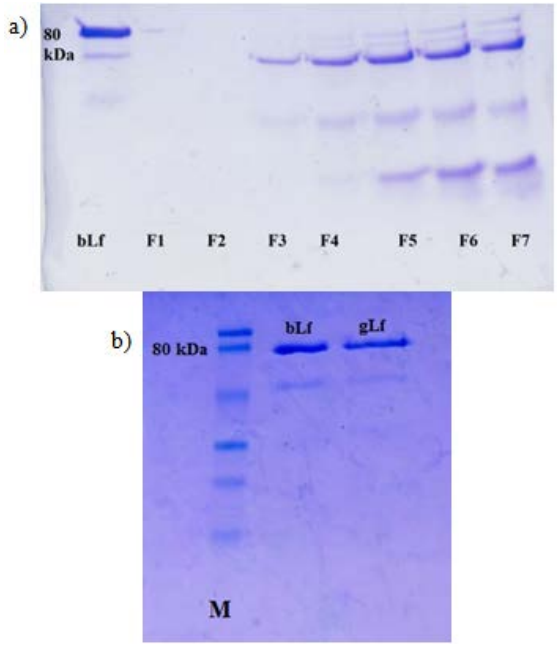

Figure 3. Electrophoretic profile (a) fractionation by ionic-gel filtration chromatography (b) the pure gLf compared with bLf

Lactoferrin was released from second fractination until fifth fractination. We tried to maximize the purification of lactoferrin by two-step chromatography. From the first trial with gel filtration, we chosed the Sephadex G-75 to combine with carboxymethyl Sephadex C-50, because the seperation using Sephadex G-75 was better than Sephadex G-100. Figure 3 showed that two steps purification gave better result than single step purification. We also compared the purified gLf with standard bLf (Figure 3 b).

Results showed the purified gLf has very similar molecular weight compared with standard bLf of 82 kDa obtained commercially. This molecular weight was the same as Korean native goat Lf which was purified by two-step chromatography, ion exchange and affinity [31]. This method was successful in purifying gLf based on their charged molecules and its molecular weight. The concentration of purified lactoferrin from our local etawa goat was 364 µg/mL as measured by BCA assay.

The pure gLf was further evaluated for its *in vitro* antiviral activity to HPV on HeLa cell line. HeLa cell is a human epithelial cells originate from cervical cancer caused by HPV, known to have HPV shedding to the culture media. The DNA virus of HPV was extracted from HeLa cells pellet using QiaAmp DNA mini blood kit (Qiagen). Table 1 showed the value of cycle threshold (Ct) by real time PCR.

Cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold. A positive inhibition of HPV replication indicated by the increased value of Ct. On the first day after incubation with Lf, the replication of HPV were still not inhibited by Lf This could be due to the lack of interaction between gLf and the HPV. However, on day-2 and day-3 an increased of inhibition of HPV replication was shown. The highest inhibition of replication was 31.37 on the third day after incubation with gLf 100 µg/mL. This indicated that Lf interacted with HPV. Common mechanisme antiviral activity of Lf, such as binding to cell membrane glycosaminoglycans, act in the early phase of the vial infections thus preventing entry of virus into the host cell, either by blocking cellular receptors or by direct binding to virus particles [3,4,18,37,38].

Table 1. Inhibition of gLf and bLf from HeLa cell line

Day	Ct value			% difference of Ct value	
	Untreatment Cell	gLf [100 µg/mL]	bLf [100 µg/mL]	gLf	bLf
1	32,97	32,09	32,49	-2,67	-1,46
2	27	31,94	28,49	18,30	5,52
3	28	36,73	31,37	31,18	12,04

4. Conclusion

The result of this study demonstrate that the most optimum method to isolate and purify the gLf from etawa goat colostrum was by two-steps chromatography using cation exchange (CM Sephadex C-50 and gel filtration Sephadex G-75). We also showed that the isolate of gLf has inhibitory activities to HPV.

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