PURIFICATION AND BIOLOGICAL ACTIVITY OF A RECOMBINANT BOVINE LACTOFERRIN FROM *PICHIA PASTORIS* KM71-3 STRAIN

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SUMMARY

Lactoferrin is a member of the transferrin protein family with function of iron transport in blood. However, lactoferrin also plays an important role in the mammalian innate immune system, showing protective effects against several microorganisms such as viruses, bacteria, fungi and parasites, and displaying anticancer activities. In the present study, some suitable conditions for affinity purification of a recombinant bovine lactoferrin (rbLF) from intracellular *Pichia pastoris* KM71-3 strain were investigated. The Ni^{2+} -chelate resin was better than the Cu^{2+} -chelate resin for affinity purification of rbLF protein. The rbLF protein was bound to Ni^{2+} -chelate resin for 18 hours in 1X PBS buffer. Antimicrobial effect of rbLF against *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis* and *Candida albicans* was measured. The rbLF showed growth inhibition with *Micrococcus luteus*, *Bacillus subtilis* at concentration of 1 mg/ml. The rbLF also exhibited inhibition effect on the proliferation of cancer cell lines, Hep2-C and FL.

Keywords: Anticancer activity, antimicrobial activity, *Pichia pastoris*, purification, recombinant bovine lactoferrin.

INTRODUCTION

Lactoferrin (LF) is a glycoprotein belonging to the transferrin family, which is capable of binding and transferring Fe^{3+} ions. Lactoferrin is present in various mammalian species including humans, cows, goats, horses,…etc. Lactoferrin consists of one polypeptide chain containing 703 amino acids folded into two globular lobes representing the C- and N-terminal regions. Both the lobes are connected to each other by a short 310-helix. Each lobe contains two equal domains known as C1, C2, N1, and N2. The domains contain one iron binding site on each lobe (Adlerova *et al*., 2008). Lactoferrin molecule contains varying numbers of sites for potential glycosylation, mostly on the surface of the molecule; in particular, bovine LF has five sites although only four are normally glycosylated. The glycan moieties in LF may contribute to some of its biological roles (Karav *et al*., 2017). Lactoferrin may also be classified as a component of the innate immune system and has antibacterial, antiviral, antiparasitic, catalytic, anticancer, and antiallergic functions and properties (Adlerova *et al*., 2008). Lactoferrin reduces the growth and proliferation of a variety of infectious agents including both Gram-positive and negative bacteria, viruses, protozoa, or fungi (González-Chávez *et al*., 2009). It is revealed that lactoferrin has antimicrobial activity against gram-negative bacteria requiring high iron like coliforms. It also acts against bacteria like *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus* species (Niaz *et al*., 2019). There are a growing number of papers indicating that bovine and human lactoferrin can have beneficial effects for the treatment of cancer (Gibbons, 2011). Lactoferrin has even been reported to inhibit the development of experimental metastases in mice (Wolf *et al*., 2003). The mechanism of anticancer activity of lactoferrin is reported such as cell membrane disruption, apoptosis induction, cell cycle arrest and cell immunoreaction (Zhang *et al*., 2014). The aim of this study is to purify recombinant bovine lactoferrin (rbLF) from the protein extract of *P. pastoris* KM71-3 and investigate the effect of rbLF on the growth of some microorganism species and cancer cell lines.

MATERIALS AND METHODS

Microbial strains and chemicals

Recombinant *Pichia pastoris* KM71-3 strain was generated by project DT.01.18/CNSHCB (Utility solution, submitted). Strains of *Escherichia coli* ATCC 11303, *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 6538P, *Candida albicans* ATCC 10231 were purchased from ATCC (USA). Cancer cell lines were supported by POLYVAC. Chemicals used in this study were purchased from Sigma (USA), Merck (Germany), IntronBio (Korea).

Purification of His-tagged recombinant lactoferrin

The IMAC resin (TOYOPEARL AF-Chelate-650M; Tosoh Corporation) was chelated with metal ions by incubation for one hour at room temperature. After chelating the resin was washed three times with 1X PBS pH 7.4. The

target protein prepared in the corresponding buffers was bound to the chelated resin by mixing the protein extract with the resin at 4°C for different amount of time (1h, 3h, 6h, 18h). Unbound proteins and other components were removed by washing the resin with the corresponding buffers three times. The target lactoferrin protein was eluted with 1X PBS containing 40 mM imidazole. The purified protein was concentrated and buffer exchanged using Amicon 10 kDa filters and checked by SDS-PAGE electrophoresis.

Growth inhibition of microorganism in the presence of rbLF (Flores-Villasenor *et al*., 2010)

To test the antimicrobial activity of recombinant bovine lactoferrin, the microbial culture of each strain was incubated in falcon tubes containing suitable media with rbLF at the final concentration of 1 mg/ml. Bacteria and yeast in media without rbLF were used as control of growth. Ampicillin (100 μg/ml) was used as control of growth inhibition. Cultures were incubated at 37°C for 18 hours with constant agitation. Growth levels were determined by measuring the optical density of the cultures at 600 nm.

Growth inhibition of cancer cell lines in the presence of rbLF (Guedes *et al*., 2018)

To test the anticancer activity of recombinant bovine lactoferrin, cell lines were adhered in wells for 24 hours. After adhering, cells were treated with medium (negative control - untreated) and media containing different concentrations of rbLF (50, 100, 150, 200 μM). Viable cells (adhesion cells) were harvested by washing out the unbound cells (death cells) after 24h, 48h and 72h treatment and counted by glass microscope slide with a grid of perpendicular lines. Experiments were duplicated and obtained data were statistically analyzed by excel tool.

RESULTS AND DISCUSSION

Purification of recombinant bovine lactoferrin from the extract of recombinant *Pichia pastoris* KM71-3

Comparison of Cu^{2+}chelate resin and Ni^{2+}chelate resin

A variety of immobilized metals are available for use in IMAC, of which copper and nickel are most commonly used in purification of his-tagged proteins and copper ion particularly shows strong interaction with proteins. In this study, the protein level in the flow-through with copper experiment (using Cu^{2+} -chelate resin) was 1.5 times lower than nickle experiment (using Ni^{2+} -chelate resin), meaning more protein was retained in the gel resin. Stronger binding results in more undesired protein left in elution fraction than nickel gel (Fig. 1, lane 6). As shown in lane 6 of Fig. 1B, the elution fraction collected by purification using copper containing significant amount of protein of 35-48kDa. The lactoferrin in this sample has a molecular mass of around 75 kDa and some smaller fragments. The presence of some smaller fragments may be due to the proteolytic activity of lactoferrin leading to itself digestion (Massucci *et al*., 2004). Nickle shows weaker affinity but also exhibits less nonspecific protein binding resulting in higher purity of protein.

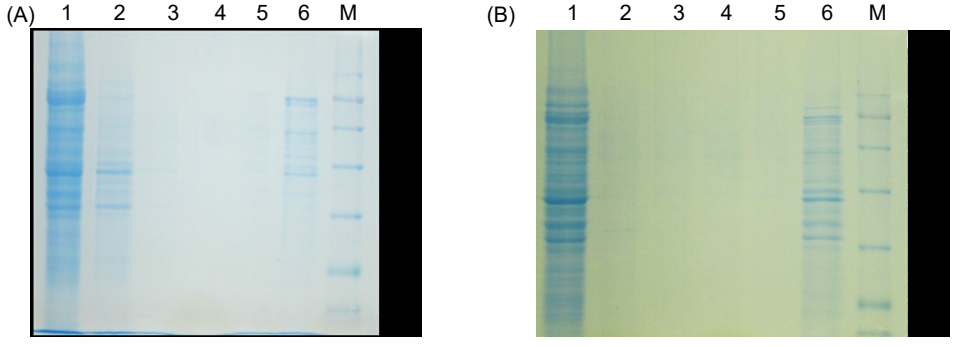

Figure 1. Purifications of recombinant lactoferrin using Ni^{2+}-chelate resin (A) and Cu^{2+}- chelate resin (B). Lane 1, total protein lysate; Lane 2, flow-through; Lane 3-5, wash fractions; Lane 6, elution fraction; M, protein marker

Selection of suitable buffer for purification of recombinant lactoferrin

Selection of suitable buffers for protein purification plays a very important role. Buffer contributes to recovery efficiency and purity of protein solution. Due to properties of lactoferrin and application purpose for functional food, three different buffer systems: 50 mM Tris-HCl pH 7.4, 1X PBS pH 7.4, and 50 mM NaPi were used to prepare protein solutions and whole purification procedure.

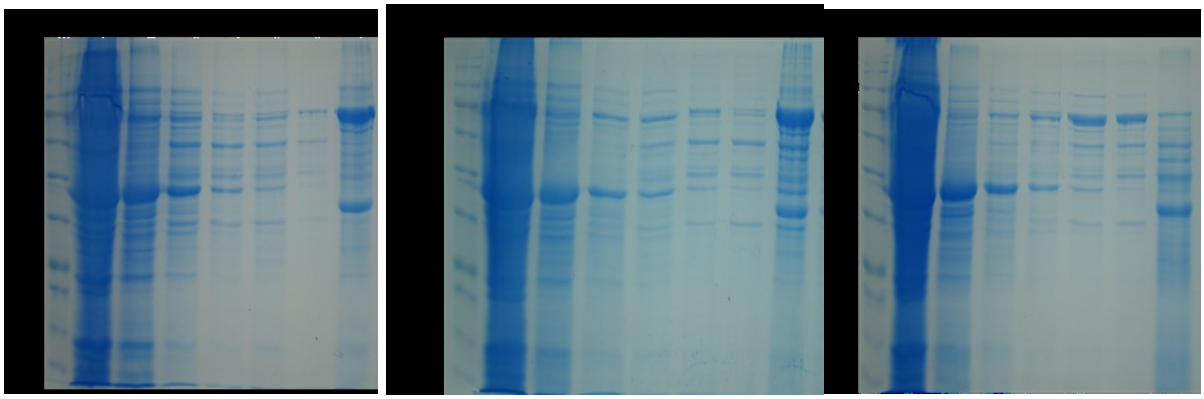

Figure 2. Purifications of lactoferrin from recombinant *P. pastoris* KM71-3 lysate using Tris buffer (A), PBS buffer (B) and NaPi buffer (C). M, protein marker; Lane 1, concentrated protein lysate; Lane 2, flow-through; Lane 3-6, wash fractions of 1-4, respectively; Lane 7, elution fraction

The obtained results showed that the intact lactoferrin protein level (75 kDa) in elution fraction by using NaPi buffer was much lower than by using Tris-HCl and PBS buffers (Fig. 2, lane 7). Intact lactoferrin of 75 kDa was washed out in the washing steps using NaPi buffer (Fig. 2C, lane 5&6). It could be seen that the binding ability of lactoferrin protein to resin in NaPi buffer is relatively weak. In comparison between using PBS and Tris-HCl buffers, the result shows that the profile of purified proteins was similar and the lactoferrin amount in elution fraction of these two conditions were significantly higher than that of NaPi buffer. The results implicated that both PBS and Tris-HCl buffers are suitable for His-tagged recombinant protein purification by using His-tag Chelate 650M resin. However, purified lactoferrin solution is will be used for further experiments such as determination of antimicrobial and anticancer activity and funtioncal food application, therefore the PBS buffer is more preferable to Tris-HCl buffer and should be chosen for further experiments.

Suitable time for binding lactoferrin to resin

In order to determine a suitable amount of time for binding lactoferrin to resins, the mixtures of lactoferrin and resin were incubated for the different periods of 1, 3, 6 and 18 hours. The result showed that binding efficiency was increased with longer binding time. The binding efficiency was the highest for 18-hour incubation within the timeframe used this study (Fig. 3).

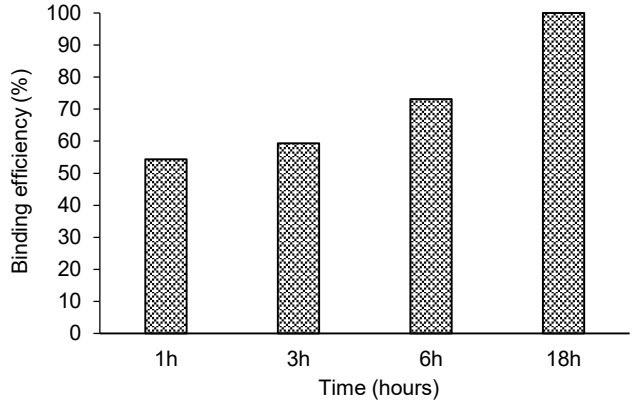

Figure 3. Incubation time for binding lactoferrin to resin. The binding efficiency was calculated based on comparison with the achieved highest condition

Effect of recombinant lactoferrin from *P. pastoris* KM71-3 on microorganism

Antimicrobial activity of recombinant lactoferrin against five different microorganisms including both Gram-positive and negative bacteria was investigated. The obtained result showed the growth of *M. luteus* and *B. subtilis* was inhibited by recombinant lactoferrin from *P. pastoris* KM71-3 strain at concentration of 1 mg/ml; while the growth of *S. aureus*, *E. coli* and *C. albicans* was not affected by recombinant lactoferrin at tested concentration. The *M. luteus* and *B. subtilis* were susceptible to rbLF with a growth inhibition of 33.1 and 24.3%, respectively. Effect of lactoferrin on the growth of *S. aureus*, *E. coli* in this study coincided with observation of Kawai *et al*. (Kawai *et al*., 2007). However, recombinant human lactoferrin from *Aspergillus awamori* exhibited inhibitory activity against *E. coli* O157:H7 at a minimum inhibitory concentration of 5 mg/ml (Conesa *et al*., 2008). It means that non-

inhibitory activity of rbLF of this study may due to low amount of protein. This judgment is supported by report of Flores-Villasenor indicating that the effect of lactoferrin on *E. coli* strains was strain and concentration-dependent manner (Flores-Villasenor *et al*., 2010).

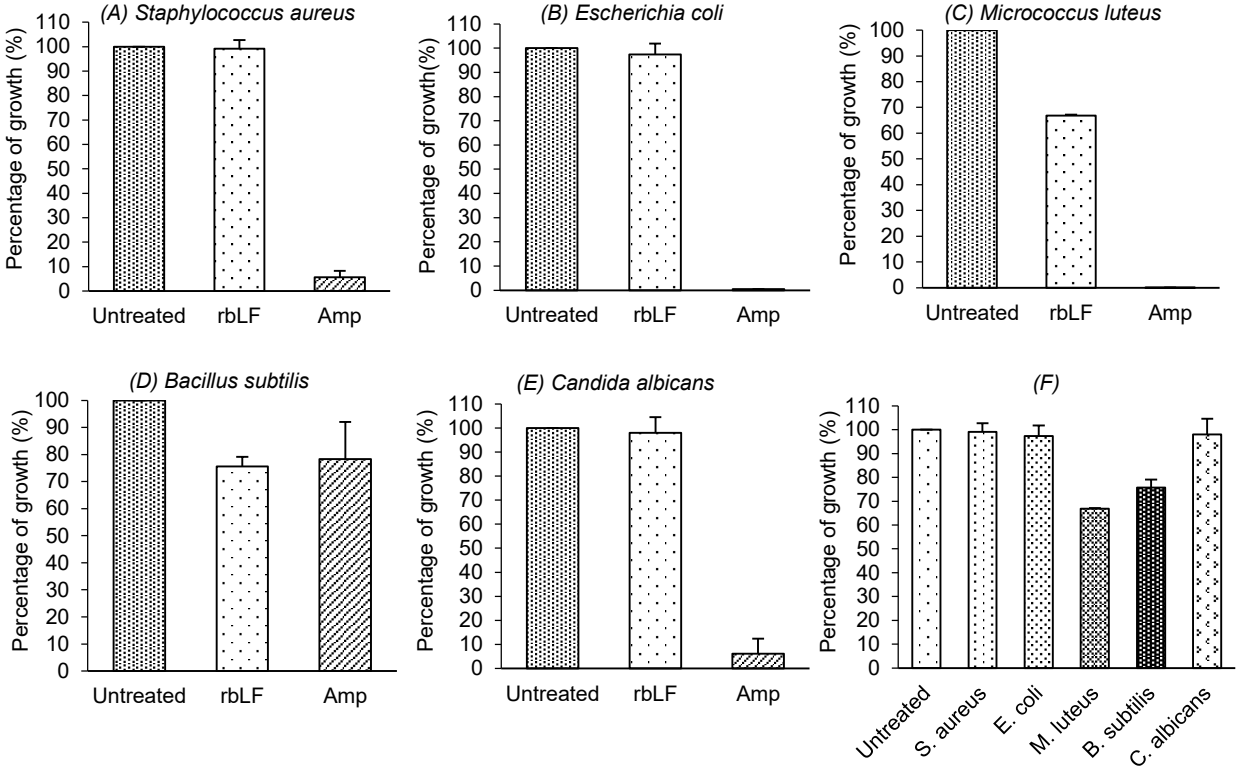

Figure 4. Antimicrobial activity of recombinant lactoferrin against *Streptaphylococcus aureus* (A), *Escherichia coli* (B), *Micrococcus luteus* (C), *Bacillus subtilis* (D), and *Candida albicans* (E). The growth level of untreated sample was considered 100% of growth and inhibition level of growth was assigned as lower percentage of growth to untreated sample. (F) Summary of all tested strains

Effect of recombinant lactoferrin from *P. pastoris* KM71-3 on cancer cell lines

In order to explore effect of recombinant lactoferrin from *P. pastoris* KM71-3 on cancer cells, two cancerous cell lines of Hep-2 and FL were cultured in medium with addition of recombinant lactoferrin at the different concentrations of 50, 100, 150, 200 and 250 μM. The effect of recombinant lactoferrin on cancerous cell line was determined by number of live cells remained after lactoferrin treatment. The obtained results showed that the effect of lactoferrin on the growth of two cancerous cell lines was concentration-dependent manner, the growth inhibition was proportional to the concentration. For Hep2-C cell line, the percentage of viable cells was 74.9% and 63.8% at concentration of 50 μM and 200 μM, respectively after 24h treatment. Similarly, the percentage of viable cells was 80.1% and 58.8% at concentration of 50 μM and 200 μM, respectively for FL cell line. For normal cell line RK13, the growth was not significantly affected by rbLF treatment (Fig. 5). The previous report also indicated that bovine lactoferrin inhibits proliferation, induces apoptosis, intracellular acidification and perturbs lysosomal acidification only in highly metastatic cancer cell lines (Guedes *et al*., 2018).

CONCLUSIONS

The suitable conditions for purification of recombinant lactoferrin from *Pichia pastoris* KM71-3 were determined including using Ni^{2+} -chelate resin, PBS buffer pH 7.4 and the binding incubation time of 18 hours. Recombinant lactoferrin rbLF exhibits antibacterial activity against *M. luteus* and *B. subtilis* strain and growth inhibition for cancerous cell lines of Hep-2C and LF. Our yeast recombinant lactoferrin rbLF from this study has potential applications such as antimicrobial and cancer agent for further application.

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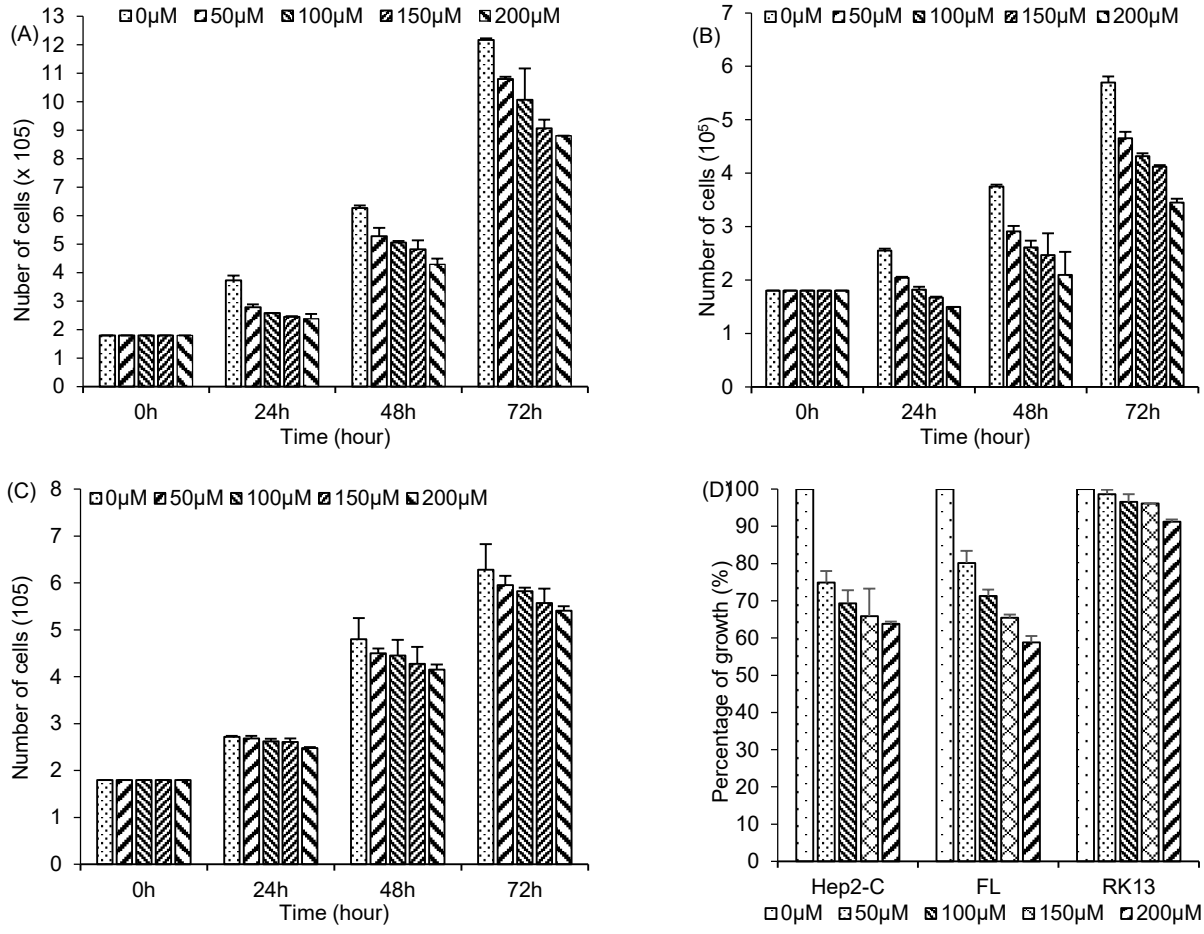

Figure 5. The effect of recombinant lactoferrin from *P. pastoris* KM71-3 on the cancerous cell lines of Hep2-C (A), FL (B) and normal cell line – RK13 (C). The growth percentage of cell lines after 24 hours treatment with rbLF (D)

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TINH SẠCH VÀ XÁC ĐỊNH HOẠT TÍNH CỦA LACTOFERRIN BÒ TÁI TỔ HỢP TỪ CHỦNG *PICHIA PASTORIS* KM71-3

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TÓM TẮT

Lactoferrin là một thành phần trong họ protein transferrin với chức năng chính là vận chuyển sắt trong máu. Tuy nhiên, lactoferrin cũng đóng một vai trò quan trọng trong hệ thống miễn dịch tự nhiên của động vật có vú; protein này thể hiện hoạt tính bảo vệ chống lại nhiều vi sinh vật khác nhau như virus, vi khuẩn, nấm và ký sinh trùng; và thể hiện cả hoạt tính kháng ung thư. Trong nghiên cứu này, một số điều kiện thích hợp để tinh sạch ái lực protein lactoferrin bò tái tổ hợp (rbLF) từ chủng nấm men *Pichia pastoris* KM71-3 đã được nghiên cứu. Gel gắn Ni^{2+} là tốt hơn so với gắn Cu^{2+} để sử dụng cho tinh sạch lactoferrin tái tổ hợp. Hoạt tính kháng khuẩn của rbLF cũng đã được đánh giá với các chủng *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis* and *Candida albicans*. Kết qủa cho thấy rbLF ức chế sự sinh trưởng của hai chủng *Micrococcus luteus*, *Bacillus subtilis* ở nồng độ 1 mg/ml. Protein rbLF cũng thể hiện hoạt tính kháng tế bào ung thư đối với hai dòng tế bào Hep2-C và FL.

Từ khóa: Hoạt tính kháng ung thư, hoạt tính kháng khuẩn, *Pichia pastoris*, tinh sạch, lactoferrin bò tái tổ hợp.

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