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Bovine lactoferrin and lactoferricin derived from milk: production and applications

M. Tomita, H. Wakabayashi, K. Yamauchi, S. Teraguchi, and H. Hayasawa

Abstract: Bovine lactoferrin is produced on an industrial scale from cheese whey or skim milk. The safety of purified lactoferrin has been confirmed from the results of a reverse mutation test using bacteria, a 13-week oral repeated-dose toxicity study in rats, and clinical studies. In order to apply active lactoferrin to various products, a process for its pasteurization was developed. Subsequently, lactoferrin has been used in a wide variety of products since it was first added to infant formula in 1986. A pepsin hydrolysate of lactoferrin is also used in infant formula. This hydrolysate contains a potent antimicrobial peptide named lactoferricin that is derived from the lactoferrin molecule by pepsin digestion. Semilarge-scale purification of lactoferricin can be performed by hydrophobic interaction chromatography. Lactoferricin also exhibits severa! biological actions and appears to be the functional domain of lactoferrin. Recent studies have demonstrated that oral administration of lactoferrin or lactoferricin exerts a host-protective effect in various animals and in humans. The results of these studies strongly suggest that the effects of oral lactoferrin are mediated by modulation of the immune system. Further elucidation of the clinical efficacy and mechanism of action of lactoferrin will increase the value of lactoferrin-containing products.

Key words: bovine, lactoferrin, lactoferricin.

Résumé : La lactoferrine bovine est produite industriellement a partir de lait écrémé ou de lactosérum de fromage. L'innocuité de la lactoferrine purifiée a été confirmée par les résultats d'un test bactérien de mutation inverse, d'une étude de toxicité de doses répétées par voie orale chez des rats et des études cliniques. Une méthode de pasteurisation de la lactoferrine active a été mise au point dans le but d'ajouter la lactoferrine a divers produits. Par la suite, la lactoferrine a d'abord été ajoutée a des laits diététiques pour bébés en 1986, puis elle a été utilisée dans une grande variété de produits. Un hydrolysat de lactoferrine, produit par la pepsine, est également utilisé dans les laits diététiques pour bébés. Cet hydrolysat contient de la lactoferricine, un peptide antimicrobien puissant obtenu par digestion de la lactoferrine par la pepsine. La lactoferricine peut etre purifiée a moyenne échelle par chromatographie par interaction hydrophobe. La lactoferricine a également plusieurs effets biologiques et elle constituerait le domaine fonctionnel de la lactoferrine. De récentes études ont démontré que l'administration de lactoferrine ou de lactoferricine par voie orale entraine un effet de protection de l'hüte chez divers animaux et chez l'homme. Les résultats de ces études suggerent fortement que les effets de l'administration de lactoferrine par voie orale sont attribuables a une modulation du systeme immunitaire. D'autres études de l'efficacité clinique et du mécanisme d'action de la lactoferrine augmenteront la valeur des produits contenant de la lactoferrine.

Mots clés : bovine, lactoferrine, lactoferricine.

[Traduit par la Rédaction]

Introduction

Various biological functions of lactoferrin (LF) have been reported during the past two decades. Antimicrobial activity of LF was reported initially and other properties, including regulation of the immune response and cellular functions, were detected subsequently. One of our research groups has found an antimicrobial domain in the N-terminal region of the LF molecule, which was named lactoferricin™ (Bellamy et al. 1992). The active peptide is released by pepsin digestion of both human and bovine LFs. Bovine LF and lactoferricin have been shown to exert various biological activities both in vitro and in vivo, including a host-protective effect. In recent years, clinical studies of bovine LF have

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been conducted in infants (Kawaguchi et al. 1989) as well as in patients with tinea pedís (Yamauchi et al. 2000c), chronic hepatitis C (Tanaka et al. 1999), and other diseases (Masci 2000).

In this article, we review the production methods for native bovine LF and lactoferrcin, their biological activities, and recent applications. In the following sections, LF and lactoferricin refer to bovine LF and bovine lactoferricin, respectively, unless otherwise indicated.

Production and application of LF and its derivatives

At present, LF is isolated and purified on an industrial scale (approximately 20-30 tons annually worldwide) from cheese whey and skim milk. The concentration of LF in cheese whey is roughly 100 mg L^{-1}. Since LF exists as a cationic protein in whey, it is readily adsorbed to a cation-exchange resin and then eluted using salt solutions. The eluted eructe LF is desalted and concentrated using ultrafiltration and diafiltration membranes, after which it is subjected to pasteurization. Purified LF powder with a purity of 95% or higher is finally obtained by freeze-drying. In an alternative process, microfiltration and spray-drying are performed instead of pasteurization and freeze-drying, respectively. In recent years, pasteurization has come to be considered as very important in order to inactivate not only bacteria but also viruses such as foot and mouth disease virus. While making efforts to develop a practica! method for the pasteurization of LF, we found that LF is stable against heat treatment under acidic conditions (Abe et al. 1991), while heat treatment at a neutral pH causes denaturation of the protein. 1t is considered that heating at a pH "" 4 and to a temperature of 90-lOO°C for 5-10 min as well as the UHT method are suitable and practica! methods for the pasteurization of LF. This pasteurization process was patented, and it has been applied to the manufacture of a wide variety of commercial products containing LF. A pepsin hydrolysate of LF is produced by treatment with porcine pepsin under acidic conditions (Saito et al. 1991). After hydrolysis has been completed, pepsin is inactivated by heat treatment. Then the reaction mixture is filtered and concentrated by reverse osmosis. Finally, the hydrolysate of LF is obtained by pasteurization and freeze-drying for use in infant formula. In addition, lactoferricin can be purified from this LF hydrolysate by two-step hydrophobic chromatography (Bellamy et al. 1992). The peptide is eluted with an acidic buffer, the eluted solution is concentrated by reverse osmosis, and finally, lactoferricin is produced by freeze-drying as a powder with over 95% purity. This production process for lactoferricin has also been patented. The toxicity of purified LF was judged to be extremely low in safety tests. From the results of single-dose, 4-week, and 13-week oral toxicity tests, the dose of LF that caused no adverse effects was found to be 2000 mg kg^{-1} day^{-1} for rats of both sexes (Yamauchi et al. *2000a).* In addition, LF did not exhibit any mutagenic potential in a bacteria! reverse-mutation test (Yamauchi et al. *2000b).* Based on the results of these safety tests and the results of clinical studies, purified LF is considered to be a highly safe food additive.

Along with increased recognition of the biological effects of LF, as described below, the applications of LF have been expanded. LF-supplemented infant formula, follow-up milk, skim milk, yogurt, chewing gum, and nutritional supplements are being marketed. In addition to foods, LF is also used in skin care cosmetics, in special therapeutic diets for the relief of inflammation in dogs and cats, and in aquaculture feed.

Host-protective effects of LF and lactoferricin

Given the wide variety of biological effects of LF, pharmaceutical- and food-related applications, including use as an immunopotentiator and for the chemoprevention of carcinogenesis, have attracted considerable attention.

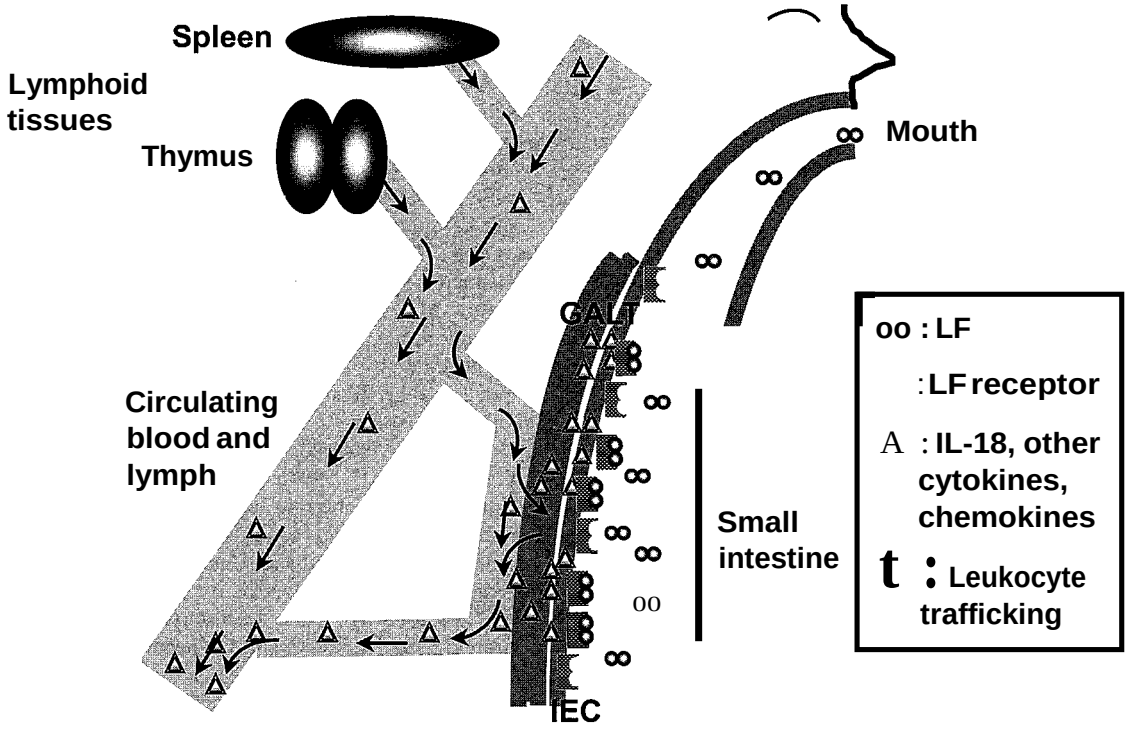
Recent studies have examined the host-protective effects of oral administration of LF in various animal models and in humans. In mouse experiments, suppression of the intestinal overgrowth and bacteria!translocation of enterobacteria as well as a protective effect against infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* have been reported (Teraguchi et al. 1994, 1995; Bhimani et al. 1999; Abe et al. 2000). LF has also been shown to improve inflammatory diseases such as dermatophytosis caused by the fungus *Trichophyton mentagrophytes* in guinea pigs and intractable stomatitis in cats (Sato et al. 1996; Wakabayashi et al. 2000), while it confers resistance against infections such as white spot disease in fish (Kakuta and Kurokura 1995).

Among the host-protective effects of LF, recent reports about cancer prevention have been attracting attention. In a rat model of colon cancer induced by subcutaneous injection of a carcinogen, oral administration of LF suppressed the formation of precancerous lesions in the large intestine and the incidence of carcinoma was also significantly reduced (Sekine et al. 1997a). Oral administration of LF also augmented natural killer (NK) cell activity in the spleen (Sekine et al. 1997b). A prevention effect on cancer has also been demonstrated in various animal models, such as carcinogen-induced cancer of the urinary bladder, esophagus, and lung in rats (Ushida et al. 1999; Masuda et al. 2000) or lung metastasis in mice with subcutaneously implanted tumors (ligo et al. 1999). Furthermore, it has been reported that oral administration of lactoferricin shows a protective effect against infection caused by MRSA or the parasite *Toxoplasma gondii* in mice (Nakasone et al. 1994; Isamida et al. 1998).

Immunomodulatory effects of oral administration of LF

Various immunomodulatory effects of orally administered LF have been reported in animal models and in humans. Debbabi et al. (1998) found an increase of IgA and IgG in the intestinal fluid of mice after oral administration of LF as well as proliferation of Peyer's patch cells and splenocytes. Recently, oral administration of LF or its pepsin hydrolysate was shown to increase the number of NK cells (asialoGMl+), CD4+ T cells, and CDS+ T cells in the peripheral blood and small intestine of tumor-bearing mice (ligo et

Fig. 1. A possible mechanism of the immunomodulatory action of LF. Ingested LF may act on IEC and GALT cells and upregulate the production of IL-18 and other cytokines and chemokines that are released into the blood.



al. 1999; Wang et al. 2000; Kuhara et al. 2001). The number of these cells in the blood, small intestine, and spleen was also increased by oral administration of LF or its pepsin hydrolysate even in non-tumor-bearing mice. Moreover, it has been shown that oral adrninistration of LF induces IL-18 secretion by epithelial cells of the small intestine in mice (Wang et al. 2000; Kuhara et al. 2001). It is known that IL-18 acts to enhance Thl cell functions and to augment NK cell activity and cytotoxic lymphocyte activity. In fact, IL-18-positive cells have been observed in the small intestines of tumor-bearing mice fed LF (Wang et al. 2000). Correspondingly, another study showed that interferon-y and IL-10 production by murine spleen cells after in vitro stimulation were increased by feeding LF to the donor mice (Nakajima et al. 1999). These findings suggest that oral administration of LF or its derivatives may modulate intestinal mucosal immunity and promote cytotoxicity, leading to an inhibitory effect on infection as well as tumor development.

A recent clinical study showed that oral administration of LF could increase the percentage of ThO and Thl cells in the peripheral blood of patients with chronic hepatitis C (Ishii et al. 2000).

Based on such information, a possible mechanism of the immunomodulatory action of oral administration of LF is shown in Fig. 1. In adult humans and animals, very little of an ingested dose of LF is thought to be absorbed from the intestine. Therefore, ingested LF is thought to act on intestinal epithelial cells (IEC) and gut-associated lymphoid tissue (GALT) cells, probably through a receptor-mediated mechanism. Intake of LF upregulates the production of IL-18, as well as that of other cytokines and chemokines, by IEC or GALT cells. These molecules are released into the blood and may then influence circulating leukocytes, or these molecules may directly stimulate leukocytes in the GALT. Further studies are required to characterize the interactions between LF or its fragments and cells in the intestinal mucosa and to elucidate the detailed mechanism of its immunomodulatory effect.

Clinical application of LF in humans

Sorne clinical trials have been conducted to evaluate the effect of oral administration of LF in humans. In a study of low birth weight infants, feeding of LF-enriched infant formula (containing LF at 1 g L^{-1}) was reported to contribute to formation of a bifidobacteria-rich flora (Kawaguchi et al. 1989). In a clinical study on tinea pedis, a fungal infection of the skin, oral administration of LF showed significant improvement of dermatological symptoms in a dose-dependent manner at a range of 0.6-2.0 g day^{-1} (Yamauchi et al. 2000). Recently, a pilot study of the effect of LF on chronic hepatitis C was reported (Tanaka et al. 1999). Patients with chronic hepatitis C were given LF orally at a dose of 1.8 or 3.6 g day^{-1} for 8 weeks. In four patients with relatively low serum concentrations of hepatitis C virus (HCV) – RNA (100 kcopy mL^{-1} or less), three patients showed a decrease in their serum alanine transaminase and HCV-RNA concentrations (Tanaka et al. 1999). These findings indicate that by choosing the appropriate indicators for evaluation, it may be possible to identify the functions of LF in humans. Finally, no adverse effects of LF were observed in any of the abovementioned clinical trials. In order to clarify the efficacy of oral administration of LF for chronic hepatitis B or C, clinical trials are underway at about 10 institutions in Japan.

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