# Production and Functional Properties of Dairy Products Containing Lactophorin and Lactadherin

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## 1. Introduction

In this chapter, we introduce the possible protective utilization of cow milk proteins, lactophorin (LP) and lactadherin (also known as periodic acid Schiff 6/7 (PAS6/7)), against human rotavirus (HRV) gastroenteritis.

Milk is the natural food of the newborn mammal, and it is endowed with protective components against pathogens, such as antibodies. Our previous studies have demonstrated that the 2 proteins, LP and lactadherin, exhibit potent inhibitory activity against HRV. HRV is the single most important etiologic agent of severe gastroenteritis in infants and young children. To determine whether cow's milk could serve as a protective food additive effective against HRV infection, this chapter discusses the potential utilizations of LP and lactadherin from normal cow's milk to protect against HRV gastroenteritis, focusing in particular on sweet whey, a byproduct of industrial-scale cheese manufacturing.

## 2. Rotavirus gastroenteritis

Infectious gastroenteritis is distinguished between bacterial and viral origin, depending on pathogenesis. Rotavirus, adenovirus, and norovirus are well-known infectious gastroenteritis pathogens of viral origin.

HRV was first discovered by Ruth Bishop et al. in 1973, and was recognized as a major cause of childhood diarrheal morbidity and mortality worldwide (Bishop et al., 1973; Bishop, 2009). The virus is transmitted by the fecal-oral route. It infects the enterocytes of the villi of the small intestine and causes gastroenteritis. The incubation period of rotavirus infection is 2-4 days, and once diarrhea occurs, recovery usually requires approximately 1 week. By the age of 5

years, nearly every child in the world has been infected with rotavirus at least once (Velázquez et al., 1996). The estimated annual incidence of rotavirus gastroenteritis is approximately 114 million episodes requiring home care and 600,000 deaths in children worldwide (Dennehy, 2008). More than 85% of these deaths occur in developing countries, South Asia, and sub-Saharan Africa (Naghipour et al., 2008; Centers for Disease Control and Prevention, 2011). In the absence of vaccination, rotavirus gastroenteritis has been estimated to cause 87,000 hospitalizations in Europe (Soriano-Gabarro et al., 2006), 55,000–70,000 hospitalizations in the USA (Parashar et al., 2006), and 78,000 hospitalizations in Japan (Nakagomi et al., 2005) among children below 5 years of age. Thus, rotavirus gastroenteritis causes large human costs in developing countries and large public medical burdens in developed countries.

In general, vaccination is the most effective method for protection against viral diseases. To reduce the aforementioned global burden posed by rotavirus gastroenteritis, the 2 oral rotavirus vaccines Rotarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq® (Merck and Co., Whitehouse station, NJ) have been licensed for use in more than 100 countries worldwide (Tate et al., 2010). Large-scale trials in Europe and North and Latin America demonstrated that these vaccines are safe and effitive (Ciarlet & Schödel, 2009; O'Ryan & Linhares, 2009). Clinical studies are ongoing in Asia and Africa to assess the safety and efficacy of the vaccines in these populations (Zaman et al., 2010; Armah et al., 2010). However, to reduce the risk of intussusceptions, the first doses of both vaccines are strictly limited between the age of 6-15 weeks, and full doses of vaccines need to be completed by ages 6-8 months (Cortese et al., 2009). Therefore, prophylactic options against HRV infection are needed.

Young mammals depend on passive immunity obtained *via* breast-feeding for resistance against infectious diseases, because their immature immune systems cannot produce antibodies immediately after birth. The mother is able to produce antibodies against infectious agents, and they are passively transmitted to the offspring *via* milk.

It has been proposed that passive protection against HRV infection could be achieved by using immunoglobulin G (IgG) from the colostrum of cows hyper-immunized with rotavirus (Ebina et al., 1992; Sarker et al., 1998). Unfortunately, the clinical use of bovine colostrum from hyper-immunized cows has been limited because of difficulties in large-scale production. Recently, skimmed and concentrated bovine late colostrum (SCBLC) obtained from normal cows at 6-7 days after parturition exhibited high potency in inhibiting human rotaviral replication *in vitro* and *in vivo* (Inagaki et al., 2010a), indicating that SCBLC is likely to play an alternative role to colostrum of cows hyper-immunized with rotavirus.

Furthermore, studies of milk components exhibiting inhibitory activity against rotavirus have also been reported. For example, supplemental dietary whey protein concentrate (WPC) (Wolber et al., 2005; Pérez-Cano et al., 2008) and macromolecular bovine whey protein fraction (MMWP) (Kvistgaard et al., 2004; Bojsen et al., 2007) exhibited protective efficacy against simian rotavirus and murine rotavirus infection *in vivo*. The inhibitory mechanism of WPC remains to be elucidated, whereas Bojsen et al. found that mucin 1 and immunoglobulins were the major rotavirus inhibitors in MMWP (2007).

## 3. Inhibitory activity of LP and lactadherin against HRV infection

Recently, we identified LP16 (16 kDa LP fragment) and bovine lactadherin (PAS6/7) as human rotavirus inhibitors in bovine milk (Inagaki et al., 2010b). In this section, we will describe the anti-viral properties of these components.

## 3.1 Anti-HRV activity of LP

LP was initially found to be a glycoprotein in the heat- and acid-stable proteose peptone (PP) fraction and was referred to as PP component 3 (Girardet et al., 1996). LP is found in bovine, cameline (Girardet et al., 2000), caprine (Sørensen et al., 1997), and ovine milk (Sørensen et al., 1997), but not in human milk (Sørensen et al., 1997).

LP is present at an average concentration of 0.3 g/l in normal bovine milk (Koletzko et al., 2005). LP consists of 2 major glycopeptides; 28 kDa (LP28) and 18 kDa (LP18) (Girardet et al., 1996). LP28 contains 5 partial phosphorylation sites (Ser$_{29}$, Ser$_{34}$, Ser$_{38}$, Ser$_{40}$, and Ser$_{46}$), 3 $O$-glycosylation sites (Thr$_{16}$, Thr$_{60}$, (Kjeldsen et al., 2003), and Thr$_{86}$), and 1 $N$-glycosylation site (Asn$_{77}$) (Girardet & Linden, 1996). It exists in various molecular forms formed via posttranslational modification (Kanno, 1989a, 1989b). LP18 has an amino acid sequence corresponding to the 54-135 C-terminal portion of LP28, and this sequence is thought to occur as a proteolytic degradation product of LP28 (Girardet & Linden, 1996). Thus far, emulsification and inhibition of lipolytic activity have been reported as the characters and functions of LP (Kanno, 1989a; Girardet et al., 1993). Recently, LP has been found to stimulate immunoglobulin production in human hybridoma cells and human peripheral blood lymphocytes (Sugahara et al., 2005). However, its biological function remains unclear.

The inhibitory activity of LP against HRV infection was identified as follows. Previously, Kanamaru et al. (1999) reported that high-M$_r$ glycoprotein fraction (F1) from cow milk whey potently inhibited HRV infection *in vitro*. They reported that F1 formed a complex with various proteins but failed to identify the inhibitory entity in F1. Ten years later, LP was identified as one of the inhibitory components of HRV replication in F1 (Inagaki et al., 2010b). In brief, F1 was initially heated at 95°C for 30 min, rendering milk antibodies inert, and then subjected to ammonium sulfate fractionation. The component with a molecular size of 16 kDa, found in a certain fraction from ammonium sulfate fractionation, exhibited inhibitory activity against HRV replication. Sequencing analysis of this substance resulted in the first 7 N-terminal amino acid residues of ILKEKHL, which is consistent with the sequence of residues 69-75 of bovine LP. Thus, LP16 exhibited a strong inhibitory activity against HRV replication.

Furthermore, a preliminary experiment revealed that LP28 and LP18 potently inhibited HRV infection, suggesting that the consensus structure of LP28 and LP18 (i.e., sequence of residues 54-135 C-terminal portion of LP) was involved in their inhibitory activities (Inagaki et al., unpublished observation). Further studies are in progress for detailed elucidation of the HRV inhibitory mechanism of LP.

## 3.2 Anti-HRV activity of lactadherin

Lactadherin is a major milk fat globule membrane component in milk. Lactadherin in bovine milk is also known as PAS6/7.

Lactadherin consists of 2 N-terminal epidermal growth facter (EGF)-like domains followed by 2 repeated C domains with homology to the C1 and C2 domains of blood clotting factors V and VIII (Mather, 2000). Interestingly, lactadherin has first EGF-like domain containing glycosylation sites, whereas human lactadherin has defects in this domain (Mather, 2000).

Lactadherin binds to integrins αvβ3 (Taylor et al., 1997; Andersen et al., 2000; Hanayama et al., 2002) and αvβ5 (Andersen et al., 2000), which are expressed by endothelial cells. However, the physiological function of lactadherin in milk is little known.

The inhibitory activity against HRV infection of human lactadherin was first identified by Yolken et al. (1992). Furthermore, a previous clinical study indicated a correlation between human lactadherin in breast milk and morbidity due to rotavirus gastroenteritis in young children (Newburg et al., 1998). These reports led us to investigate non-immunoglobulin component(s) of rotavirus inhibitor in bovine milk.

One report indicated that bovine lactadherin did not have anti-HRV activity (Kvistgaard et al., 2004). The study was performed using the human Wa strain rotavirus infected to Caco-2 cells and a short-term (1 h) incubation of cells with lactadherin. On the contrary, the inhibitory activity of bovine lactadherin against HRV infection was reported by Inagaki et al. (2010b). The study was performed using the human MO strain rotavirus infected to MA104 cells and demonstrated that long-term (22 h) incubation of cells with lactadherin resulted in significant antiviral effects. The reasons for the inconsistent results are unclear because of distinct experimental conditions. However, it has been reported that lactadherin binds to MA104 cells via integrin αvβ3 (Taylor et al., 1997; Andersen et al., 2000; Hanayama et al., 2002), which is known as one of the cell receptors for rotavirus (Guerrero et al., 2000). Therefore, the interaction between lactadherin and cell surface components is likely important for its antiviral activity. Thus, the inhibitory mechanisms of lactadherin remain controversial.

## 4. Utilization of sweet whey proteins against HRV gastroenteritis

Sweet whey is manufactured as a byproduct of cheese production. Thus, its production increases as the consumption of cheese expands. However, its routine disposal will become a significant problem in the dairy industry. Therefore, the extended utilization of sweet whey should be pursued. Based on the above findings that LP and lactadherin exhibit inhibitory activities against HRV infection, we attemped to investigate the potential utilization of sweet whey as a protective food additive against HRV gastroenteritis.

### 4.1 Microfiltration retentate fraction (MFRF) from sweet whey

To concentrate LP and lactadherin, we attempted to examine the presence of both inhibitory components in sweet whey, which was produced during cheese manufacturing. As shown in Fig. 1, the concentrate was collected as the MFRF. Then, it was pasteurized by a high-temperature short-time method sterilization (HTST) method consisting of heating at 72°C for 15 s, followed by spray drying (referred to as Dried MFRF).

Fig. 2A shows the result of two-dimensional electrophoresis of Dried MFRF stained with Coomassie Brilliant Blue. Dried MFRF contains α-lactalbumin (α-LA, Mw: 14,100 Da) and β-lactoglobulin (β-LG, Mw: 18,200 Da) as the major protein components. The existence of LP and lactadherin could be observed, although not as obviously as the major components, indicating that the inhibitory components appeared to be contained in Dried MFRF. When immunochemical detection using the specific monoclonal antiserum for each protein (Aoki et al., 1994) was performed, as shown in Fig. 2B and 2C, LP and lactadherin could certainly be detected in Dried MFRF.
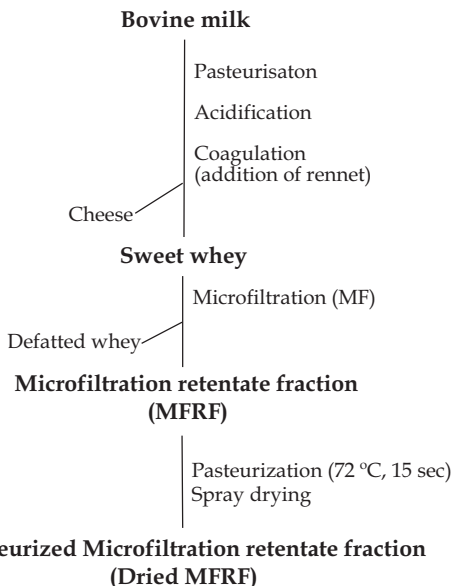
**Bovine milk**

| Pasteurisaton
| Acidification
| Coagulation
| (addition of rennet)

Cheese

**Sweet whey**

| Microfiltration (MF)

Defatted whey

**Microfiltration retentate fraction
(MFRF)**

| Pasteurization (72 °C, 15 sec)
| Spray drying

**Pasteurized Microfiltration retentate fraction
(Dried MFRF)**

Fig. 1. Flow chart for the production of Dried MFRF



(A)                    (B)                    (C)
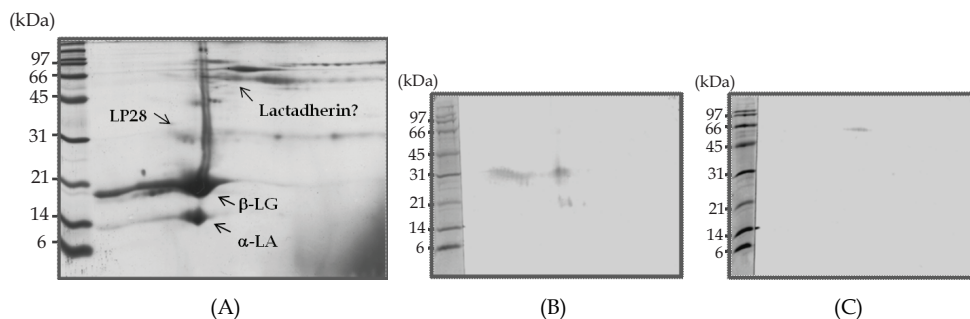
Fig. 2. Dried MFRF contains LP and Lactadherin. A. Two-dimensional protein profile of Dried MFRF. The horizontal dimension was isoelectric focusing (pI, 3.0-10.0), and the second dimension was 15% polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie Brilliant Blue. The molecular weights of the standards (broad range, Bio-Rad) are indicated in kDa on the left. B. Immunochemical detection of LP. Two-dimensional PAGE was performed as in panel A, and then samples were transferred onto a polyvinylidene difluoride (PVDF) membrane and immunostained for LP using the monoclonal anti-LP 1C10 primary antibody (Aoki et al., 1994), followed by horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody. The molecular weights of the standards (broad range, Bio-Rad) are indicated in kDa on the left. C. Immunochemical detection of lactadherin. Two-dimensional PAGE and western blotting were performed as in panel B. Samples were immunostained for lactadherin with the monoclonal anti-lactadherin 3F12 primary antibody (Aoki et al., 1994), followed by HRP-conjugated goat anti-mouse IgG secondary antibody. The molecular weights of the standards (broad range, Bio-Rad) are indicated in kDa on the left

### 4.2 Inhibitory activity of Dried MFRF against HRV infection

Next, we investigated the inhibitory activity of Dried MFRF against HRV infection. A replication inhibition (neutralization) assay for HRV was performed using MA104 cells (African rhesus monkey kidney cell line) following a procedure described previously (Inagaki et al., 2010b) with slight modifications. Our previously published focus reduction assay for rotaviral infection was performed using a suspension of MA104 cells, and a preincubated virus/milk sample mixture was incubated further for 22 h with the cells before fixation. In this study, a confluence monolayer of MA104 cells was established in wells of a glass slide, and a virus/milk sample mixture was inoculated for 1 h and removed from the monolayer before further advancing the viral infection to exclude the influence of the milk sample on MA104 cells by prolonged incubation.

As shown in Fig. 3, MFRF was found to potently inhibit the replication of HRV MO strain (serotype G3P[8]) with an MIC of 3.1 µg/ml. Furthermore, even after pasteurization by HTST method, the neutralizing activity of Dried MFRF remained, with an MIC of 4.7 µg/ml. This activity might also be attributed largely to the heat-resistant character of LP. The colostrums whey from the hyper-immunization of pregnant cows with human rotavirus (rotawhey) was used as a positive control. Rotawhey contains a high level of specific anti-human rotavirus antibodies, and it exhibited a robust inhibitory activity, with an MIC of 0.012 µg/ml (Fig. 3). Bovine lactoferrin also exhibited an inhibitory activity, although weak, with an MIC of 180 µg/ml (Fig. 3). The MIC value of Dried MFRF indicated that it has great potential as a protective food additive against HRV infection.
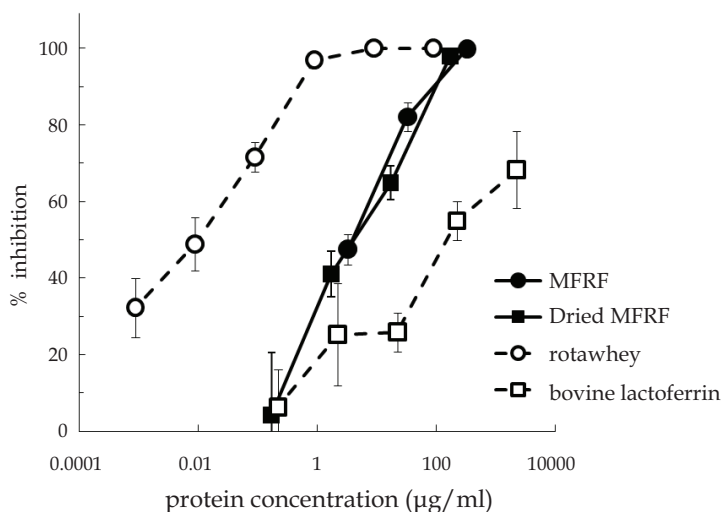


Fig. 3. MFRF exhibits inhibitory activity after pasteurization treatment

MA104 cells were plated into the wells of a 24-well heavy Teflon (HT)-coated slide (AR Brown, Tokyo) and grown to full confluence. A suspension containing infectious virus at a titer of $1 \times 10^5$ - $1 \times 10^6$ fluorescent cell focus-forming units (FCFU)/ml was treated with 20 µg/ml trypsin (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After appropriate dilution

with Eagle's minimum essential medium (E-MEM) containing 2% fetal calf serum to give a titer of approximately $10^3$ FCFU per 100 μl, aliquots were mixed with equal volumes (100 μl) of one-half serially diluted samples in microtubes for 1 h at 37°C. The diluted mixtures (20 μl/well) were added to the confluent monolayer of MA104 cells. The control produced approximately 100 infected foci per well without the test samples of milk. The cells were further cultured for 1 h at 37°C in an atmosphere of 5% $CO_2$. After removal of the inoculums, the cells were washed once with E-MEM to remove unbound virus, followed by incubation at 37°C in an atmosphere of 5% $CO_2$. After 17 h of incubation, the cells were fixed with cold methanol for 10 min. Infected cells were detected by an indirect immunofluorescence assay using the PO-13 monoclonal anti-pigeon rotavirus antibody (Minamoto et al., 1993) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG serum. The foci numbers of infected cells were measured by observation of fluorescence microscopy. Neutralizing activity was expressed as the percentage reduction in the foci numbers of infected cells as compared with infected cells without milk sample. The minimum inhibitory concentration (MIC), the minimum concentration inducing a 50% reduction in infected cells, was calculated for each sample from a logarithmic regression of the concentration-dependent percentage focus reduction. The inhibitory activity of each sample is expressed as a percentage of infected cells as compared to control cells (100%). The experiments were performed in triplicate at least 3 times, and representative results for each sample are given as the mean (SD).

### 4.3 Analysis of the protective components in Dried MFRF

To verify and further characterize the effective components of Dried MFRF regarding protection against HRV infection, we attempted to fractionate Dried MFRF by size exclusion chromatography on Sephacryl S-500 HR. As shown in Fig. 4, 3 fractions were collected according to the elution pattern of Dried MFRF.
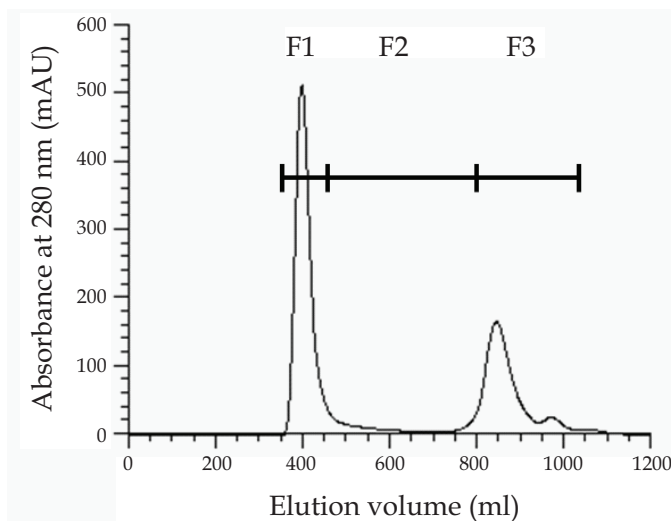


Fig. 4. Fractionation of Dried MFRF by Size Exclusion Chromatography on Sephacryl S-500 HR

The column (60 × 5.0 cm, GE Healthcare UK Ltd., Little Chalfont, UK) was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, 2 mM EDTA, and 0.02% NaN₃. Dried MFRF was dissolved in elution buffer at a concentration of 5 mg/ml, and 30 ml were added to a Sephacryl S-500 HR column. The flow rate was 10 ml/min. Eluted fractions were freeze-dried after dialysis against distilled water.

Next, to investigate the protein components in the fractions, we attempted to resolve the fractions by two-dimensional PAGE. The results are shown in Fig. 5. We confirmed by immunoblot analysis that only F1 contained lactadherin (result not shown). LP was mainly detected in F2 and slightly present in F1. Although 2 major whey proteins, α-LA and β-LG, were detected in each of the three fractions, the vast majority of them detected in F3. Lactadherin and LP28 were present as minor components in F1 and F2, respectively. α-LA and β-LG were present as major components in F3.
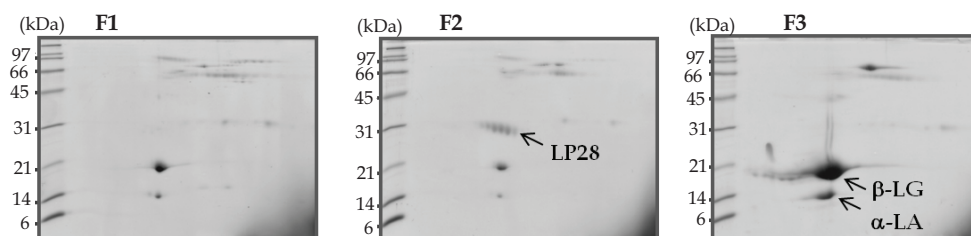


Fig. 5. Protein profiles of size exclusion chromatography fractions. Two-dimensional PAGE profiles of each fractions. The horizontal dimension was isoelectric focusing (pI, 3.0-10.0), and the second dimension was 15% PAGE. The gel was stained with Coomassie Brilliant Blue. The molecular weights of the standard (broad standard, Bio-Rad) are indicated in kDa on the left

As mentioned above, IgG was identified as a rotavirus inhibitor in bovine milk (Ebina et al., 1992; Sarker et al., 1998). Accordingly, to address the contribution of IgG to the anti-HRV activity of Dried MFRF, we attempted to separate IgG in each fraction from other components by using affinity chromatography on a HiTrap Protein G HP column (5 ml, GE Healthcare UK Ltd.). A typical elution pattern of F3 is shown in Fig. 6. The bound fraction was IgG, and the unbound fractions from each fraction were collected as F1′, F2′, and F3′. We found that IgG was removed from F2 and F3, although a small portion remained, as shown in Fig. 6. Conversely, we did not observe the elution of IgG from F1 (results not shown). These results indicated that IgG might represent a minor component in Dried MFRF. In this manner, we obtained 4 fractions: F1′ (fraction containing lactadherin), F2′ (fraction containing LP28), F3′ (fraction containing α-LA and β-LG as major components) and IgG collected from F3.

F3 was dissolved in 20 mM sodium phosphate buffer (pH 7.0) at a concentration of 1 mg/ml. The column was equilibrated with the same buffer, and the column was connected with and controlled by the ÄKTA prime system (GE Healthcare UK Ltd.). The flow rate was 2 ml/min. Proteins were monitored at 280 nm (solid line). The unbound fraction was collected as F3′. The bound fraction, IgG, eluted with a step 100% elution buffer (0.1 M glycine-HCl, pH 2.7) (dotted line). The eluted IgG fractions were neutralized with 1 M Tris-HCl (pH 9.0). Each fraction was freeze-dried after dialysis against distilled water.
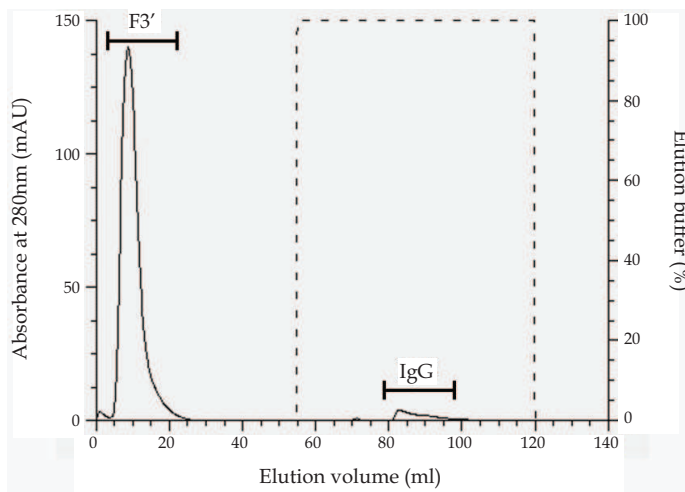
Fig. 6. Fractionation of F3 by Affinity Chromatography on HiTrap Protein G HP column

In the following focus reduction assay, IgG exhibited inhibitory activity against HRV MO infection, with an MIC of 0.27 μg/ml (Fig. 7). F2′ exhibited similar inhibitory activity level as IgG, with an MIC of 0.32 μg/ml (Fig. 7). F1′ exhibited slightly weaker inhibitory activity than did F2′, with an MIC of 1.2 μg/ml (Fig. 7). Although F3, before proteinG affinity chromatography, exhibited a strong inhibitory activity (result not shown), F3′ lost this activity after chromatography, resulting in an MIC of 20,000 μg/ml (Fig. 7). Taken together, the inhibitory components in Dried MFRF should include at least lactadherin, LP, and IgG. These components exhibited very similar activity, although the former 2 could not be
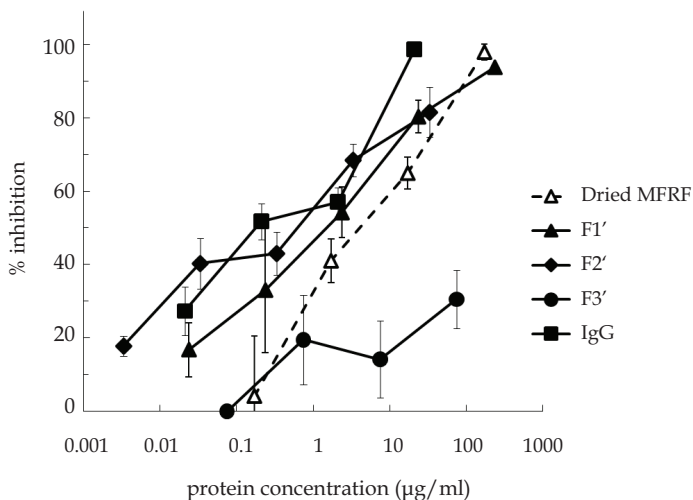


Fig. 7. The Fractions Obtained by Protein G Affinity Chromatography and Their *in Vitro* Inhibitory Activity against HRV MO Strain

purified from Dried MFRF in this study. Our previous study demonstrated that the MICs of lactadherin and LP16 in pure form were 0.016 and 1.8 µg/ml, respectively (Inagaki et al., 2010b). As the precise content of these active components in Dried MFRF is at present not clear, we conclude that their contribution to the inhibitory efficacy against HRV infection of Dried MFRF is likely to be comparable.

Inhibitory activity was determined as described in the legend to Fig. 3. The inhibitory activity of each sample was expressed as the percent decrease in foci numbers of infected cells as compared to the foci numbers of control cells, which were treated with PBS in place of the milk sample (100%). The experiments were performed in triplicate at least 3 times, and representative results for each sample are given as the mean (SD).

### 4.4 Inhibitory activity of Dried MFRF against various types of HRV

Furthermore, we investigated the protective efficacy of Dried MFRF against other types of HRV besides the MO strain. Rotavirus has two independent serotypes (G and P types), and they are defined by VP7 and VP4, respectively. Epidemiological studies on rotavirus showed that strains with G-types of G1, G2, G3, and G4 and those with P-types of P[4] and P[8] are the most prevalent causes of rotavirus gastroenteritis in humans (Gentsch et al., 2005; Santos and Hoshino, 2005; McDonald et al., 2009). Furthermore, the rotavirus G/P-type distribution varies from year-to-year (O'Ryan, 2009). As shown in Fig. 8, Dried MFRF also exhibited inhibitory activity against the Wa strain (serotype G1P[8]) and the Hochi strain (serotype G4P[8]), with MICs of 2.8 and 3.2 µg/ml, respectively. Therefore, Dried MFRF can be concluded to have potential as a protective food additive against several serotypes of HRV.
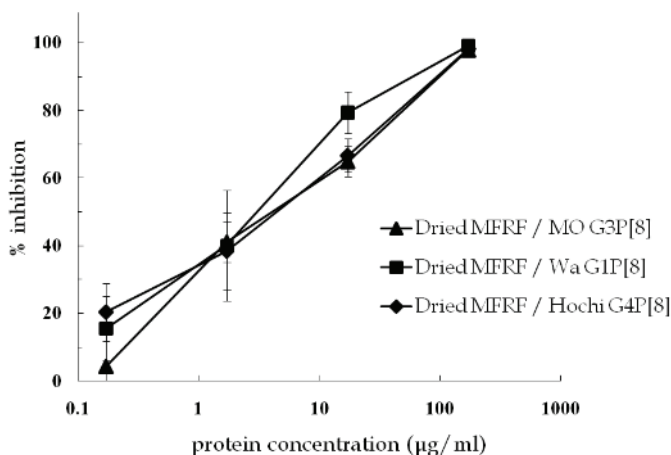


Fig. 8. Dried MFRF Exhibits Inhibitory Activities against Various Types of HRV

Inhibitory activity was determined as described in the legend to Fig. 3. The inhibitory activity of each sample was expressed as the percent decrease in foci numbers of infected cells as compared to the foci numbers of control cells, which were treated with PBS in place of the milk sample (100%). The experiments were performed in triplicate at least 3 times, and representative results for each sample are given as the mean (SD).

## 4.5 Protective efficacy of Dried MFRF against HRV-induced diarrhea in suckling mice

Finally, we investigated whether a single administration of Dried MFRF exhibits prophylactic efficacy against HRV-induced diarrhea *in vivo*. As shown in Fig. 9, in the PBS group, 10 of the 11 mice developed diarrhea 48 h post inoculation (hpi), and all mice recovered from diarrhea by 96 hpi. In the Dried MFRF (2.5 mg) group, only 2 of 16 mice developed diarrhea at 48 hpi, and all mice recovered from symptoms by 72 hpi. In the Dried MFRF (1.0 mg) group, 4 of 11 mice developed diarrhea at 48 hpi, and all mice recovered by 72 hpi. This result clearly indicated that Dried MFRF is a promising candidate for a prophylactic food additive against HRV infection.
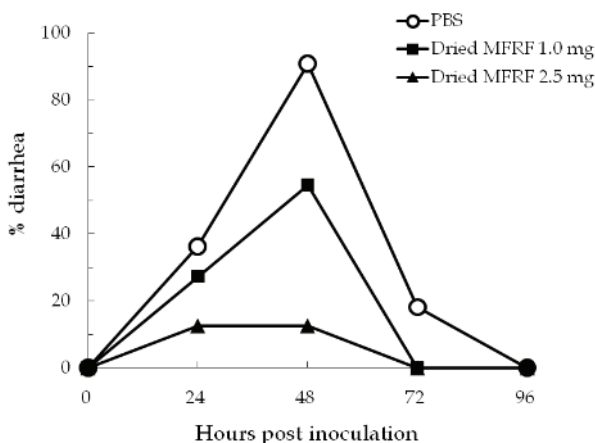


Fig. 9. Dried MFRF Exhibits Preventive Efficacy against HRV-Induced Diarrhea in Suckling Mice

Pregnant BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Litters of 5-day-old mice were orally administered with PBS (n = 11), 1.0 mg of Dried MFRF (n = 11), or 2.5 mg of Dried MFRF (n = 16) for 60 min before inoculation with $2.5 \times 10^5$ FCFU of the HRV MO strain. Stools were examined daily to assess diarrhea for 4 days after viral inoculation. Liquid-like mucous yellow stool was considered diarrhea.

## 5. Summary

Milk contains essential components for child growth. In this chapter, we introduced the inhibitory activity of LP and lactadherin against HRV infection, and examined the possibility of MFRF, which is obtained as a byproduct of cheese manufacturing, as an alternative therapeutic option against HRV gastroenteritis.

Dried MFRF exhibited inhibitory activity against several types of HRV *in vitro*. Furthermore, we demonstrated that prophylactic oral administration of Dried MFRF once before inoculation of HRV prevented the development of diarrhea in suckling mice *in vivo*. Finally, we concluded that Dried MFRF contained LP, lactadherin, and IgG as rotavirus inhibitors. As the anti-HRV activity of LP was not affected by heating at 95°C for 30 min (Inagaki et al., result not shown), the anti-HRV activity of MFRF would be stable to partial heat sterilization.

Recently, it was reported that one-third of all pediatric rotavirus gastroenteritis patients are children between 3 and 6 years of age, an age group outside the primary target of rotavirus vaccine in Japan (Ito et al., 2011; Nakanishi et al., 2009). Thus, these epidemiological studies indicated the need for not only vaccination but also alternative preventive procedures against HRV infection. In conclusion, Dried MFRF, in which the non-immunoglobulin components including LP and lactadherin are concentrated, is a promising candidate prophylactic food additive against HRV infection. Dried MFRF was also found to be a potent inhibitor of several types of bovine rotavirus derived from field breeds (Inagaki et al., results not shown). Rotavirus gastroenteritis is an important issue in livestock animals as well. Taken together, Dried MFRF is very useful as a protective food additive against rotaviral infection.

## 6. Acknowledgements

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**Food Additive**

Edited by Prof. Yehia El-Samragy

A food additive is defined as a substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value. Food additives are natural or manufactured substances, which are added to food to restore colors lost during processing. They provide sweetness, prevent deterioration during storage and guard against food poisoning (preservatives). This book provides a review of traditional and non-traditional food preservation approaches and ingredients used as food additives. It also provides detailed knowledge for the evaluation of the agro-industrial wastes based on their great potential for the production of industrially relevant food additives. Furthermore the assessment of potential reproductive and developmental toxicity perspectives of some newly synthesized food additives on market has been covered. Finally, the identification of the areas relevant for future research has been pointed out indicating that there is more and more information needed to explore the possibility of the implementation of some other materials to be used as food additives.

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