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HIROYOSHI INOUE*, MARIKO YAMASAKI*,
D. SHANTHANA LAKSHMI*, TOSHIYUKI KOJO*,
SHO-ICHI YAMAGISHI#

EFFECTS OF SUPERSONIC TREATMENT OF LACTOFERRIN SEPARATION FROM WHEY WASTE USING REVERSED MICELLAR SYSTEM

A cationic oleic acid reversed micellar system was used to simplify and enhance the purification of bovine lactoferrin (Lfn) from whey. The rate of Lfn extraction by the application of 30 kHz supersonic treatment to the organic solvent phase ($P < 0.01$) was by 1.5 time higher compared to non-supersonic treatment. An increase in water content and the light scatter were observed in the organic phase when the supersonic treatment was applied. An increase in the efficiency of Lfn extraction by supersonic treatment of an organic phase was attributable to increased water dispersion in the organic solvent phase and an increase in the orientation of the cationic oleic acid towards microparticles of water in organic solvent phase.

Keywords: *lactoferrin, whey, reversed micellar system, supersonication, lipid*

1. INTRODUCTION

The cheese-manufacturing industry produces 330 million tons of whey waste worldwide each year. Lactoferrin (Lfn) is one of the major glycosylated proteins found in whey waste. Bovine-derived Lfn is a single-chain polypeptide with a molecular weight of about 80 kDa. The researchers are interested in Lfn because of its antimicrobial activity and its role in the modulation of iron metabolism during inflammation [1]. Recent research has revealed a large number of other possible functions of Lfn and many of them do not appear to involve iron binding [2]. However, Lfn is normally detected only in the external secretions and the secondary granules of neutrophils in the adult animal. The concentration of this protein in serum is normally very low (less

Radioisotope Institutes for Basic and Clinical Medicine*, Department of Medicine#, Kurume University School of Medicine, 67 Asahi-machi, Kurume, 830-0011, Japan. E-mail: hiroin@med.kurume-u.ac.jp; TEL: +81-942-317584; FAX: +81-942-317702

than 1 μg/cm^3) probably because of the neutrophil degranulation as suggested by an increase in Lfn concentration in serum during infection or inflammation [3] and during exercises [4].

The isoelectric point of Lfn varies between 7.8 and 8.0. Thus, Lfn is positively charged at the whey pH ranging 6.0 to 7.0, while all other whey proteins are negatively charged. Many milk manufacturers make use of these characteristics of Lfn and whey in order to separate them on a commercial scale using a cation-exchange resin. However, the existing methods require a large facility for Lfn separation which is not efficient (only 50%) because it is absorbed in a deeper layer resin. High salt concentrations do not prevent Lfn from adsorption. In addition, a small amount of immunoglobin G (IgG), which has as isoelectronic point at pH 5.1–7.4, is also separated with Lfn by means of existing method.

We have developed the system for Lfn separation using reversed micelles with a negatively charged lipid and surfactant as shown in figure 1 [5]. The reversed micellar system is composed of nm-sized water and surfactant aggregates dispersed in an organic solvent. Water-soluble biomolecules, such as protein, disintegrate into the microparticles of water. Therefore, facilitating the transformation from the biomolecules into the microdroplets of water would allow an effective extraction, and the organic solvent would act to protect the biomolecules from denaturation and enzyme deactivation [6], [7].

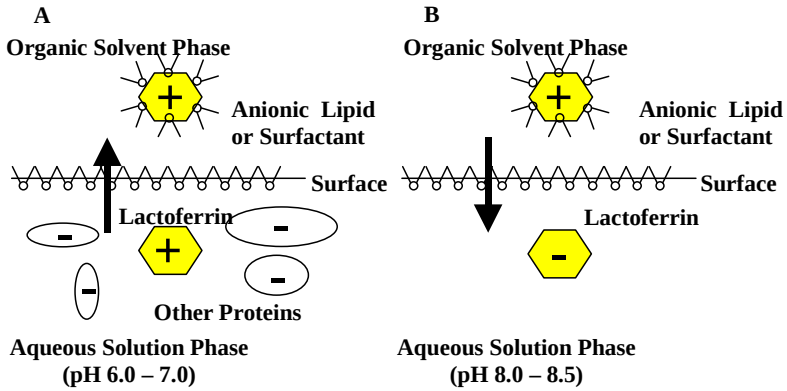

Fig. 1. A schematic representation of the extraction procedures used to separate lactoferrin from whey proteins

In the present study, a continuous reversed micellar system was investigated using a liquid membrane apparatus. Although large amounts of whey could not be instantly treated, Lfn could be separated in an oleic acid sodium salt–isooctane system. The contamination of oleic acid and sodium salt in the extracted Lfn did not pose a problem for food safety, although the other synthesized surfactants could. Our method is inexpensive,

requires simple equipment, and the lipids and organic solvents can be reused. The effect of supersonic treatment on the reversed micellar system was examined to determine whether this would enhance the Lfn extraction.

2. METHODS

Whey was a gift from Omu Milk Industries, Inc. (Omuta, Japan). Oleic acid sodium salt, isooctane, decanol, HCl, and NaOH were of reagent grade and supplied by Nacalai Tesque Inc. (Kyoto, Japan).

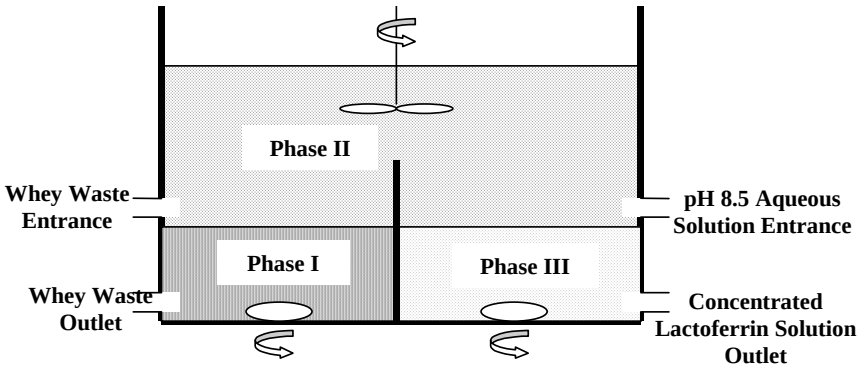

Fig. 2. Apparatus for continuous lactoferrin extraction

A schematic representation of the apparatus is shown in figure 2. Phase I contained 0.5 mmol of oleic acid sodium salt per 1 dm^3 of whey waste. Phase II contained 200 mmol of decanol in 1 dm^3 of isooctane solution. Phase III contained an aqueous solution of pH 8.5. 100 cm^3 of aqueous solution were placed in both phases I and II, and 200 cm^3 of organic solvent were placed in phase III. All phases were stirred at 90 rpm. The extraction of Lfn was evaluated by sampling 0.001 cm^3 of phase III solution 10, 20, 30, 40, 50, 60, 90, and 120 min after setting up and determining the concentration of Lfn by the Bovine Lactoferrin ELISA Quantitation Kit (Bethyl Laboratories Inc., TX). The concentration of IgG in phase III was measured by Bovine IgG-FC ELISA Quantitation Kit (Bethyl Laboratories Inc., TX) and HPLC method. The water content of the phase II was measured by Karl-Fisher titration.

A CAPCELL PAK C_8SG300 S5 column (Shiseido Co. Ltd., Japan) was used for reversed-phase HPLC system (Gilson, Inc., WI). Solvents A and B were prepared as CH_3CN/0.5 mol dm^{-3} NaCl/trifluoroacetic acid = 50/450/0.15 and CH_3CN/0.5 mol dm^{-3} NaCl/trifluoroacetic acid = 500/450/0.3, respectively. The elution of Lfn was carried out over 20 min (0.8 cm^3/min) at 30 °C using a linear gradient of 50%–90% solvent B. The elution of Lfn was monitored at 220 nm.

The supersonic treatment was applied to phase II at output of 50 W and a frequency of 10, 20 and 30 kHz. The dynamic light scattering experiment was performed to measure the extent of the reversed micelle formation with a DLS-700 Dynamic Light Scattering Spectrophotometer (Otsuka Electronics Co. Ltd., Japan). The sample was contained in a 1 cm^2 spectrophotometer cell and placed in an aluminum oven at 25 °C. Dynamic light scattering measurements were carried out using 488 nm light emitted from a 10 mW argon ion laser at a 90° scattering angle. All experiments were carried out at 25 °C ±1 °C.

The values obtained are given as the means without standard errors. The significance of the difference between values was determined using a two-tailed paired Student's *t*-test. The values of $P < 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

Figure 3 shows the concentration of lactoferrin present in the extract obtained in phase III (figure 1) as a function of time. The separation of protein from a bulk aqueous solution and its passing to the reversed micellar solution is governed by steric, electrostatic, hydrophobic, and other molecular properties of the protein and micelle interactions [6]. The interactions between Lfn, surfactants, and lipids were important for effective extraction of Lfn from whey in the reversed micellar system [8].

It was important to stir each phase in the continuous extraction apparatus until the liquid surface ruffled. Lfn was scarcely transferred into the phase III when the surfaces were calm. The amount of the extracted Lfn was totally dependent on the treatment time when the whey containing 105 μg of Lfn in 1 cm^3 was used as shown in figure 3. This result was also checked by HPLC. The IgG that was negatively charged in the whey was not measured by the ELISA method in our experiments. The extraction rate of Lfn was approximately 0.92 $ng \cdot cm^{-3} \cdot min^{-1}$ in the apparatus presented here. The extraction of Lfn was approximately 1.38 $ng \cdot cm^{-3} \cdot min^{-1}$ when supersonication was applied. Extraction of Lfn after supersonic treatment was 1.5 times as effective as that obtained without supersonic treatment ($P < 0.01$). The micelles leave the cavitation area due to supersonic treatment. The supersonic treatment was performed in the organic phase of our reversed micellar system (figure 3).

However, Lfn extraction was not enhanced by an increase in the frequency of the supersonic treatment (no statistical significance; figure 4). In general, a typical industrial high-power generator produces the supersonic frequencies ranging from 20 to 120 kHz. The generated supersonic energy interacts with the liquid media to generate implosions producing the cavitation. High-intensity supersonic treatment can create micro-vapour/vacuum bubbles, which grow to maximum sizes proportional to the supersonic frequencies applied to the liquid medium. The created bubbles then imploded. The higher the frequency, the smaller the cavitation in the medium.

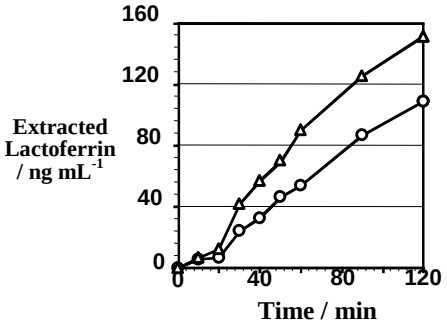

Fig. 3. Lactoferrin present in phase III of the extraction system (figure 1) is shown as a function of time. Circle and triangle signs indicate the experiments without and with 30 kHz supersonic treatment, respectively. Values are the means of five samples

The amount of bubbles created increases greatly when the supersonic frequency applied to the solution exceeds 20 kHz [9]. The size of a bubble is approximately 50–200 μm in diameter at 20 kHz. However, these values being appropriate for an aqueous solution could not be applied to the organic solvent used in our experiment. The formation of cavities is attributable to the natural molecular bonding forces of the liquid medium. The strength of the molecular interactions in water is greater than that of organic solvents, with the exception of solvents with extremely high elasticity [10]. In the water-saturated isooctane/decanol, which has low elasticity, the cavitation may be produced even at 10 kHz. The extraction of Lfn from whey did not depend on the frequency of supersonication used here (figure 4).

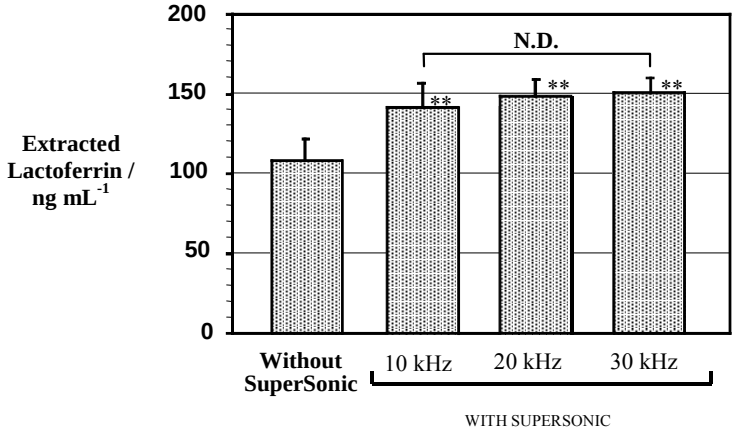

Fig. 4. The effect of supersonication frequency on lactoferrin extraction. The values were determined after 2 h extraction, and the indicated mean ±SDs are represented by vertical bars. $^{**}P < 0.01$ versus "without SuperSonic" group. N.D. indicates no statistical significance

The table shows the effect of supersonic treatment on the water content of phase II in the system (figure 2). The water content was increased remarkably by supersonic treatment. The application of 10 kHz, 20 kHz and 30 kHz supersonication increased the water content by 9.6%, 9.6% and 12.5%, respectively. It has been reported that a change in pH [11], [12] and also nonionic surfactant [6] can increase the water content of organic solvent, and thus facilitate the extraction of Lfn. This is shown in the table and correlated with the increases in Lfn shown in figure 4.

Table

The effects of supersonic treatment on the water content of phase II

Supersonic	Without treatment	10 kHz	20 kHz	30 kHz
Water content	1.04 mol dm^{-3}	1.14 mol dm^{-3}	1.14 mol dm^{-3}	1.17 mol dm^{-3}

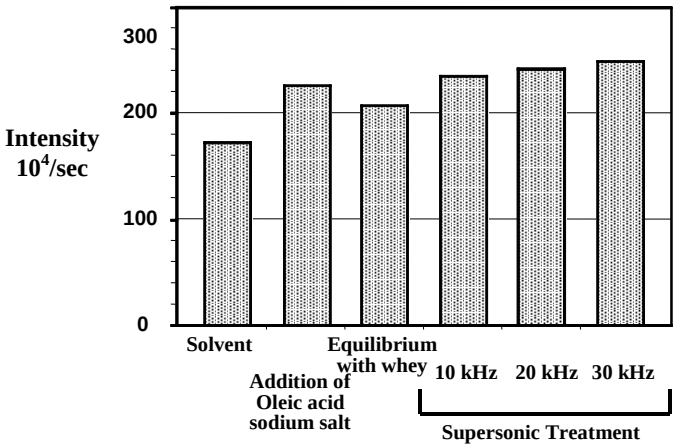

Fig. 5. Intensity of light scattering in the organic solution of phase II. "Solvent" indicates a water-saturated isooctane containing 200 mmol dm^{-3} decanol; "addition of oleic acid sodium salt" indicates that oleic acid sodium salt was added to the solution in the first column. "Equilibrium with whey" indicates that the solution in the secondary column was in equilibrium with whey. "Supersonic treatment" indicates that the solution in the third column was treated by supersonication

The formation of reversed micelles in phase II with or without supersonic treatment was observed in light scattering analysis shown in figure 5. The light scattering in the solution of organic phase II was increased by 32% when 0.5 mol of oleic acid sodium salt was added to 1 dm^{-3} of solution. The reversed micelles were formed by systematic orientation of the oleic acid in the microparticles of water formed in the organic solvent. The light scattering intensity was decreased by 8.5% when the re-

versed micellar phase of oleic acid was in equilibrium with whey. This may have resulted from a partial disruption of the oleic acid in the reversed micelles permitting the entry of the low-molecular weight Lfn protein. The application of supersonic treatment to the reversed micellar system increased the light scattering intensity (figure 5). The light scattering intensity increased as the extraction of Lfn improved (figure 4) when the frequency applied was raised from 10 kHz to 30 kHz. This suggested that the supersonic treatment of the organic reversed micellar phase increased the water content as shown in the table and facilitated the orientation of the oleic acid. In the reversed micellar system used in this study, Lfn was successfully extracted from whey. The supersonic treatment at the frequency range of 10 – 30 kHz increased the extraction rate of Lfn by about 50%. Thus, high efficiency of Lfn extraction obtained due to low-frequency supersonic treatment was really economical.

4. CONCLUSION

Lfn extraction was increased by supersonic treatment in the continuous reversed micellar apparatus used in this study. An increase in the efficiency of Lfn extraction by supersonic treatment of the organic phase was attributable to increased water dispersion in the organic solvent phase and an increase in the orientation of the cationic oleic acid towards the microparticles of water in organic solvent phase.

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