Selective Precipitation of Fat Globule Membranes of Cheese Whey by Saponin and Bile Salt

Der-Chyan Hwang and Srinivasan Damodaran*

Department of Food Science, University of Wisconsin−Madison, 1605 Linden Drive, Madison, Wisconsin 53706

A simple method to flocculate milk fat globule membrane (MFGM) fragments of cheese whey has been studied. Treatment of whey with 0.05% saponin (from *Gypsophila* sp.) and 0.01% bile salt under acidic conditions caused flocculation of MFGM fragments. The optimum pH and temperature for efficient flocculation were found to be 3.75−5.0 and 20 °C, respectively. Centrifugation of the treated whey resulted in a clear whey supernatant which contained almost all whey proteins and less than 0.15% total fat on protein weight basis. Only saponins that contained sugar chains attached to the triterpenoid aglycon were able to cause flocculation of MFGM membranes in the presence of bile salts. Among bile salts, sodium cholate was more effective than sodium deoxycholate.

Keywords: *Cheese whey; lipids removal; saponin; bile salts; flocculation; precipitation*

INTRODUCTION

The demand in the food industry for low-cost, functional, and nutritionally excellent proteins for use in processed foods provides an opportunity to expand the utilization of whey proteins. The factors that limit the use of whey protein products, such as whey protein concentrates (WPC) and isolates (WPI), are several. These include the economics of production of whey proteins with low lactose and mineral contents, flavor stability of WPC during storage and processing, and poor functionality, such as foaming, emulsification, and gelation properties, of commercial WPC prepared by the ultrafiltration method. The root cause of these problems can be attributed to lipids present in the whey. Cheese whey contains about 0.02% lipids (Marshall, 1986). The majority of the phospholipids are derived from milk fat globule membrane (MFGM) fragments that remain dispersed in a stable colloidal state. Triglycerides found in centrifuged whey are also associated with MFGM fragments in the form of butyrophilin−TG complex coat material (Keenan et al., 1988). The turbidity of the whey is mainly due to light scattering caused by these micrometer-sized membrane fragments. During ultrafiltration, they foul the membrane and greatly reduce the flux rate. In addition, because of retention of MFGM in the retentate, WPC prepared by ultrafiltration usually contains about 5-15% fat (Morr et al., 1973). Oxidation of these predominantly phospholipids during storage and processing impairs the flavor stability of WPC- and WPC-containing food products (DeBoer et al., 1977). These lipids also adversely affect the emulsifying and foaming properties of whey proteins (Morr, 1986).

Thus, it is evident that development of methods or processes to remove and/or reduce the fat content of WPC is imperative to solving several of the problems that limit utilization of whey proteins in processed foods. Several approaches to reduce the fat content of the whey prior to ultrafiltration have been examined in the past (Attebery, 1971; Fauquant et al., 1985). Attebery (1971) reported that addition of 0.075 M calcium ion to cheese whey at pH above 6.0 and at 60 °C resulted in formation of a precipitate which contained almost all of the original fat in the whey. Variations of this original approach have been reported (Fauquant et al., 1985; Pierre et al., 1992; Maubois et al., 1987). Maubois et al. (1987) reported that addition of 1.2 g/kg calcium to whey at 2 °C, followed by rapid heating to 50 °C and holding for 8 min, caused precipitation of lipids. Although this method effectively removes lipids, the economics of this process is not known.

In the present study, we show that MFGM fragments can be selectively flocculated and precipitated by adding saponin and bile salt under appropriate conditions.

Rationale of the Approach. The colloidal stability of MFGM fragments in cheese whey is mainly due to high surface charge density contributed by the phosphate groups of the bilayer membrane. The high hydrophilicity of the membrane surface, coupled with electrostatic repulsion between the membrane particles, is responsible for the stability of these particles against flocculation. It follows then that if the hydrophobicity of the membrane surface is increased by attaching hydrophobic molecules on the surface of the membrane, above a critical hydrophobicity/hydrophilicity ratio the membrane particles should be able to overcome electrostatic repulsive forces and should spontaneously flocculate via hydrophobic interactions.

The hydrophobicity of the membrane surface can be increased by attaching saponin molecules. Saponins are steroidal or triterpenoid aglycons with one or more sugar chains (Price et al., 1987). Saponins are naturally present in food plants, and the general structure of triterpenoid-type saponin is shown in Figure 1. In most triterpenoid-type saponins glycosylation occurs at carbon atoms 3 and 28 of the aglycon moiety (Price et al., 1987). It is known that saponins possess hemolytic activity. The hemolytic activity is mainly due to interaction of the aglycon moiety with cholesterol in cell membranes, which results in the formation of "holes" in the membrane (Segal et al., 1966). Recent findings indicate that, depending upon their structures, saponins may have two modes of interaction with lipid bilayers. These are interaction of the aglycon moiety with membrane cholesterol molecules (which involves insertion of the aglycon moiety into the bilayer) and interaction of the sugar chain with the specific sugar binding groups

* Author to whom correspondence should be addressed.

0021-8561/94/1442-1872$04.50/0 © 1994 American Chemical Society

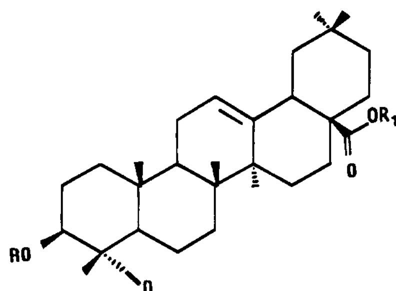

Figure 1. General structure of triterpenoid-type saponins. R and R_1 are either H atoms or sugar chains.

on the membrane surface. Saponins with no sugar moiety freely insert themselves into the cell membrane and cause hemolysis. Saponins with one sugar chain attached at either the 3- or 28-position may interact with the cell membrane either via interaction of the aglycon moiety with cholesterol molecules or through binding of the sugar moiety with the membrane glycoproteins. It is reasonable to expect that under normal conditions both of these modes of interaction will have equal probability. In such a case, those that interact via the aglycon moiety will create holes in the membrane but will not increase the hydrophobicity of the membrane surface, whereas those that interact via the sugar moiety will have the aglycon moiety exposed at the membrane surface and thus may increase the hydrophobicity of the membrane surface. Saponins with two sugar residues at the 3- and 28-positions show very little hemolytic activity compared with saponins with one sugar chain or no sugar chain (Oleszek, 1990). This is apparently because of inaccessibility of the aglycon moiety to the membrane cholesterol molecules due in part to steric hindrance from the hydrophilic sugar chains. Recent studies have shown that saponins containing two sugar chains (from *Panax ginseng*) caused agglutination of phospholipid vesicles and erythrocytes but had no hemolytic activity (Fukuda et al., 1985). It has been shown that the mechanism of agglutination involves interaction of the sugar chains with membrane phosphotidylcholines and possibly also with membrane glycoproteins.

Since the properties of MFGM fragments are similar to those of cell membranes, it is conceivable that when saponins with one or two sugar chains bind to the MFGM surface, they should increase the surface hydrophobicity of the membrane surface and also should cause agglutination of the membrane particles. The flocculation and precipitation of these MFGM–saponin complexes can be greatly enhanced further by adding sterol-type molecules, e.g., bile salts (derivatives of cholesterol), which are known to interact with the aglycon moiety of saponins.

Using the above rationale, we investigated the ability of saponins and bile salt to flocculate and precipitate MFGM fragments of cheese whey.

MATERIALS AND METHODS

Clarified Cheddar cheese whey was obtained fresh from a local cheese industry. The pH of the fresh sweet whey was about 6.2. The whey was used immediately upon arrival. Saponins (extracted from *Gypsophila* and *Quillaja* species), bile salts (50% sodium cholate plus 50% sodium deoxycholate), sodium cholate, and sodium deoxycholate were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Flocculation of Lipids. The following general procedure was used to study flocculation of milk fat globule membrane fragments of cheese whey by saponin and bile salts: The temperature of cheese whey was brought up to the experimental temperature by incubation in a water bath. The pH of the sweet whey was adjusted to the experimental pH. Stock solutions of saponin and bile salts (10%) were prepared in water. The pHs of these stock solutions were about 6.0 and 8.6, respectively. An aliquot of saponin was first added to the whey, followed by the addition of an aliquot of bile salt stock solution. Since only microliter amounts of saponin and bile salt stock solutions were added, neither the pH nor the volume or composition of the whey was significantly changed by these additions. The treated whey was incubated at the experimental temperature for 10 min. At the end of the incubation period, the solution was centrifuged at 1116*g* for 5 min by using a laboratory clinical centrifuge (International Equipment Co., Needham Heights, MA). The turbidity of the supernatant was measured at 500 nm by using a Beckman DU-68 spectrophotometer. A control, containing no added saponin and bile salt, was performed in parallel. The difference between turbidities of the control and the saponin/bile salt treated whey supernatant represented the efficiency of precipitation and removal of MFGM fragments from the whey.

Unless and otherwise mentioned, saponin from *Gypsophila* species (Sigma) and bile salt (a mixture containing 50% sodium cholate and 50% sodium deoxycholate) were used in all experiments. All experiments were done in triplicate.

Analytical Methods. The protein content and the protein profile of the whey before and after treatment with saponin and bile salt were determined by using high-performance liquid chromatography (HPLC) using a TSK G2000SW size exclusion column (7.5 × 300 mm) (LKB-Pharmacia, Piscataway, NJ) and a UV detector with a 254 nm filter. The untreated whey was first filtered through a 0.2 μm membrane filter to remove MFGM fragments. A 20 μL aliquot of the filtrate was injected into the HPLC column and was eluted with 20 mM phosphate buffer at a flow rate of 1.0 mL/min. The protein profile of the whey supernatant obtained after the saponin/bile salt treatment was determined similarly by injecting 20 μL of the supernatant into the HPLC column.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 5–20% linear gradient slab gels was performed as described elsewhere (Laemmli, 1970). Gels were run at a constant voltage of 50 V for about 12 h and stained with Coomassie brilliant blue R. The molecular weight marker proteins were from Sigma. Protein samples for SDS–PAGE were prepared by mixing whey supernatants with 2-fold concentrated sample buffer solution containing 1% SDS. The mixture was then heated in boiling water for 5 min prior to electrophoresis.

To determine the lipid content, first the untreated and treated wheys were exhaustively dialyzed against water to remove lactose and salts and then lyophilized. The lipid content of these lyophilized samples was determined according to the Mojonnier method (Newlander and Atherton, 1977).

Ultrafiltration. Ultrafiltration of sweet whey was performed using a bench-top ultrafiltration unit (Tri-Clover Inc., Kenosha, WI) fitted with a U4-E500 spiral wound membrane (Desalination Inc., Escondido, CA).

RESULTS

Preliminary experiments indicated that the optimum pH for flocculation of MFGM fragments by saponin and bile salt was below 5.0. To determine the minimum concentrations of saponin and bile salt needed to cause flocculation of MFGM fragments, a 2 × 2 factorial design experiment involving saponin concentration in the range 0–0.15% and bile salt concentration in the range 0–0.1% was carried out at pH 4.5. As shown in Figure 2, addition of either saponin or bile salt alone did not cause flocculation of MFGM fragments and its removal upon centrifugation. Addition of bile salt alone to the whey in fact resulted in an increase in turbidity of the centrifuged supernatant, suggesting that in the

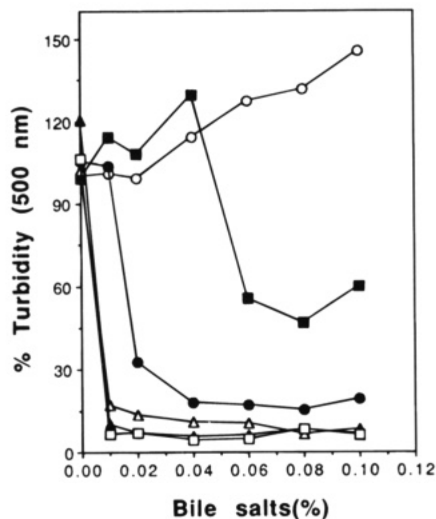

Figure 2. Effects of saponin and bile salt concentrations on precipitation and removal of MFGM fragments from cheese whey at pH 4.5 and 15 °C. Saponin concentrations were (○) 0%, (●) 0.02%, (△) 0.03%, (▲) 0.05%, (□) 0.075%, and (■) 0.1%. The range of standard error for all curves was ±0.2 to ±1.5 (n = 3).

absence of saponin bile salt formed colloidal aggregates at pH 4.5. Addition of saponin alone did not change the turbidity of the centrifuged whey in the time scale studied. However, when the solution was incubated overnight at room temperature, flocculation of MFGM membranes was observed, which upon centrifugation at 1116g for 5 min resulted in a clear whey supernatant; this indicated that flocculation (or agglutination) of MFGM fragments by saponin alone was a slow process. Both saponin and bile salt were required to induce rapid flocculation of MFGM fragments. Maximum removal of MFGM fragments occurred when the bile salt concentration was at or above 0.01% and the saponin concentration was at or above 0.05%. The minimum concentrations of saponin and bile salts that caused maximum removal of MFGM fragments at pH 4.5 and 15 °C were found to be 0.05% and 0.01%, respectively (Figure 2). Under these conditions, more than 85% of the turbidity-causing MFGM fragments were flocculated and removed upon centrifugation. The resulting supernatant was very clear (Figure 3).

The sequence in which saponin and bile salts were added to whey was critical to the efficiency of flocculation of MFGM. Flocculation of MFGM was more efficient when saponin was added first, followed by bile salt addition. The flocculation of MFGM membranes also was observed when a premixed saponin/bile salt stock solution was added to the whey at pH 4.5. However, when bile salt was added first, followed by saponin, to the whey at pH 4.5, no flocculation of MFGM occurred (data not shown). In this case, the supernatant obtained after centrifugation was more turbid than the control whey, probably because of spontaneous aggregation of bile salts into colloidal-size stable particles at pH 4.5.

To determine whether loss of whey proteins occurred during flocculation of MFGM fragments by saponin and bile salt, the protein profiles of the untreated and saponin/bile salt treated whey supernatant were determined by using HPLC (Figure 4). The HPLC profiles were identical except for a peak at the void volume of the column for the untreated whey (Figure 4). This high molecular weight species might be attributed to smaller than 0.2 μm size MFGM fragments that were not

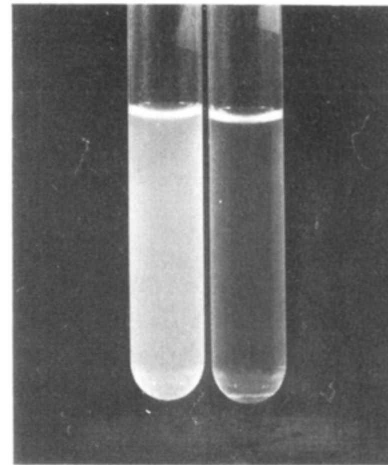

Figure 3. Photographs of untreated and saponin (0.05%)/bile salt (0.01%) treated whey.

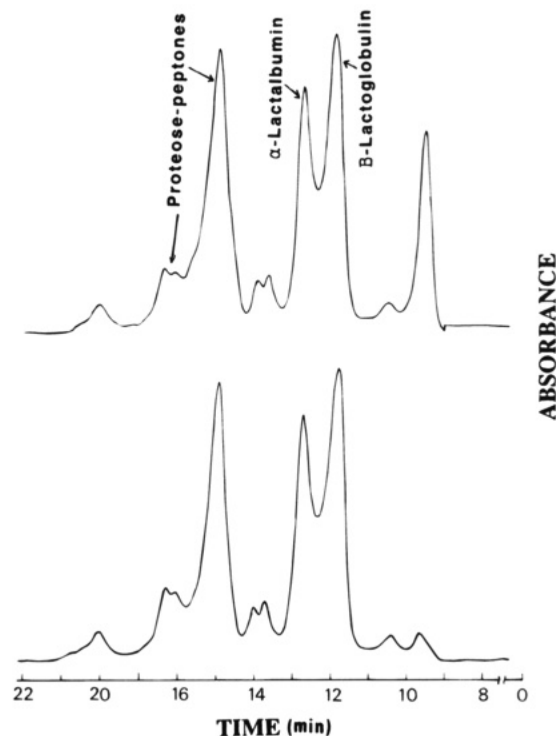

Figure 4. High-performance liquid chromatography elution profiles of untreated (A, top) and saponin (0.05%) and bile salt (0.01%) treated (B, bottom) whey. The column was eluted with 20 mM phosphate buffer, pH 6.8, at a flow rate of 1 mL/min.

retained by the 0.2 μm filter membrane. The fact that the rest of the elution profiles were the same, in terms of the number of peaks as well as the area (intensity) under the peaks, suggests that no soluble whey proteins were lost during flocculation and removal of MFGM fragments by the saponin/bile salt treatment. SDS–PAGE of untreated and saponin/bile salt treated whey supernatants is shown in Figure 5). Both the treated and untreated whey supernatants contained the same number of protein bands, and the intensities of the protein bands, especially the β-lactoglobulin and α-lactalbumin bands, were similar. However, quantitation of the protein bands using densitometry indicated that 16% of total protein was lost after the saponin/bile salt treatment. It should be noted that both the treated and untreated whey supernatants contained IgG, indicating that IgG was not lost during the saponin/bile salt treatment.

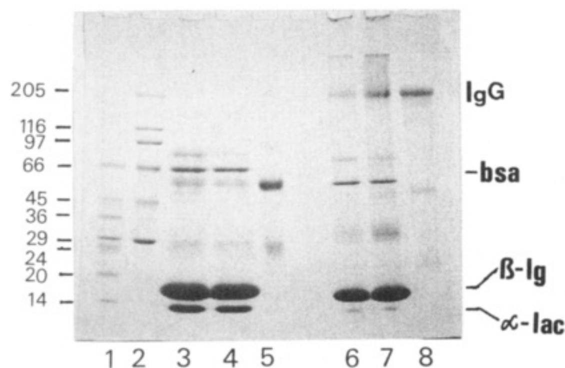

Figure 5. SDS–PAGE of whey. Samples 1–5 contained 5% β-mercaptoethanol in the sample buffer, and samples 6–8 were not treated with β-mercaptoethanol. Samples 1 and 2 were low and high molecular weight markers. Samples 3 and 4 were untreated and saponin/bile salt treated whey. Sample 5 was pure IgG. Samples 6 and 7 were untreated and saponin/bile salt treated whey, respectively, with no β-mercaptoethanol treatment. Sample 8 was IgG with no β-mercaptoethanol treatment.

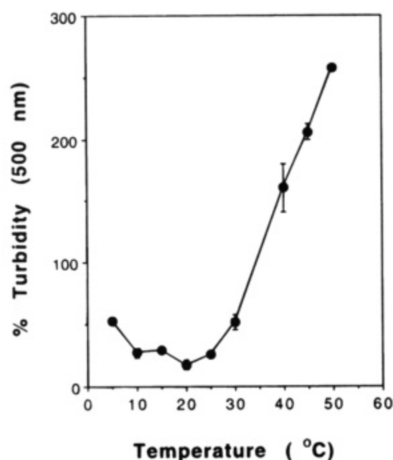

Figure 6. Effect of temperature on removal of MFGM fragments by saponin/bile salt treatment at pH 4.5. Saponin and bile salt concentrations were 0.05% and 0.01%, respectively.

Lipid analysis showed that the dialyzed and freeze-dried whey proteins (free of ash and lactose) from untreated whey contained 1.68% fat, whereas the dialyzed and freeze-dried proteins from the saponin/bile salt treated whey contained 0.15% fat on dry weight basis.

The effect of temperature on the efficiency of flocculation and removal of MFGM fragments by the saponin/bile salt treatment is shown in Figure 6. The optimum temperature for flocculation of MFGM was found to be in the neighborhood of 20 °C. Below and above 20 °C, the turbidity of the supernatant increased. Above 30 °C, the saponin/bile salt treated whey supernatant was more turbid than the untreated whey. This might be partly due to the greater tendency of bile salts to form colloidal aggregates at elevated temperatures at pH 4.5. It is also probable that at high temperatures bile salts might have greater tendency to form mixed co-micelles with saponin (Malinow, 1985), which may then form stable colloidal aggregates at pH 4.5.

The pH optima for flocculation of MFGM fragments by the saponin/bile salt treatment were in the pH range 3.75–5.0 (Figure 7). In this pH range, about 90% of the turbidity-causing materials in the whey were removed when treated with 0.05% saponin and 0.01% bile salt. Above pH 5.0, the turbidity of the treated supernatant increased sharply and reached a maximum value at pH 6.0, which was comparable to turbidity of the control whey. The data indicated that partial neutralization of surface charges of the MFGM fragments and protonation of the carboxyl group of bile salt were essential for complex formation between MFGM, saponin, and bile salt.

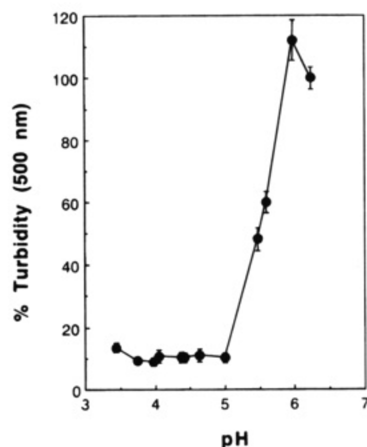

Figure 7. Effect of pH on removal of MFGM fragments by the saponin/bile salt treatment. The temperature was 20 °C, and saponin and bile salt concentrations were 0.05% and 0.01%, respectively.

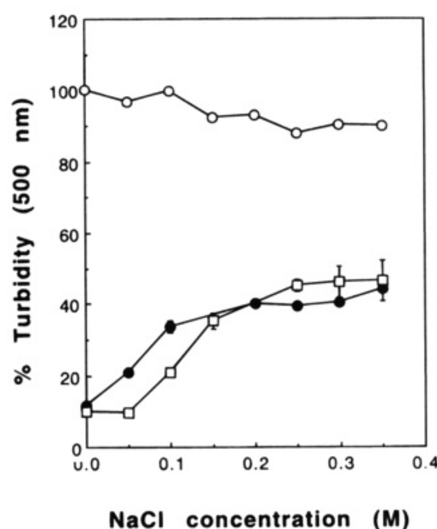

Figure 8. Effect of NaCl concentration on removal of MFGM fragments by the saponin/bile salt treatment at pH 4.5 and 20 °C. Saponin and bile salt concentrations were 0.05% and 0.01%, respectively. (○) Whey treated with NaCl only; (●) whey treated with NaCl, saponin, and bile salts in that order; (□) whey treated with saponin, bile salt, and NaCl in that order.

Addition of NaCl to the whey either prior to or after the saponin/bile salt treatment decreased the efficiency of flocculation and removal of MFGM at pH 4.5 (Figure 8). The turbidity of the supernatant of the saponin/bile salt treated whey increased from 10% to about 40% of the original turbidity with increasing NaCl concentration from 0 to 0.2 M. Increase of NaCl concentration above 0.2 M did not further increase the turbidity of the treated whey. The negative effect of NaCl on flocculation of MFGM fragments by saponin/bile salt treatment cannot be simply attributed to nonspecific electrostatic effect, because neutralization of charges of both MFGM fragments as well as bile salt should in fact enhance flocculation via hydrophobic interactions. Moreover, since the pH optima for flocculation of MFGM fragments by saponin/bile salt treatment exist in a wide

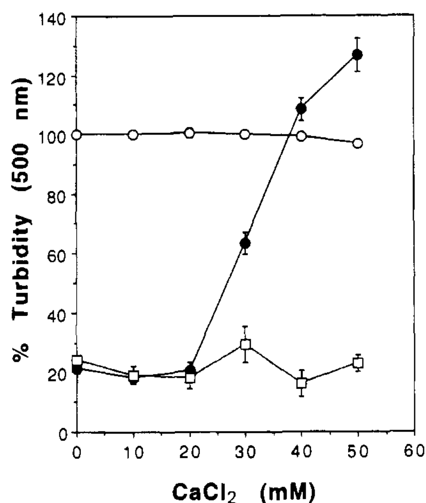

Figure 9. Effect of $CaCl_2$ concentration on removal of MFGM fragments by the saponin/bile salt treatment at pH 4.5 and 20 °C. Saponin and bile salt concentrations were 0.05% and 0.01%, respectively. (○) Whey treated with $CaCl_2$ only; (●) whey treated with $CaCl_2$, saponin, and bile salts in that order; (□) whey treated with saponin, bile salt, and $CaCl_2$ in that order.

range from pH 3.75 to 5.0 (Figure 7), salt-induced shifts in the p*K*s of phosphate and carboxyl groups are unlikely to significantly affect the efficiency of flocculation. On the other hand, it is likely that NaCl might promote formation of mixed micellar aggregates between saponin and bile salt via a salting-out mechanism, and thus decrease their tendency to interact with and flocculate MFGM fragments.

The effect of $CaCl_2$ on the efficiency of removal of MFGM fragments by the saponin/bile salt treatment is shown in Figure 9. The sequence of addition of saponin, bile salt, and $CaCl_2$ had a significant effect on flocculation and removal of MFGM fragments. When $CaCl_2$ was added after additions of saponin and bile salt in that order, apparently there was no effect of $CaCl_2$ on flocculation and removal of MFGM. However, when $CaCl_2$ was added first, followed by additions of saponin and bile salt in that order, the turbidity of the centrifuged whey supernatant progressively increased with increasing $CaCl_2$ concentration above 20 mM. At 50 mM $CaCl_2$ concentration, the turbidity of the treated whey was higher than that of the untreated whey (Figure 9). On the other hand, addition of $CaCl_2$ only to the whey did not change the turbidity of the whey. The data suggested that once a complex was formed between MFGM fragments and saponin/bile salt, addition of $CaCl_2$ to this complex did not affect the flocculating tendency of the complex. However, when more than 20 mM $CaCl_2$ was initially added to the whey, it either prevented formation of MFGM–saponin–bile salt complex or promoted spontaneous formation of saponin–bile salt mixed micellar aggregates before they could interact with MFGM fragments.

Preconcentration of whey by ultrafiltration increased the minimum saponin concentration required to cause maximum flocculation of MFGM fragments but did not affect the bile salt concentration (Figure 10). For example, the minimum saponin concentration required was about 0.15% for the 2-fold and 3-fold concentrated wheys, compared to 0.05% for the single-fold whey. In all of these cases, the minimum bile salt concentration required was 0.01%.

The bile salt used in this study contained both sodium cholate and sodium deoxycholate at 50% levels. To

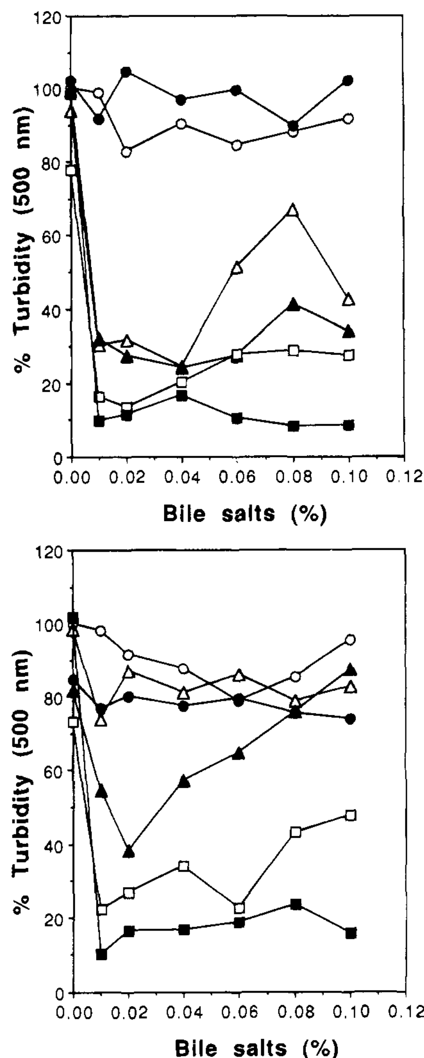

Figure 10. Effect of preconcentration of whey by using ultrafiltration on the removal of MFGM fragments by the saponin/bile salt treatment at pH 4.5 and 25 °C. (A, top) 2-fold concentrated whey; (B, bottom) 3-fold concentrated whey. Saponin concentrations were (○) 0%, (●) 0.03%, (△) 0.05%, (▲) 0.075%, (□) 0.1%, and (■) 0.15%. The range of standard errors for all curves was ±0.2 to ±2.4 (n = 3).

determine whether structural differences between the oxy and deoxy forms of cholate affected their effectiveness to cause flocculation of MFGM fragments, studies were conducted with pure sodium salts of cholate and deoxycholate. The results at 0.01% level are shown in Figure 11. Among these two bile salts, sodium cholate was more effective in causing flocculation of MFGM fragments. The data also suggested that pure sodium cholate might be as effective at 0.005% as the bile salt (1:1 mixture) at 0.01%.

DISCUSSION

The results of this study showed that milk fat globule membrane (MFGM) fragments of cheese whey could be flocculated by adding 0.05% saponin and 0.01% bile salts to the whey at pH 4.5 and 20 °C. The flocculated MFGM–saponin–bile salt complex was easily removed from the whey by centrifugation. The resulting whey supernatant was clear and contained almost all of the proteins and less than 0.15% fat on protein weight basis.

The biological source of saponin influenced its effectiveness to cause flocculation of MFGM membranes. Saponins from *Quillaja saponaria* were practically

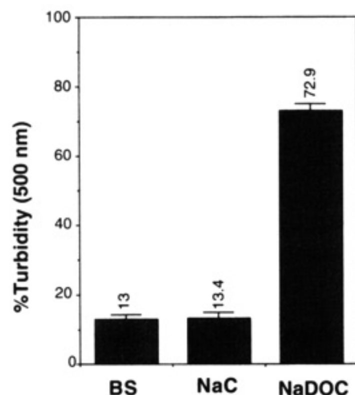

Figure 11. Effect of bile salt structure on the efficiency of removal of MFGM fragments. The concentrations of bile salt and saponin used were 0.01% and 0.05%, respectively. BS, a 50:50 mixture of sodium cholate and sodium deoxycholate; NaC, sodium cholate; NaDOC, sodium deoxycholate.

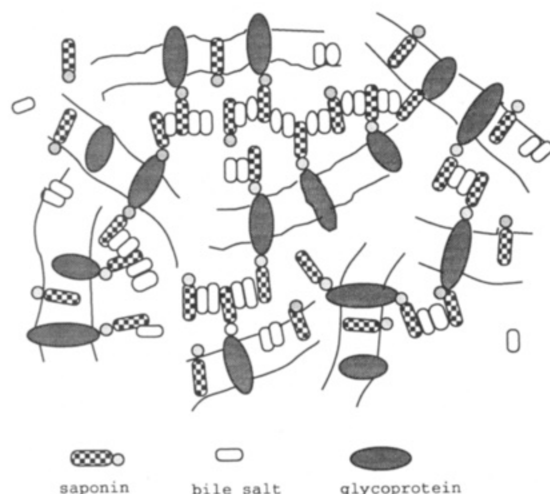

Figure 12. Schematic representation of mechanism of flocculation of MFGM fragments by the saponin/bile salt treatment.

ineffective in causing flocculation of MFGM membranes (data not shown) compared to saponins from *Gypsophila* species. This was probably due to their structural differences. Saponins from *Gypsophila* contain two sugar chains at the 3- and 28-positions, whereas the major saponins from *Q. saponaria* species do not contain sugar chains (Price et al., 1987). As discussed earlier, only saponins with at least one sugar chain can bind to membrane glycoproteins or phosphotidylcholines via the sugar chain, expose the aglycon moiety at the membrane surface, and thus increase the surface hydrophobicity of the membrane. Moreover, interaction of bile salts with this exposed aglycon moiety is possible only when it is exposed at the membrane surface.

The mechanism of flocculation of MFGM fragments by saponin and bile salts is complex, and it apparently involves electrostatic, hydrophobic, and hydrogen bonding interactions. On the basis of the results of this study, a general mechanism for the flocculation (agglutination) of MFGM membranes by saponin and bile salts is proposed (Figure 12): When saponin from *Gypsophila* is added to whey at pH 4.5, the sugar chains at the 3- or 28-position of the aglycon moiety bind to membrane glycoproteins, phosphotidylcholines, and membrane glycolipids. When bile salt is added to this solution, the steroid (cholate) interacts and forms a complex with the aglycon moiety of saponin. At pH 4.5, protonation of the carboxyl group of cholate insolubilizes the saponin–bile salt complex; since the MFGM fragments are bound to saponins, flocculation of the saponin–cholate complex at pH 4.5 *de facto* causes flocculation and precipitation of the MFGM membranes as well.

The method described here to selectively precipitate and remove MFGM fragments from cheese whey has several advantages over other methods. First of all, fat removal is achieved at ambient temperature, which facilitates recovery of proteins in their native state. The ash content of the resulting WPC or WPI should be very low since no NaCl of $CaCl_2$ addition is involved in the method. In addition, since a clear whey supernatant is obtained after the saponin/bile salt treatment, it is possible that 15–20-fold concentration of the whey could be achieved without a major reduction in the flux rate during ultrafiltration. This, however, needs to be studied. The residual amount of bile salt in the whey supernatant, determined according to the method of Gordon et al. (1963), was about 4% of the total amount added to the whey, indicating that 96% of the added bile salt was removed in the MFGM–saponin–bile salt complex. Determination of the residual amount of saponin in the whey supernatant was found to be difficult because of interference from other whey constituents. However, since saponins are naturally found in many food plants, such as beans and spinach (Price et al., 1987), residual amounts of saponin in WPC should not be a major concern. Furthermore, both saponin and bile salt residues can be readily removed by diafiltration of the whey retentate.

ACKNOWLEDGMENT

This research was supported by a grant from the National Dairy Promotion and Research Board and the Center for Dairy Research at the University of Wisconsin–Madison.

LITERATURE CITED

Attebery, J. M. Removing lipid materials from whey. U.S. Pat. 3,560,219, 1971.

DeBoer, R.; DeWit, J. N.; Hiddink, J. Processing of whey by means of membranes and some applications of whey protein concentrates. *J. Soc. Dairy Technol.* **1977**, *30*, 112–120.

Fauquant, J.; Vieco, E.; Brule, G.; Maubois, J.-L. Clarification of sweet wheys by thermocalcium aggregation of residual fatty material. *Lait* **1985**, *65*, 1–20.

Fukuda, K.; Utsumi, H.; Shoji, J.; Hamada, A. Saponins can cause the agglutination of phospholipid vesicles. *Biochim. Biophys. Acta* **1985**, *820*, 199–206.

Gordon, B. A.; Kaksis, A.; Beveridge, J. M. R. Separation of bile acid conjugates by ion exchange chromatography. *Can. J. Biochem.* **1963**, *41*, 77–89.

Keenan, T. W.; Mather, I. H.; Dylewski, D. P. Physical equilibria: Lipid phase. In *Fundamentals of Dairy Chemistry*, 3rd ed.; Wong, N. P., Jenness, R., Keeney, M., Marth, E. H., Eds.; Van Nostrand Reinhold: New York, 1988; pp 511–582.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **1970**, *227*, 680–685.

Malinow, M. R. Effects of synthetic glycosides on cholesterol absorption. *Ann. N.Y. Acad. Sci.* **1985**, *23*, 454.

Marshall, K. R. Industrial isolation of milk proteins: Whey Proteins. In *Developments in Dairy Chemistry—1*; Fox, P. F., Ed.; Elsevier Applied Science: New York, 1986; p 339.

Maubois, J. L.; Pierre, A.; Fauquant, J.; Piot, M. Industrial fractionation of main whey proteins. *Int. Dairy Fed. Bull.* **1987**, *212*, 154–159.

Morr, C. V. Functional properties of milk proteins and their use as food ingredients. In *Developments in Dairy Chemistry—1*; Fox, P. F., Ed.; Elsevier Applied Science: New York, 1986; p 375.

Morr, C. V.; Swenson, P. E.; Richter, R. L. Functional characteristics of whey protein concentrates. *J. Food Sci.* **1973**, *38*, 324–330.

Newlander, J. A.; Atherton, H. V. Babcock, Gerber, Mojonnier tests for fat. In *The Chemistry and Testing of Dairy Products*; AVI Publishing: Westport, CT, 1977; p 103.

Oleszek, W. Structural specificity of alfalfa (*Medicago sativa*) saponin haemolysis and its impact on two haemolysis-based quantification methods. *J. Sci. Food Agric.* **1990**, *53*, 477–485.

Pierre, A.; Legraet, Y.; Fauquant, J.; Piot, M.; Durier, C.; Kobilinsky, A. Role of physicochemical factors in whey clarification. *Lait* **1992**, *72*, 405–420.

Price, K. R.; Johnson, I. T.; Fenwick, G. R. The chemistry and biological significance of saponins in foods and feedingstuffs. *CRC Crit. Rev. Food Sci. Nutr.* **1987**, *26*, 27.

Segal, R.; Mansour, M.; Zaitschek, D. V. Effect of ester groups on the haemolytic action of some saponins and sapogenins. *Biochem. Phamocol.* **1966**, *15*, 1411–1416.

Received for review December 21, 1993. Revised manuscript received May 25, 1994. Accepted June 27, 1994.[⊗]

⊗ Abstract published in *Advance ACS Abstracts*, August 1, 1994.