Characterization of Proteinaceous Membrane Foullants from Whey Ultrafiltration

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ABSTRACT

Cheddar cheeses were made using calf rennet and Mucor pusillus protease. The resultant wheys were processed with a pilot-scale, plate and frame UF system, which contained negatively charged, 10-kdal (nominal) cutoff, polysulfone membranes. In addition, whey from cheese making using calf rennet was treated with Mucor pusillus protease prior to UF. After whey UF, the proteinaceous fraction of the membrane foullants was isolated and analyzed by SDS-PAGE.

Alpha-lactalbumin and several peptides in whey adsorb preferentially onto the surface and foul the UF membranes. Foullant isolated from UF membranes used to process whey from cheese making using calf rennet contained significant amounts of 13.5-kdal and 18 to 22-kdal peptides that appear to be casein proteolysis products. These peptides were not detectable in the unprocessed wheys or in the membrane foullants from UF of whey form cheese making using Mucor pusillus protease. Permeate flux during UF of whey from cheese making using calf rennet was significantly lower than permeate flux of whey from cheese making using Mucor pusillus protease.

When the whey from cheese making with calf rennet was treated with Mucor pusillus protease before UF, permeate flux was improved by 40% when compared with that of untreated whey. Membrane foullants from UF of this whey did not contain the 13.5 and 18 to 22-kdal peptides.

Our results indicate that low permeate flux during UF of whey from cheese making using calf rennet is associated with adsorption of various peptides to the UF membrane. Mucor pusillus protease fragmented many of these peptides in whey from cheese making using calf rennet and increased permeate flux to levels comparable to permeate flux during UF of whey from cheese making using Mucor pusillus protease.

INTRODUCTION

In a previous study it was shown that permeate flux during whey UF depended on the type of milk coagulant used in the cheese making process (24). Permeate flux during UF of whey from Cheddar cheese manufactured with a microbial coagulant isolated from Mucor pusillus was approximately 1.5 times the permeate flux of whey from Cheddar cheese manufactured with the milk coagulant, calf rennet (CR). Because these coagulants are proteases, it was suggested that differences in permeate flux between the two wheys during UF were related to differences in the types of proteolysis products resulting from the coagulants' enzymic activity on milk proteins (24).

Proteins in whey associated with membrane foulling have been studied extensively. Patel and Merson (22) reported that during UF of cottage cheese whey, the membrane foullants were primarily casein and whey proteins. Lee and Merson (17) studied deposit formation during UF of individual whey protein solutions. They found that β-lactoglobulin and γ-globulin caused the most significant flux decline.
Cheryan and Merin (4) studied fouling during UF of individual protein fractions from cottage cheese whey and found α-lactalbumin had the strongest gel layer-forming tendencies and exhibited the lowest initial UF flux.

Caseins have also been implicated as membrane foulants during whey UF. DeWit and DeBoer (7) found that removing casein fines from whey by clarification increased permeate flux twofold during UF. Hayes et al. (11) characterized a casein associated with membrane fouling during whey UF as similar to κ-casein and referred to it as "I-S casein" (casein soluble at pH 4.6).

Cheese wheys should contain very little casein. However, factors such as poor milk quality, poor cheese making practices, or poor milk handling procedures can increase the amount of casein and casein proteolysis products in whey (2, 3, 16).

Little work has been reported that relates casein proteolysis products to membrane fouling. Hickey and Hill (12) have published the only report dealing with casein proteolysis products and their relationship to UF performance. A peptide prepared from casein (amino acids 1 to 23 of α1-casein, approximately 2.5 kdal) did not affect flux. However, the glycomacropeptide from the action of rennin on κ-casein (approximately 8 kdal) caused a 25 to 35% reduction in permeation. Unfortunately, these studies were done on relatively hydrophilic cellulose acetate UF membranes.

Most UF systems used for whey processing today utilize hydrophobic polysulfone membranes and membranes from different suppliers may have different surface charge characteristics. These membranes may have different affinities for individual milk proteins and casein proteolysis products.

Therefore, due to the significant influence of milk coagulants used in cheese making on permeate flux during whey UF (24) and the need for more information on effects of specific milk proteins and their proteolysis products on membrane fouling, this study was undertaken. The objectives were to: 1) identify proteins in membrane foulants from UF of sweet whey resulting from cheese making using calf rennet and M. pusillus protease, and 2) determine if treating whey from cheese making using calf rennet with M. pusillus protease prior to UF influences membrane fouling and permeate flux.

### MATERIALS AND METHODS

#### Whey Production, Handling, and Ultrafiltration Processing Conditions

Three types of Cheddar cheese wheys were studied: CR whey (whey resulting from Cheddar cheese making using calf rennet), M. pusillus whey (whey resulting from Cheddar cheese making using Mucor pusillus protease), and M. pusillus-"treated" CR whey (CR whey treated with M. pusillus protease prior to UF). Laboratory procedures for production and handling of CR whey and M. pusillus whey prior to UF are described elsewhere (23, 24).

*Mucor pusillus* protease-treated CR whey was CR whey that was incubated with *M. pusillus*. Fresh CR whey at 38.5°C was heated to 49°C and passed through a cream separator (DeLaval Model 619) to reduce the fat content to ≤0.05% as measured by the Mojonnier method (20). Separated whey was cooled to 39°C and *M. pusillus* protease was added (90 ml single strength per 454 kg of whey) and incubated for 60 min. After this treatment, the whey was pasteurized (63°C for 30 min), cooled to 57°C held for 30 min, and then cooled to the UF running temperature of 52°C to duplicate conditions utilized in earlier experiments (24). Untreated CR whey and *M. pusillus* whey were subjected to the same pasteurization and cooling prior to UF. All wheys were processed with a pilot-scale, plate and frame UF system (Dorr-Oliver, Inc., Stamford, CT) containing negatively charged polysulfone membranes with a 10-kdal cutoff. The UF process conditions and flux determination methods are described elsewhere (23, 24).

After each whey UF trial, the UF system was flushed with 10 L of 49°C deionized water (no applied back pressure) to remove residual whey from the system. An additional 40 L of deionized water at 49°C was then recirculated at high speed through the system at no applied back pressure for 5 min. The water flush and high speed water recirculation ensured that any residual whey in the system or whey components loosely associated with the membrane surface were removed from the membrane surface prior to foulant sampling. Hence, the material sampled represented foulants firmly adsorbed on the membrane surface. At the end of 5 min of water recirculation, the system was...
shut off, disassembled, and one polysulfone plate was removed for destructive sampling of the membrane.

Electrophoretic Analysis

One half of one side of a fouled UF membrane plate (.17 m²) was removed by carefully cutting it from the plate. This section of membrane was then cut into 1-cm × 6-cm strips and placed into a screw cap test tube containing 10 ml of buffer (10 mM Tris HCl at pH 6.8, 1% SDS, 20% glycerol, and .02% bromphenol blue). The tube was capped and placed into a boiling water bath for 5 min to fully extract the foulants from the membranes. Membrane pieces remaining after extraction were removed from the buffer solution (reextraction of the membrane pieces did not yield any more protein). Hence, the membrane pieces were discarded and .0772 g of reducing agent, dithiothreitol (DTT) were added to the buffer solution (to dissociate disulfide bonded protein polymers). Next, the solution was placed in a boiling water bath for 2 min. Membrane foulant sample solutions were held frozen (−20°C) prior to electrophoretic analysis. Immediately prior to electrophoresis, samples were thawed, boiled for 5 min, and loaded on the electrophoresis gel. A 10 to 20% gradient discontinuous SDS-PAGE procedure (26) was used for quantitative milk protein analysis.

RESULTS AND DISCUSSION

Electrophoretic Analyses of Ultrafiltration Membrane Foulsants

Calf Rennet and Mucor pusillus Trials. To characterize the chemical nature of the proteinaceous fraction of membrane foulants, SDS-PAGE was performed. The SDS-PAGE patterns of molecular weight standards, milk, CR and M. pusillus wheys, CR and M. pusillus whey membrane foulants, and the CR and M. pusillus proteases are shown in Figure 1. Molecular weight standards are shown in lane 1 of Figure 1 for reference.

![Figure 1](image-url)
The electrophoretic pattern of milk is shown in lane 2 (Figure 1). Seven major milk proteins were resolved clearly. They were from top (-) to bottom (+) bovine serum albumin, \( \alpha_c \)-casein (\( \alpha_c1 \) and \( \alpha_c2 \)-casein), \( \beta \)-casein, \( \kappa \)-casein, \( \beta \)-lactoglobulin, \( \alpha \)-lactalbumin, and a proteolytic fragment of \( \beta \)-casein (26). Molecular weights were assigned to all unknown peptides in the membrane foulants based on the plot of log (molecular weight) vs. log (percent total acrylamide) developed from SDS-PAGE of molecular weight protein standards and a milk sample as described by Verdi et al. (26).

Lanes 3 and 4 (Figure 1) are typical electrophoretic patterns of fresh CR whey and \textit{M. pusillus} whey. There were no observable differences between the electrophoretic patterns. Proteins typical of sweet cheese whey were observed; namely, bovine serum albumin, \( \beta \)-lactoglobulin, and \( \alpha \)-lactalbumin.

Typical examples of electrophoretic patterns of the proteinaceous membrane foulants from CR and MP whey UF are shown in lanes 5 and 6, respectively (Figure 1). Both membrane foulants contain large amounts of \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin. However, there are some obvious qualitative differences in the electrophoretic patterns of the two membrane foulants. Prominent protein bands exist in the membrane foulant from UF of CR whey that are not present in the \textit{M. pusillus} whey membrane foulant. These proteins have molecular weights of approximately 18 to 22 kdal and 13.5 kdal. Membrane foulant from UF of MP whey also contains two distinct protein bands less than 13.5 kdal that are not detected in membrane foulant from UF of CR whey.

The only difference between the two Cheddar cheese wheys was the type of coagulant used in cheese making. The protein bands found in commercial preparations of the two coagulants (Figure 1, lanes 7 and 8) do not explain the observed differences in electrophoretic patterns of the UF membrane foulants from CR whey and \textit{M. pusillus} whey. In other words, the proteins observed in the electrophoretic patterns of the CR and MP protease preparations used for cheese making were not detectable in the electrophoretic patterns of membrane foulants from UF of CR and \textit{M. pusillus} whey, respectively (Figure 1, lanes 5 and 6).

Differences in protein present in membrane foulants from UF of CR and MP whey had to originate from subtle differences in CR and MP whey. Because no detectable qualitative differences in the electrophoretic patterns of the two types of fresh whey were observed, it is likely that the peptides of approximately 18 to 22 kdal and 13.5 kdal were present in the CR whey at concentrations too low to be detected. However, during UF these peptides were selectively adsorbed to the membrane and easily detected in the membrane foulant.

A quantitative analysis of proteins separated by SDS-PAGE (Table 1) revealed that the relative distribution of proteins in the two wheys was similar to previous reports (13, 15). No significant differences in the distribution of proteins between the two wheys were detected. The ratio of \( \alpha \)-lactalbumin to \( \beta \)-lactoglobulin was approximately .24 in both wheys.

In contrast, many differences were detected between the types of proteins present in the two membrane foulants. \( \beta \)-Lactoglobulin was a significant proportion of the foulant. This is in agreement with previous studies (18). Lee et al. (19) reported the presence of polymers of \( \beta \)-lactoglobulin in the membrane foulants analyzed by SDS-PAGE. In our study, disulfide-bonded protein polymers could not be measured because of the chemical dissociating agent DTT used in the SDS-PAGE system. One of the reasons why SDS and DTT are used in this electrophoresis system is to cause protein to migrate and be resolved as monomers (10). Lee et al. (19) used a similar gel electrophoresis system of Weber and Osborn (28), which included SDS and the reducing agent \( \beta \)-mercaptoethanol, and thus, their results indicating the presence of polymers of \( \beta \)-lactoglobulin are hard to explain.

Removal of the proteinaceous membrane foulants from the membranes in their native state was not possible in the present study. Hence, any protein-protein interactions in the foulants would have been destroyed during our foulant removal and sample preparation procedure. However, this technique does assure that a representative sample of the individual protein monomers involved in membrane fouling are removed completely from the membrane surface and well characterized by SDS-PAGE.

\( \alpha \)-Lactalbumin was an important foulant. But, more importantly, the ratio of \( \alpha \)-lactalbu-
TABLE 1. Densitometric quantitation of proteins in whey and UF membrane foulants separated by SDS-PAGE.

<table>
<thead>
<tr>
<th>Samples</th>
<th>CR(^2) Whey</th>
<th>\textit{Mucor pusillus} Whey</th>
<th>CR Whey membrane foulant</th>
<th>\textit{M. pusillus} Whey membrane foulant</th>
<th>\textit{M. pusillus} treated CR Whey membrane foulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>X SD</td>
<td>X SD</td>
<td>X SD</td>
<td>X SD</td>
<td>X SD</td>
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<td></td>
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<tr>
<td>Lactoferrin</td>
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<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>. .</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
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<td>Immunoglobulin</td>
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<td>ND</td>
<td>. .</td>
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<tr>
<td>(\beta)-Lactoglobulin ((\beta)-LG)</td>
<td>78.0</td>
<td>6.0</td>
<td>78.3</td>
<td>2.0</td>
<td>31.7</td>
</tr>
<tr>
<td>(\alpha)-Lactalbumin ((\alpha)-LA)</td>
<td>18.9</td>
<td>5.0</td>
<td>18.2</td>
<td>2.0</td>
<td>31.1</td>
</tr>
<tr>
<td><strong>Proteolysis products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 to 22 kdal</td>
<td>ND</td>
<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>10.1</td>
</tr>
<tr>
<td>Unknown #1 (13.5 kdal)</td>
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<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>18.2</td>
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<tr>
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<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>2.1</td>
</tr>
<tr>
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<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>ND</td>
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<tr>
<td>Other</td>
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<td>. .</td>
<td>ND</td>
<td>. .</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Protein ratio</strong></td>
<td></td>
<td></td>
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<td>(\alpha)-LA:(\beta)-LG</td>
<td>.24</td>
<td>. .</td>
<td>.23</td>
<td>. .</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^1\)n = 3. 
\(^2\)CR = Calf rennet. 
\(^3\)Not detectable (less than .1% of total area). 
\(^4\)Molecular weight less than 13.5 kdal.
min to β-lactoglobulin in the two foulants was much higher (foulant from UF of CR whey 1:1; foulant from UF of M. pusiUus whey 6.8:1) than in the two fresh wheys (.24:1). Hence, α-lactalbumin is preferentially adsorbed to the membrane surface. Similar findings have been reported for membrane foulants from milk UF (25).

The preferential adsorption of α-lactalbumin on polysulfone UF membranes is difficult to explain at this time. It has been suggested that calcium may play a role (23, 25). The UF membranes used in this study were negatively charged and at the pH of sweet whey, α-lactalbumin also has a net negative charge. Thus, divalent calcium ions may act to form salt bridges between α-lactalbumin and the membrane. The strong calcium-binding sites in α-lactalbumin, identified by Kronman et al. (14), may be important.

Significant quantities of intermediate to low molecular weight peptides were also evident in the membrane foulants. Recall, these polypeptides were not detected in the two fresh wheys by SDS-PAGE. The 13.5-kdal peptide (unknown 1) accounted for 18% of the protein in the CR membrane foulant, but it was not detected in CR whey prior to UF. The results reemphasize that these peptides were present at such low concentrations that they could not be detected in the electrophoretic patterns of fresh wheys, but preferential adsorption of these peptides on UF membranes resulted in their detection in the membrane foulants.

These peptides are thought to be produced by proteolysis of caseins by the milk coagulants (8, 23). Casein proteolysis products may act as direct foulants. However, they may form complexes with other whey proteins that may foul UF membranes and increase membrane resistance to permeation. Thus, the higher proportion of β-lactoglobulin in membrane foulant from UF of CR whey may be related to the corresponding higher levels of casein proteolysis products present in foulants from UF of CR whey.

Comparison of Calf Rennet and Mucor pusillus Whey Trials to Mucor pusillus-Treated Calf Rennet Whey Trials

The significance of the 13.5-kdal and 18 to 22-kdal peptides identified as UF membrane foulants from CR whey UF becomes more evident by examining the results obtained from UF processing of M. pusillus-treated CR whey. The distribution of proteins in membrane foulant from UF of MP treated CR whey is shown in Table 1. Three features of this distribution are very important. First, membrane foulant from UF of MP treated CR whey does not contain the 18 to 22 or 13.5-kdal peptides, which represented almost 30% of the total membrane foulant from UF of CR whey (Table 1). Second, the ratio of α-lactalbumin to β-lactoglobulin in foulant from UF of MP-treated CR whey (4.6:1) more closely resembles the ratio of these proteins in membrane foulant from UF of MP whey (6.8:1) than membrane foulant from UF of CR whey (1:1). Finally, unknown 3, which was not found in membrane foulant form UF or CR whey and represented 3% of the total membrane foulant from UF of M. pusillus whey, was almost 15% of the membrane foulant from UF of M. pusillus-treated CR whey.

Average permeate flux and 95% confidence intervals over the 130 min of UF processing for the CR whey treated with M. pusillus protease is shown in Figure 2. Permeate flux results for CR whey and M. pusillus whey reported previously (24) are shown for comparison. Treatment of CR whey with M. pusillus protease prior to UF improved substantially permeate flux (40%) compared with untreated CR whey.

Figure 2. Average permeate flux and 95% confidence intervals over the 130 min process time during UF of calf rennet whey (■), Mucor pusillus whey (●), and calf rennet whey treated with M. pusillus protease (○).
permeate flux. After 130 min process time, the CR whey permeate flux was lower ($\alpha = .05, n = 3$) than both M. pusillus whey permeate flux and M. pusillus-treated CR whey permeate flux (Figure 2). The level of M. pusillus protease added to the CR whey was equivalent to the amount that would be present in M. pusillus whey. A much lower level of M. pusillus protease addition may be capable of producing the desired flux improvement with calf rennet whey. No attempts were made to optimize the amount of M. pusillus protease used or conditions for best activity.

Based on these results, the difference in the electrophoretic patterns of membrane foulant from UF or CR whey and membrane foulant from UF of M. pusillus whey can be interpreted. The absence of the 13.5-kdal and 18 to 22-kdal peptides in the membrane foulant from UF of M. pusillus-treated CR whey was associated with an increase in permeate flux compared with untreated CR whey. Apparently, M. pusillus protease can hydrolyze the 18 to 22-kdal and 13.50-kdal peptides in CR whey to lower molecular weight products which do not increase resistance to permeation. The fact that these peptides are present in CR whey membrane foulants and are absent in M. pusillus-treated CR whey membrane foulants supports this interpretation. However, M. pusillus protease may produce an entirely different set of peptides than CR, which may have a lower affinity for the membrane surface (lower fouling tendency). Regardless, these observations demonstrate the importance of trace amounts of specific peptides in membrane fouling and permeate flux during whey UF.

It is generally thought that proteolytic cleavage by these milk coagulants is highly specific for the phenylalanine-methionine (position 105-106) bond of $\kappa$-casein during the primary phase of milk coagulation (8, 9, 27). Hence, it is important to note the wide array of trace proteolytic fragments that appeared as important components of the membrane foulants from the UF of whey produced by these milk coagulants, particularly calf rennet.

Most literature on flux decline has focused on concentration polarization phenomena (5). However, recently, membrane fouling by interfacial phenomena (i.e., adsorption of proteins on membrane surfaces) has received increased attention (1, 6, 21, 23, 25). Results from this study emphasize the importance of casein proteolysis products in fouling and flux decline during the UF of CR whey.

The mechanism by which these peptides contribute to flux decline is unknown. Perhaps these peptides adsorb directly onto the membrane via hydrophobic or ionic interactions to cause resistance to permeation. Proteolysis products may also interact with other constituents of whey (e.g., $\beta$-lactoglobulin) after adsorption to a membrane surface and further increase the resistance of the fouled layer to permeation.

CONCLUSIONS

Alpha-lactalbumin and casein proteolysis products preferentially adsorbed on to polysulfone membranes and were associated with flux decline during whey UF. Differences between UF flux of CR and M. pusillus whey were related to differences in the protein composition of their respective UF membrane foulants. Ultrafiltration of M. pusillus whey resulted in a membrane foulant that contained very little casein proteolysis products. Ultrafiltration of CR whey resulted in a membrane foulant that contained significant quantities of casein proteolysis products. Peptides of approximately 13.5 kdal and 18 to 22 kdal were the most prominent peptides in the membrane foulant from UF or CR whey and were not present in membrane foulant from UF of M. pusillus whey. Restored UF permeate flux and absence of these peptides in membrane foulant from UF of M. pusillus-treated CR whey, support the proposed significance of casein proteolysis products in membrane fouling and flux decline during whey UF. This study provides further knowledge concerning the role of specific milk proteins in membrane fouling and flux decline during whey UF. A better understanding of these processes should lead to the design of UF membranes systems with improved performance for the dairy industry.

ACKNOWLEDGMENTS

We wish to acknowledge Dorr-Oliver, Inc., Stamford, CT for supplying UF equipment and
supplies. Technical assistance from M. Chapman, P. Fleming, R. Rasmussen, and M. Smithers and Cornell University Dairy Plant personnel was extremely valuable.

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