Ultrafiltration of Partially Hydrolyzed Rice Bran Protein to Recover Value-Added Products[1]

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ABSTRACT: Rice brans were treated with a protease together with a disulfide bond-breaking agent (Na_2SO_3) to achieve a 2–4% peptide bond hydrolysis (DH). Ultrafiltration (UF) with 3 kDa molecular weight cut-off (MWCO) membrane led to substantial loss of feed protein due to permeation. Using 1 kDa MWCO membrane increased protein yields, but it was not effective in purifying the protein hydrolysates despite the increase in membrane area and operating time. The efficiency of this UF process can be improved using a larger MWCO membrane (e.g., 2 kDa MWCO), which may facilitate complete removal of phytate. Based on disparity of molecular sizes, use of phytase may also increase purity of protein retentates and allow the recovery of functional inositol phosphates in permeates. The presence of Na_2SO_3 during proteolysis to 2% DH of preheated bran (100°C, 10 min) repaired the damage caused by preheat treatment by increasing protein recovery but increased the concentration of small peptides in hydrolysates, i.e., <1 kDa, particularly for highly aggregated proteins. Heat treatment is necessary to stabilize rice bran, but the sulfite treatment may be avoided to increase UF yield and purity of protein retentates and allow higher DH values for hydrolysis of stabilized brans. Accordingly, this UF process can be an efficient method for recovering high-value components from rice bran, an underutilized rice milling co-product, for many industrial applications.

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Rice bran is an underutilized co-product of rice milling, but it can be a source of inexpensive, high-quality protein for food use. A large portion of the protein in rice bran cannot be solubilized after sequential extraction with water, salt, alcohol, and weak acids. This insoluble protein is called residue protein (1). Residue proteins in rice bran are composed of high molecular weight glutelin polypeptides that are highly aggregated and/or cross-linked by disulfide bridges (1). The approach taken to solubilize rice proteins has been limited to alkali extraction (2–6). Although treatment with high concentrations of alkaline solutions solubilized up to 98% of the total rice proteins, use of high alkali concentration with food proteins is undesirable. It may result in reduced nutritional value, disappearance of lysine, cysteine, and serine, and the formation of lysinoalanine. High alkali concentration also can cause some amino acid racemization and other undesirable reactions. In addition, alkaline-treated foods have been implicated in kidney damage in rats due to the appearance of lysinoalanine (7). Our work is focusing on developing efficient methods to recover the protein of rice bran. These methods may lead to the creation of new value-added, innovative products from rice bran and other rice milling co-products.

In previous studies, proteases were used to enhance the solubility of rice bran proteins. With proteolysis, extraction yield increased significantly as percentage of peptide bond hydrolysis or degree of protein hydrolysis (DH) increased, reaching 92% at a 10% DH value (8). However, extensive enzymatic hydrolysis of most proteins is often accompanied by a bitter taste (9). Accordingly, we used limited proteolysis of rice bran proteins, which was carried out in the presence of a small concentration of sodium sulfite, a disulfide-breaking agent, to boost protein recovery with limited proteolysis. At 2% DH, the presence of sodium sulfite substantially increased protein recovery and yielded protein hydrolysates of a broader range of medium-sized peptides (8).

The objective of this research was to evaluate the effectiveness of ultrafiltration in recovering and purifying rice bran protein hydrolysates. These hydrolysates were prepared by treatment of rice brans from two different rice varieties with a protease to 2 and 4% DH values in a small concentration of sodium sulfite.

MATERIALS AND METHODS

Materials. Alcalase 2.4 L was from Novo Nordisk, Inc. (Franklinton, NC). Alcalase is from *Bacillus subtilis,* generally recognized as safe (GRAS), according to the manufacturer, Novo Nordisk. Defatted rice bran was obtained from the kernels of Cypress and Toro II varieties by milling and defatting with ethyl ether according to the methods of Hamada (1). The 2,4,6-trinitrobenzenesulfonic acid, bicinchoninic

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acid (BCA) protein assay reagent, blue dextran, and protein standards for high-performance liquid chromatography (HPLC) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were reagent-grade or the highest purity obtainable.

Protease treatment of rice brans. Protein hydrolysis was carried out using pH-stat titration at pH 9 and 50°C based on the method of Hamada (8). Suspensions of brans (5.0 g proteins in 250 mL water) from two rice varieties, Cypress and Toro-2, were heated to 50°C, and their pH values were adjusted to 9.0. Sodium sulfite (0.005 M) and alcalase 2.4 L (0.1 or 0.05 g) were added for proteolysis to 2 and 4% DH, respectively. After treatment to desired DH value, the protease was inactivated by a 10-min heat treatment at 85°C (8). The 2–4% DH protein hydrolysates of the bran were recovered by mixing and centrifugation. Also, defatted rice bran suspensions were subjected to heat (100°C for 10 min) before they were treated with Alcalase to 2% DH (but not 4% DH) in 0.005 M of sodium sulfite at pH 9 and 50°C, according to the method described above. Duration of hydrolysis to 2 and 4% DH was 2 and 3.5 h for the protein of Cypress bran and 2.5 and 4.25 h for the protein of Toro-2, respectively. Hydrolysis of preheated Cypress and Toro-2 brans took 1.5 and 1.7 h, respectively. Controls were prepared by repeating proteolysis experiments using deactivated protease for the same duration and conditions used to prepare protein hydrolysates from rice bran. A small portion of the combined supernatants from the controls and protease-treated experiments was lyophilized.

Ultrafiltration of the protein hydrolysates. An Amicon hollow-fiber concentrator Model CH4 (Amicon Corp., Lexington, MA) was used for ultrafiltration of the rice bran protein hydrolysates according to the method of Hamada (10). After sampling, the combined supernatants from the proteolysis experiment (800-mL protein hydrolysate solution containing 3.8–4.3 g protein, pH 9.0) was ultrafiltered. Membranes were 1 ft^2 spiral-wound membrane with molecular weight cut-off (MWCO) of 3 kDa and 3 ft^2 spiral-wound membrane with MWCO of 1 kDa. They were operated at 25°C in a concentration mode at a permeate flux rate of 0.22 and 0.022 L/m^2/min for 60 and 90 min, respectively. The feed pressures for the 3 and 1 kDa membranes were 9 and 29 psi, respectively. The net driving force for the membranes (difference between feed pressure and permeate pressure) was 2.5 and 4 psi, respectively.

Chemical analyses of bran and protein hydrolysates. Protein content of the rice brans, before and after protease treatment, and lyophilized hydrolysates was determined by the combustion method using Leco FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI). The nitrogen conversion factor for proteins and protein hydrolysates was 5.95. The protein contents of protein hydrolysate solutions were determined by the macro method of Lowry *et al.* (11) using BCA protein assay reagents. Rice bran protein hydrolysates and retentates with known protein contents were used as standards. DH value of protein hydrolysates was determined by 2,4,6-trinitrobenzenesulfonic acid to analyze evolved free amino groups (12).

Size exclusion HPLC analysis. Delta Prep 3000 (Waters Corporation, Milford, MA) was used for size-exclusion analysis according to the method of Hamada (8). The protein contents of injected protein hydrolysates and eluted fractions were determined by the BCA protein assay reagents.

Functional properties. Retentates of protein hydrolysates at 2 and 4% DH from the two brans were tested for protein solubility (13) and emulsifying activity (14) at pH 5 and 7.

Statistical analysis. Multifactor analysis of variance of variables, determined in duplicate, was performed using Statgraphics Plus, a software package from Statgraphics Corp. (Rockville, MD).

RESULTS AND DISCUSSION

Extraction yield and molecular weights of hydrolyzed rice bran proteins from two rice varieties. Protein content of the defatted Cypress and Toro-2 brans was 16.3 and 14.5%, respectively. Analysis of free amino groups in the protein hydrolysates revealed that the DH values of rice bran protein hydrolysates prepared using Cypress and Toro-2 brans were 2.2 and 4.0%, respectively. The protein content of lyophilized Cypress protein hydrolysates at 2 and 4% DH was 32.0 and 31.1%, respectively. Lyophilized 2 and 4% DH Toro-2 protein hydrolysates had 28.0 and 27.6% protein, respectively. Protein recovery from defatted rice brans of the two varieties without active protease treatment (control) and after protease treatment to 2 and 4% DH is presented in Table 1. Protein recovered from protein hydrolysates was greater than protein recovered from their corresponding unhydrolyzed controls. With proteolysis, significantly less protein was extracted from Toro-2 bran than that of Cypress at 2% DH. Protein recovery from rice bran increased with increasing DH value to 4%. Calculation of the percentage increase in protein recovery as a result of proteolysis was based on comparing the recovery of protein hydrolysates to their respective controls. For Cypress and Toro-2 brans, protease treatment resulted in 18 and 27% increase in protein recovery at 2% DH and in 22 and 32% increase in protein recovery at 4% DH, respectively. The increase in percentage solubility was significantly more for the bran sample of Toro-2 than that of Cypress.

The use of enzymatic proteolysis significantly enhances the recovery of rice bran protein especially in the presence of disulfide bond-breaking agents. Sodium sulfite disrupts disulfide bonding and extensive aggregation of rice bran protein to

TABLE 1
Protein Recovery from Rice Bran After Protease Treatment in Sodium Sulfite[a]

Protein hydrolysate	Protein recovery (%)			
	Cypress		Toro-2	
	Control	Treated	Control	Treated
2% DH	68	80	59	75
4% DH	71	86	63	83

[a]DH, degree of peptide bond hydrolysis.

maximize the recovery of protein from rice bran (8). Small concentrations of cysteine and sodium sulfite are used to break disulfide bonds in food proteins (15). It is clear that the recovery of rice bran protein hydrolysates was influenced by the variety of rice bran. Although less protein was extracted from Toro-2 bran than that of Cypress, the percentage increase in protein recovery as a result of proteolysis (based on comparison with corresponding controls) was much more for the bran sample of Toro-2 than that of Cypress. This could be explained by the difference in the protein structure of brans from the two varieties. We previously suggested that the insolubility of the glutelins of Cypress and Toro-2 bran was largely due to their strong aggregation through hydrophobic interactions and cross-linking through disulfide bonds, respectively (1). Protease access to protein substrate was probably more in Toro-2 protein than Cypress protein because of more partial breakage of the extensive disulfide bonding in Toro-2 bran proteins.

Figure 1 presents the size-exclusion HPLC fractionation of supernatants of rice bran protein hydrolysates. Molecular weights of eluting fractions were obtained from a calibration curve and are presented in Table 2. Although hydrolyzing Cypress bran proteins to 2 and 4% DH yielded a small percentage of protein hydrolysates with high-molecular-weight polypeptides of more than 90 kDa, most of these hydrolysates had medium-size to small peptides with molecular weights of 10–90 kDa (Fig. 1). On the other hand, molecular weight distribution for Toro-2 rice bran hydrolysates was broadly more even than that of Cypress bran protein hydrolysates (Fig. 1). Most of the peptides of 2 and 4% Toro-2 hydrolysates were also medium- to small-size peptides (Fig. 1). The much broader range of molecular weights for Toro-2 hydrolysates may be due to more breakage of disulfide bonding, leading to more access of protein subunits to the protease. Subsequently, this might have resulted in more proteolysis in Toro-2 protein by disulfide bond breakage than that in Cypress protein. This may also explain why a fundamental proportion of the protein hydrolysates had smaller molecular weight peptides due to sodium sulfite presence.

TABLE 2
Molecular Weights of Eluting Size-Exclusion Fractions of Supernatants (Crude Hydrolysates) and UF Retentates of Rice Bran Protein Hydrolysates[a]

Fraction number	Retention time (min)		MW (kDa)
	Start	End	
1	17	24	>150
2	24	27	150–90
3	27	34	90–50
4	34	41	50–10
5	41	46	10–3
6	46	54	3–1
7	54	75	<1

[a]Relative molecular weight of chromatograms presented in Figure 1 as calculated from elution volumes using a calibration curve. UF, ultrafiltration.

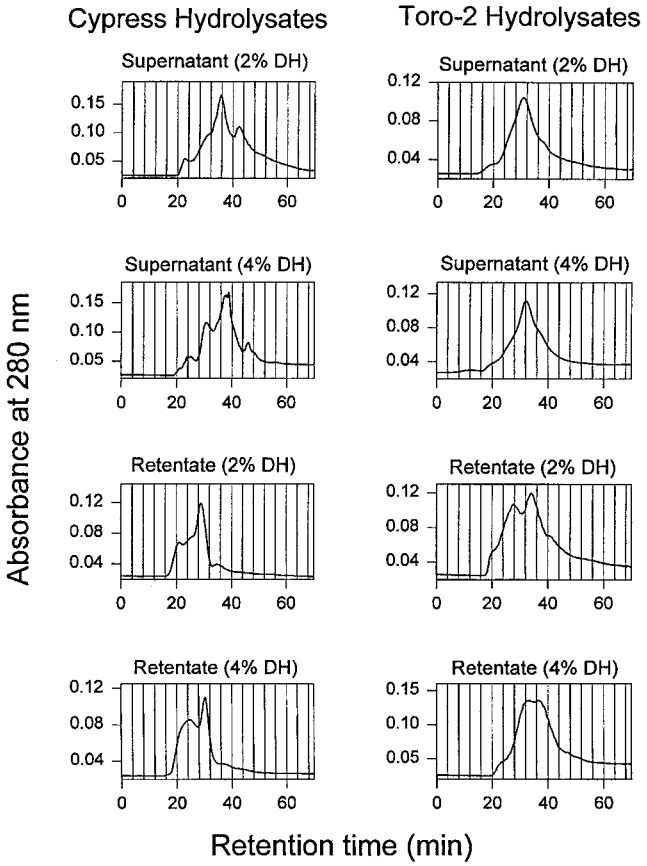

FIG. 1. Size-exclusion high-performance liquid chromatography fractionation of feed (supernatants) and retentates from protein hydrolysates. DH, degree of peptide bond hydrolysis.

At 2% DH, sodium sulfite may have increased the access of individual subunits to protease more for Toro-2 protein than Cypress protein. This could have resulted in more small peptides of molecular weights <3 kDa in Toro-2 hydrolysate. Because of less breakage of disulfide bonding, as hydrolysis progressed to 4% DH, the number of polypeptide bonds cleaved per each single subunit was more for Cypress protein and accordingly yielded more peptide fractions with molecular weight of less than 3 kDa. This may also explain why concentration of small peptides in Toro-2 hydrolysates was not changed when the DH increased from 2 to 4% DH. When hydrolyzing the proteins in Cypress bran to 2% DH, the presence of the sulfite resulted in protein hydrolysates of a broader range of medium-size peptides having M_r of 11–68 kDa (8). However, the small peptides of molecular weight less than 2 kDa without and in the presence of sodium sulfite were 9 and 14%, respectively. Although sodium sulfite is allowed by the U.S. Food and Drug Administration in a low concentration, it may not be desirable in food applications, particularly for consumers allergic to sodium sulfite. Therefore, removal of the small amounts of sodium sulfite used in this study was sought by ultrafiltration. This separation should be quite attainable with the use of proper membrane configuration (16).

Partial purification of protein hydrolysates by ultrafiltration with 3 kDa membrane. Protein hydrolysates extracted

from rice brans were subjected to ultrafiltration (UF) to purify and to remove sodium sulfite. Protein contents of lyophilized retentates at 2 and 4% DH were 44.7 and 42.1% for Cypress hydrolysates and 39.6 and 38.4% for Toro-2 hydrolysates, respectively. Accordingly, UF increased protein content of the freeze-dried 2 and 4% hydrolysates from 32 to 43% for Cypress hydrolysates and from 28 to 39% for Toro-2 hydrolysates. However, UF led to substantial loss of protein of feed (supernatant) due to permeation (passed through the membrane), up to 24 and 35% for the hydrolysates of Cypress at 2 and 4% DH, respectively. Due to permeation, ultrafiltration led to 25% loss of the protein of both 2 and 4% DH Toro-2 hydrolysates. Accordingly, for Cypress bran protein, increasing DH from 2 to 4% led to a 46% increase in the permeated peptide fraction with molecular weight of less than or around 3 kDa. On the other hand, there was no change in the amount of Toro-2 peptide fraction with an average molecular weight of 3 kDa or less.

Cypress supernatants (crude hydrolysates or feeds) contained 18 and 34% of the total protein in 2 and 4% hydrolysates with molecular weight average of less than 3 kDa, respectively (Fig. 1). It appears that all of these peptides were removed since the percentage permeates for Cypress were 24 and 35%, respectively. Similarly, the loss of protein in Toro-2 hydrolysates due to permeation was 25% for both 2 and 4% DH hydrolysates; 27% of the total protein of these hydrolysates had molecular weight average of less than 3 kDa (Fig. 1). Therefore, UF with the 3 kDa MWCO membrane removed all small peptides of less than 3 kDa from all hydrolysates. The differences in the amount of permeated protein between the two brans could have been due to different response to disulfide-breaking agents among the two bran proteins as explained above. This also may explain why permeation of Toro-2 hydrolysates was not changed when the DH increased from 2 to 4%. Another critical factor influencing the permeation of small peptides through the membrane was the broad pore size distribution of the membrane. The broad pore size distribution of the membrane (16) may account partially for the permeation of protein species with molecular weights much greater than 3 kDa through the membrane. Since protein loss for both 2 and 4% hydrolysates was substantial, the use of a membrane with MWCO of less than 3 kDa should be tried to overcome this problem to improve retentate recovery.

As for the UF feeds (supernatants), retentates were separated by size-exclusion HPLC into six fractions (Fig. 1) with the majority of proteins being in fractions 1–4. UF resulted in the production of higher molecular weight aggregates, shifting the molecular weight of peptides to higher ranges in the retentates as compared with crude hydrolysates (Fig. 1). In comparing size fractions of feeds (supernatants) and retentates, retentates gained some of the polypeptides near the void volume. The very high molecular weight Fraction 1 increased three- and sixfold in corresponding retentates of Toro-2 and Cypress protein hydrolysates. Assembled aggregates in the retentates were presumably formed from the high- and low-molecular weight peptides of the original feeds due to the removal of disulfide-breaking agent.

Functional properties of partially purified protein hydrolysates. Protein solubility of retentates at pH 5 and 7 increased with increasing protein hydrolysis (Table 3). Protein hydrolysates at 4% DH also exhibited greater emulsifying activity at pH 7 and under mildly acidic conditions, pH 5, than the 2% hydrolysates (Table 4). Solubility and emulsifying activity increase with increasing protein hydrolysis (17). Both percentage solubility and emulsifying activity increased 32 and 20% at pH 5 and 7 when DH increased from 2% to 4%, respectively, for both protein hydrolysates. This is expected because of the positive correlation between solubility and emulsifying activity, as solubility of the protein is a prerequisite for emulsifying activity (18). The increase in solubility of the 2 and 4% hydrolysates of the two brans may reflect different changes in protein conformation due to increased proportions of the soluble high molecular weight polypeptides in 4% hydrolysates. Adler-Nissen (9) attributed a considerable increase in the protein solubility index of protein hydrolysates to increased proportions of soluble high molecular weight aggregated fractions.

Protein extraction from heat-treated brans by limited proteolysis in presence of sodium sulfite. About 70 and 57% of the total proteins of Cypress and Toro-2 brans were extracted after proteolysis to 2% DH without heat treatment or the use of sodium sulfite, respectively. The recovery of protein, hydrolyzed to 2% DH, from the two brans was reduced to 42 and 35%, respectively, by the heat treatment. After heat treatment of Cypress and Toro-2 brans and proteolysis to 2% DH in the presence of sodium sulfite, 74% of the protein was retrieved from both brans. After it was lyophilized, the latter protein hydrolysates from Cypress and Toro-2 rice brans contained 22.1 and 17.6% protein, respectively. The protein content of these protein hydrolysates was lower than those 2% unheated hydrolysates described above. Evidently, the lower protein content was because heat treatment resulted in the extraction of more nonprotein solubles, e.g., phytic acid, than that of unheated bran. Heat treatment of bran was applied since it is usually used to stabilize its oil (19). Extrusion treatment decreased the solubility of rice bran protein (6). However, the presence of Na_2SO_3 during the proteolysis of heated rice brans substantially increased protein recovery (8), particularly for Toro-2 hydrolysate.

TABLE 3
Solubility of Retentates Recovered from Protein Hydrolysates by Ultrafiltration[a]

Protein hydrolysate	Solubility (%)			
	Cypress		Toro-2	
	pH 5	pH 7	pH 5	pH 7
2% DH	36	41	33	43
4% DH	44	49	38	53

[a]Means within each column differed significantly ($P < 0.05$). See Table 1 for abbreviation.

TABLE 4
Emulsifying Activity[a] of Retentates Recovered from Protein Hydrolysates by Ultrafiltration[b]

Protein hydrolysate	Emulsifying activity			
	Cypress		Toro-2	
	pH 5	pH 7	pH 5	pH 7
2% DH	0.12	0.14	0.13	0.18
4% DH	0.17	0.21	0.18	0.21

[a]Measured as described in Reference 14.
[b]Means within each column differed significantly ($P < 0.05$). See Table 1 for abbreviation.

Partial purification of protein hydrolysates by ultrafiltration with 1 kDa MWCO membrane. UF of heated 2% DH protein hydrolysates was carried out with a 3 ft^2 spiral-wound membrane having MWCO of 1 kDa, which was operated in a concentration mode under the same conditions used for the 1 ft^2 3 kDa MWCO but for 1/2 h longer. The yield of proteins after UF with this membrane is presented in Table 5. In comparison to the 3 kDa MWCO membrane, this UF increased protein yield. Protein contents of lyophilized Cypress and Toro-2 retentates were 21.3 and 17.3%, respectively. Compared to feed, protein contents stayed unchanged in retentates, presumably due to the leak out of small peptides (1 kDa) and equal amount of nonnitrogenous materials. For instance, sodium salts of phytic acid from rice bran and its degradation products including inositol penta- and tetraphosphates have an approximate molecular weight range from 600 to 1000. This membrane process has the potential of being scaled up (10) for the production of a large quantity of these value-added ingredients from underutilized rice bran. However, clearly, the 1 kDa membrane was not effective in purifying the protein hydrolysates despite the increase in membrane area and operating time. The efficiency of this UF process may be improved using a larger than the 1 kDa MWCO membrane. Since the molecular weight of phytic acid is around 1 kDa, use of a membrane with MWCO of 2 kDa may facilitate its complete removal by UF.

Also, a phytase may be added before UF for this process to become more efficient for recovering the proteins of rice bran. Phytase may allow the full recovery from permeate dephosphorelated phytic acid, preferably those functional inositol phosphate isomers known to have high value as pharmaceutical and antioxidant products. Specific enzymatic treatment of phytic acid of rice bran prior to UF can produce these special inositol phosphates. Examples of these specific phytases are the isolates of alkaline phytase prepared by Barrientos *et al.* (20) from lily pollen and by Liu (21) from rice bran.

As shown above, presence of the disulfide-breaking agent increased concentrations of small peptides dramatically with increasing protein hydrolysis, particularly for highly aggregated proteins, i.e., from Cypress bran. The use of a disulfide bond-breaking agent during proteolysis repaired the damage caused by heat treatment by increasing the recovery of both bran proteins. However, the use of sulfite resulted in more formation of substantial levels of small peptides. At 2% DH, the loss of protein from Cypress and Toro-2 hydrolysates due to permeation through the 1 kDa MWCO membrane was 13 and 19%, respectively. As did heat treatment, using disulfide bond-breaking agents denatured the protein and caused a similar effect on the molecular size of the hydrolysates (8). During enzymatic proteolysis to small DH values of heat-denatured proteins, they are fragmented to intermediate molecular weight polypeptides before further hydrolysis to smaller molecular weight peptides with progress of a hydrolysis. However, use of sodium sulfite in combination with heat treatment may have increased protease access to the protein subunits even more. The increased access may have intensified protein hydrolysis in individual subunits, leading to the production of more small peptides. Since heat treatment is necessary to stabilize rice bran and sodium sulfite was used to assist in solubilization, the sulfite treatment can be avoided to reduce the formation of small peptides, thereby improving yield and purity of retentates. This may allow higher DH values for hydrolysis of stabilized brans.

TABLE 5
Recovery of Protein in Supernatants (Crude Hydrolysates) and UF Retentates of 1 kDa Membrane

Sample	% Protein recovery	
	Cypress	Toro-2
Supernatant	73.6	73.6
Retentate	64.2 (87.2)[a]	59.4 (80.8)[a]

[a]% Values in parentheses are percentage UF retention; UF permeation = 100 – % UF retention. See Tables 1 and 2 for abbreviations.

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