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Microfiltration-based separation of milk proteins and their enzymatic hydrolysis using papain

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ABSTRACT

In recent years, consumer preferences have shifted towards healthier food choices, emphasizing quality and nutritional benefits. This study aims to utilize microfiltration to produce milk with a lower protein concentration, thereby improving its suitability for individuals with milk protein allergies. Microfiltration of fat-free UHT milk using ceramic membranes (Membralox – 5, 1.4, and 0.8 μm) demonstrated that a 0.8 μm pore size has efficient protein fractionation at high permeation rates, lowering the permeate protein concentration to <10 g L^{-1} ($R_{Protein}$ = 69.4%). Membranes with larger pore sizes (1.4 and 5 μm) exhibited only marginal protein selectivity, indicating their suitability primarily for cold-sterilization purposes. Enzymatic hydrolysis with papain was effective on the 0.8 μm permeate, yielding low-molecular-weight peptides and eliminating allergenic proteins based on molecular-weight reduction. Microfiltration (MF) can be employed as a pre-treatment to reduce protein content, facilitating enzymatic hydrolysis.

KEYWORDS

membrane filtration, papain, enzymatic hydrolysis, molecular weight distribution, protein concentration

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1. INTRODUCTION

In the 21st century, the consumer demands for food have significantly altered. The consumption structure has been changed due to the development of living standards, which has the effect of increased attention to food quality and physical fitness (Wen et al., 2024). The consumer demand is growing for food products that contribute to the prevention of various chronic conditions. Intake of essential nutrients — including carbohydrates, proteins, lipids, vitamins, and minerals — within a balanced dietary pattern is essential for the optimal functioning of physiological processes and the maintenance of overall health (Chen et al., 2024). Biologically, milk is defined as a liquid secreted by mammals that serves as the primary source of nutrition for new-borns, providing all essential nutrients required for early development (Csurka et al., 2021). Milk and dairy products contain a wide range of macro and micronutrients that are essential for maintaining overall health and supporting balanced growth. The milk proteins provide all essential amino acids and exhibit excellent digestibility and have a protective role against chronic diseases (Antunes et al., 2022). Milk-derived proteins are classified among the eight major food allergens, because they contain both linear and conformational epitopes. The immunological responses depend on the protein molecular weights, with a significant immune reaction observed between 15 and 40 kDa (Hayes, 2018). Avoiding dairy products presents several challenges due to the high protein content of cow's milk, which is approximately 30–39 $g \cdot L^{-1}$ (Garau et al., 2021). Milk proteins are predominantly composed of caseins (80%) and whey proteins (20%). The major components of the whey are the α-lactalbumin and β-lactoglobulin, which together represent approximately 70–80% of the total whey proteins (Davoodi et al., 2016). Cow's milk protein allergy (CMPA) is an immunological hypersensitivity to specific milk proteins, particularly casein subtypes (α_{S1}-, α_{S2}-, β-, and κ-casein) and whey proteins such as α-lactalbumin (α-LA), β-lactoglobulin (β-LG), bovine serum albumin, and various immunoglobulins (Emmert et al., 2023; Nath et al., 2021). The food allergies (FAs) can be divided into two main categories like immunoglobulin (Ig)E-mediated or non-IgE mediated. Compared the two main categories, the IgE-mediated reactions are the most common (Giannetti et al., 2021). Thermal (heat and enzymatic hydrolysis) and non-thermal (high-pressure and ultrasound) methods are known for reducing milk protein allergy with different effectiveness in reducing food allergenicity. The future of milk allergen reduction may lie in enzymatic hydrolysis by the targeting of the allergenic epitopes (Zeng et al., 2024; Mierzejewska and Kubicka, 2003). Cow milk allergenicity was investigated with different types of enzymes such as Protamex, Flavourzyme, Neutrase, Papain, and Pepsin. Plant proteases (actinidin, bromelain, ficin and papain) have been used in dairy industry with growing interest due to their improved techno-functionality and less allergenicity. Furthermore, ACE-inhibitory activities significantly increased (70%) compared to trypsin (65%) (Kaur et al., 2024; Liang et al., 2021). Papain is a member of the cysteine protease family. This enzyme is known for its ability to break down proteins without contributing to the development of bitterness in food products (Amri and Mamboya, 2012; Nath et al., 2019). This enzyme has a relatively high level of thermal stability with optimal enzymatic activity at the pH range of 5–8 (Alpay and Uygun, 2014). Within the food industry, papain is commonly applied in the production of various protein hydrolysates. Bioactive peptides are biologically active molecules that remain closed within parent proteins and are released upon protein cleavage. Bioactive peptides have a wide range of physiological effects including antimicrobial, antihypertensive, antioxidant activities, blood-lipid-lowering effect, opioid role, antiobesity ability.

Milk proteins are good sources of bioactive peptides. Milk derived bioactive peptides are locked inside the milk protein and can be released by enzymatic hydrolysis and microbial fermentation. Recently, developing bioactive peptides from milk proteins has received great attention (Akbarian et al., 2022; Zeng et al., 2024). Membrane technology is a separation method by employing specific semi-permeable membrane filters to concentrate or fractionate a liquid by selectively allowing some compounds to pass through while retaining the others (Kumar et al., 2013). Microfiltration (MF) is a pressure-driven membrane separation technique with pore sizes ranging from 0.1 to 10 μm, and is widely applied for particle removal, clarification, water purification and the separation of biological components (Altinkaya, 2024). Membrane-based separation techniques offer numerous benefits compared to traditional processes, such as low-temperature operation, high separation efficiency, high permeate flux, low energy consumption, environmental friendliness (Lemmer et al., 2018). Microfiltration (MF) can be applied as a pre-treatment to isolate casein micelles from serum proteins (SP), resulting a casein enriched fraction (retentate) (Mercier-Bouchard et al., 2017; Reig et al., 2021). The purpose of this study was to examine the effect of ceramic microfiltration pore size (0.8, 1.4, and 5 μm) and papain concentration (0.016 and 0.024 $g \cdot 200\ mL^{-1}$) on the protein fractionation of fat-free UHT milk and the subsequent enzymatic production of bioactive, hypoallergenic peptide fractions.

2. MATERIALS AND METHODS

2.1. Collection of UHT milk samples

Ultra-heat-treated milk Riska UHT 0.1% (Alföldi Tej Kft, Székesfehérvár, Hungary) and Auchan UHT 0.1% (Auchan Magyarország Kft, Budaörs, Hungary) were received from local supermarkets in Budapest, Hungary. Due to the relatively large time span of the proposed experiments first, Auchan UHT 0.1% milk was used with the 0.8 μm membrane but later it was not available when 1.4 and 5 μm membranes were under investigation, therefore another product (Riska UHT 0.1% milk) was considered as feed liquid. Both products comply with EU standards for UHT milk, and their declared protein, lactose, and fat contents show no relevant differences. Thus, the substitution ensured comparable feed characteristics for the membrane filtration experiment. The concentrations for protein, lactose, and fat in the milk samples were provided by the manufacturers.

In the case of Auchan UHT 0.1% milk, the respective concentrations were reported as $43.5 \pm 0.16\ g \cdot L^{-1}$ protein, $47 \pm 0.15\ g \cdot L^{-1}$ lactose, and $1 \pm 0.02\ g \cdot L^{-1}$ fat. For Riska UHT 0.1% milk, protein content was $31 \pm 0.16\ g \cdot L^{-1}$, while lactose and fat concentrations remained the same as in the Auchan sample. Samples were kept under refrigerated conditions at 10 °C. The detailed process flow diagram is shown in Fig. 1. The process begins with microfiltration (MF – 5, 1.4, and 0.8 μm), which fractionates the milk into two primary streams. The retentate is enriched in casein micelles and other high–molecular weight proteins, whereas the permeate contains the low–molecular weight soluble fraction, including whey proteins, lactose, and minerals. In the second stage, both streams are subjected to enzymatic hydrolysis (papain) under controlled conditions. The objective of this step is to cleave milk proteins into smaller peptides and free amino acids, thereby modifying their techno-functional and potential bioactive properties. As a result of this two-step process, two compositionally and functionally differentiated dairy half-products are obtained.

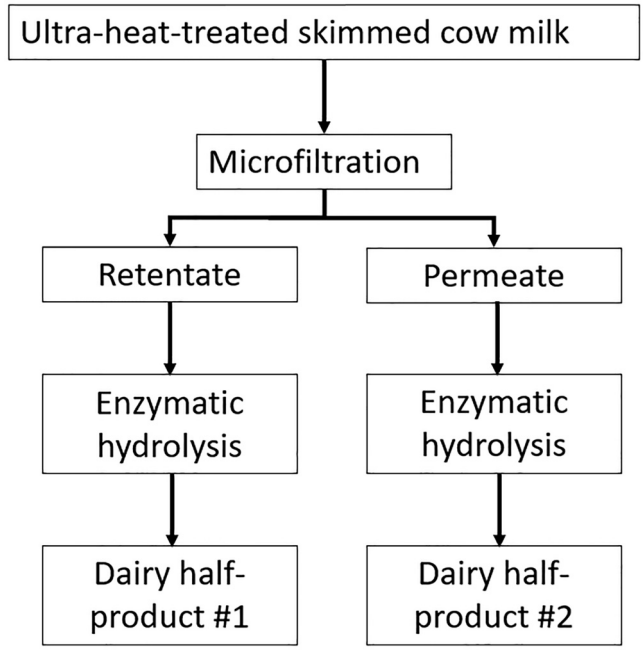

Fig. 1. Process flowsheet for MF separation and hydrolysis of milk proteins

2.2. Milk protein separation by membrane technology

Protein separation of UHT milk was performed using Membralox tubular ceramic membranes (Pall Corporation, Crailsheim, Germany) with an active filtration area of $5 \cdot 10^{-3}$ m^2 and nominal pore sizes of 5, 1.4, and 0.8 µm. The membranes were assembled into a stainless steel cross-flow module (see Fig. 2). Each membrane had a length of 250 mm, with an internal diameter of 7 mm and an external diameter of 10 mm. The recirculation flow rate (200 L·h^{-1}) was regulated via a centrifugal pump (Verder Hungary Kft, Budapest, Hungary). Flow rates of the processing fluids were adjusted using a rotameter positioned at both the retentate channel and the bypass inlet of the membrane unit. Transmembrane pressure (TMP) was monitored by pressure gauges installed at the inlet and outlet of the module. To enhance hydrodynamic conditions, a static turbulence promoter – a stainless steel (Inox, Backi Petrovac, Vojvodina, Serbia, SS316) double-helix ribbon – was inserted during each filtration.

The static turbulence promoter specifications were as follows: pitch-to-diameter ratio of 2, 6.5 mm diameter, 241 mm total length, 1.2 mm thickness. TMP of 3 bars and recirculation-flowrate of 200 L·h^{-1} were used during the filtration. The microfiltration experiments were carried out at room temperature (25 °C), under constant temperature conditions. At the beginning of the experiment, 1.2 L of UHT skim milk was used, and a volume concentration factor (VCF, in rel. unit) of 2 was applied throughout the process. The volume concentration factor was determined according to Equation (1).

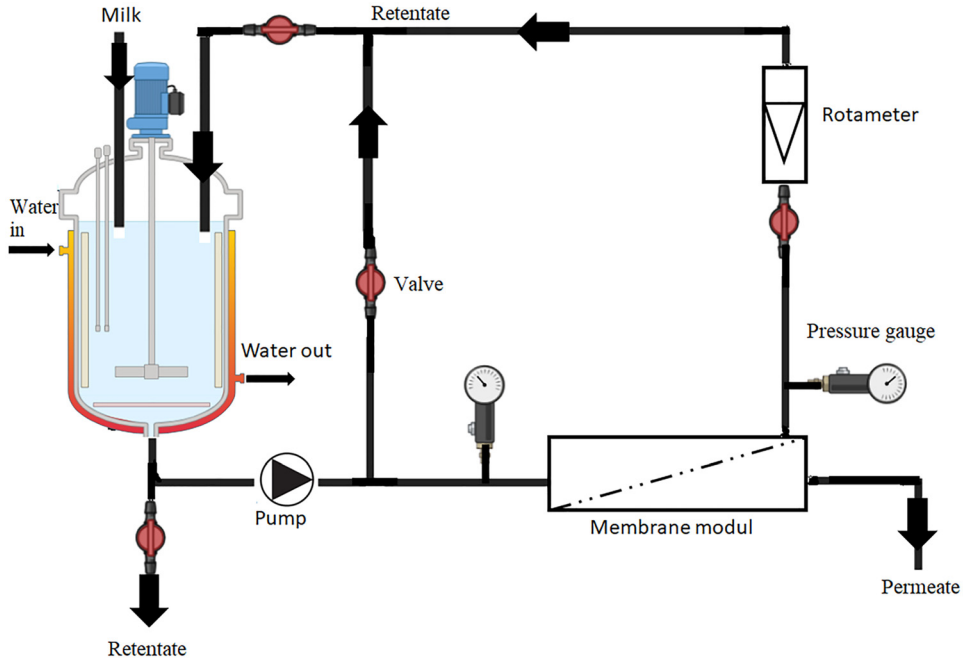

Fig. 2. Process flowsheet for membrane filtration (MF – Cross flow module)

$$VCF = \frac{V_F}{V_R} \quad (1),$$

where 'V_F' denotes the initial feed volume, 'V_R' represents the volume of the retentate. Permeate was collected at different time intervals under constant volume operation. The permeate flux (J) was determined using the following equation Equation (2).

$$J = \frac{V}{A \cdot t} \quad (2),$$

where 'J' denotes the permeate flux during filtration (L $m^{-2} \cdot h^{-1}$), 'V' is the permeate volume (L), 'A' refers to the membrane surface area (m^2), and 't' represents the filtration time (h). Reduction of permeate flux (ΔJ, %) was calculated from initial permeate flux with Equation (3).

$$\Delta J\% = \left(\frac{J_{initial} - J_{final}}{J_{initial}}\right) \cdot 100 \quad (3),$$

where 'ΔJ' represents the percentage decline in permeate flux, '$J_{initial}$' is the initial permeate flux (L $m^{-2} \cdot h^{-1}$), and 'J_{final}' corresponds to the final permeate flux (L $m^{-2} \cdot h^{-1}$). Retention of protein was calculated with Equation (4)

$$R_i = \left(1 - \frac{C_{Pi}}{C_{Ri}}\right) * 100 \quad (4),$$

where 'R_i' denotes the retention (%) of component *i*, 'C_{Pi}' is the concentration of component *i* in the permeate ($g \cdot L^{-1}$), and 'C_{Ri}' refers to its concentration in the retentate ($g \cdot L^{-1}$).

2.3. Membrane cleaning

Following each microfiltration (MF) experiment, the membrane underwent a sequential cleaning procedure. Initially, a 1% (v/v) Ultrasil P3-11 solution (Ecolab®, Budapest, Hungary) was circulated for 30 min, followed by a 5-min rinse with deionized water. Subsequently, the membrane was rinsed again with deionized water for 5 min and treated with 1% citric acid solution for 30 min. During the cleaning procedure the TMP and flow rate were maintained at 0.8 bar and 200 $L \cdot h^{-1}$, respectively. In contrast, during deionized water rinsing steps, a TMP of 3 bars was applied, while maintaining the same flow rate of 200 $L \cdot h^{-1}$.

2.4. Enzymatic hydrolysis of cow milk protein

Enzymatic hydrolysis of cow's milk proteins was carried out using papain enzyme produced by HIMEDIA® (HiMedia Laboratories GmbH, Einhausen, Germany, enzyme catalogue number EC 232-627-2). Papain is extracted from the species *Carica papaya* L. (papaya). The enzyme appears as a fine, white, solid powder, with a solubility of 10 $mg \cdot mL^{-1}$ in water. At 25 °C and with a moisture content of 2%, its pH ranges between 4.80 and 6.20. The enzymatic activity is approximately 30,000 USP units $\cdot mg^{-1}$ from the manufacturer's certified product specification sheet. During the experiments, a temperature of 50 °C was selected from the activation temperature range to prevent heat-induced precipitation of the thermosensitive milk proteins. Having hydrolysis completed, the enzyme was deactivated by heating to 70 °C for 30 min.

2.5. Analytical method

2.5.1. Estimation of protein concentration by Lowry method. Estimation of the total protein was performed by the Thermo Scientific™ Pierce™ Modified Lowry Protein Assay Kit (Rockford, IL 61105, USA - Cat. No. 23240).

All reagents are sensitive to the light and temperature, therefore they were stored at 4 °C. Due to the high expected protein concentrations, samples were diluted 1:100 (100 µL sample + 9.9 mL water) and homogenized. Each of the samples was measured in triplicate ($n = 3$), resulting in a total of ten data points including the blank. The samples were incubated under ambient conditions (~25 °C) for 10 min in the dark. Subsequently, 0.5 mL 2N Folin-Ciocalteu Phenol Reagent (Rockford, IL 61105, USA - Cat. No. 23240) reagent was added, followed by a second homogenization and incubation (~25 °C) step for 30 min under the same light-protected conditions. Colorimetric determination was performed with wavelength 750 nm using a JENWAY (Scientific & Technical Instruments (STI), UK) M6850 dual-beam spectrophotometer (Model 73.6850) (Waterborg and Matthews, 2003).

2.5.2. Sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE). A molecular weight marker (Bio-Rad Precision Plus Protein™ Dual colour Standards, 1610374, USA) along

with casein (20–30 kDa) and whey proteins — specifically α-lactalbumin (14.4 kDa) and β-lactoglobulin (18 kDa) — were applied as reference standards. The separation process was carried out on gels composed of 15% polyacrylamide and 6% stacking layers, following the method established by Laemmli (1970). Electrophoresis was performed using the vertical mini-PROTEAN 3 system (Bio-Rad Laboratories, Hercules, CA, USA), employing 0.75 mm spacers, at a constant voltage of 200 V for approximately 40–55 min at room temperature. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). Gel imaging and analysis were conducted using the BIO-RAD Gel Doc 2000 imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The software-assisted analysis included the quantification of protein band intensity and molecular weight estimation based on standard protein markers.

2.5.3. Statistical analysis of permeate flux. For the statistical analysis of the permeate flux mathematical model was developed according to Equation (5):

$$J = J_0 + (J_{SS} - J_0) \cdot \left(1 - e^{-K \cdot t}\right) \quad (5),$$

where 'J' is the permeate flux at the given time (L $m^{-2} \cdot h^{-1}$), 'J_0' is the initial flux for the measurement L $m^{-2} \cdot h^{-1}$); 'J_{ss}' is the steady-state flux during the measurement (L $m^{-2} \cdot h^{-1}$); 'K' is the flux decrease rate (s^{-1}), and 't' is the time corresponding to the given flux value (h) (Varga et al., 2018).

Based on Equation (5) and time–flux data 'J_0', 'J_{ss}', and 'K' values for filtration were calculated via nonlinear regression in SPSS (ver. 29.0) (IBM, Armonk, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Partial reduction of milk protein by microfiltration (MF)

As illustrated in Fig. 3, an increase in the volume concentration factor (VCF, in rel. unit) led to a noticeable decline in permeate flux. This decrease is primarily attributed to the formation of

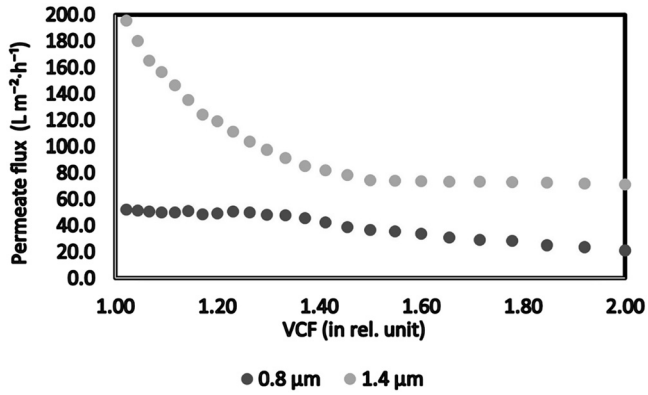

Fig. 3. Permeate flux (J) during membrane filtration of UHT milk (using the 0.8 and 1.4 µm MF membranes)

concentration polarization on the membrane surface. The initial permeate flux decreased from 195 to 71 L $m^{-2} \cdot h^{-1}$ for the 1.4 µm membrane and from 52 to 21 L $m^{-2} \cdot h^{-1}$ for the 0.8 µm membrane, both measured at a VCF of 2 (Fig. 3). These changes represent a flux reduction of 63.5% for the 1.4 µm membrane and 59.6% for the 0.8 µm membrane.

Additionally, the use of a static turbulence promoter helped to induce tangential flow on the membrane surface. This hydrodynamic effect reduced solute deposition on the membrane surface, thereby enhancing the permeate flux during filtration (Fig. 4). These results can be interpreted in the context of existing literature on milk microfiltration and enzymatic hydrolysis. The observed high protein retention by the 0.8 µm membrane aligns with previous studies reporting that ceramic membranes with pore sizes below 1 µm efficiently retain casein micelles while allowing whey proteins and lactose to permeate (Mercier-Bouchard et al., 2017; Tomasula et al., 2011).

The initial and steady-state permeate flux of the 5 µm membrane was found to be approximately two orders of magnitude higher than that of the 1.4 µm membrane, which can be attributed to its large pore size and reduced hydraulic resistance. Despite the rapid permeation, concentration polarization develops at the membrane surface, leading to a local accumulation of proteins and other colloidal components. Additionally, fouling caused by particle deposition and adsorption on the membrane surface progressively reduces the effective pore area. Consequently, the permeate flux decreases along the filtration process, following a similar trend observed for the 0.8 and 1.4 µm membranes, although the absolute flux values remain significantly higher. This behaviour illustrates that even membranes with very large pores are subject to mass transfer limitations and fouling phenomena during microfiltration of protein-rich liquids such as milk (Gul et al., 2021).

3.2. Statistical analysis of permeate flux during microfiltration (MF)

A nonlinear regression was used to evaluate the performance of different membrane types (0.8, 1.4, 5 µm) based on the hydrodynamic parameters 'J_0', 'J_{SS}', and 'K', which represent initial flux, steady-state flux, and a resistance-related parameter. The models demonstrated varying levels of fit depending on membrane type (Varga et al., 2018). The regression model for the 0.8 µm membrane demonstrated a good fit with an R^2 value of 0.850. The ANOVA

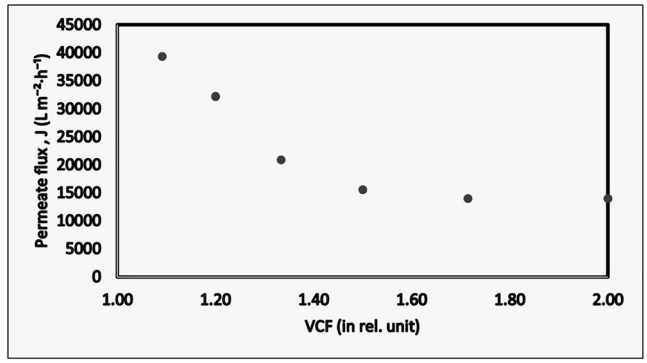

Fig. 4. Permeate flux (J) during membrane filtration of UHT milk (using the 5 µm MF membrane)

results showed a significant regression ($F(3.21) = 667.1$, $P < 0.001$), with a total corrected sum of squares of 1.447 and residual sum of squares of 216.5 (Tables 1 and 2).

For the 1.4 µm membrane, the model had an excellent fit with an R^2 of 0.998, indicating that nearly all the variability in 'J_0' is accounted for by the model. The low residual mean square (2.519) and high regression sum of squares relative to the corrected total confirm the model's high explanatory power. The ANOVA results showed a significant regression ($F(3.21) = 39794$ $P < 0.001$), with a total corrected sum of squares of 34413.6 and residual sum of squares of 52.906 (Tables 3 and 4).

Table 1. Parameter estimates for the hydrodynamic parameters (for the 0.8 µm membrane)

Parameter	Estimate	Std. error	95% Confidence interval	
			Lower bound	Upper bound
J_0	49.405	2.528	44.148	54.662
J_{SS}	23.429	1.030	21.288	25.570
K	17.112	3.538	9.753	24.470

Table 2. ANOVA for the 0.8 µm microfiltration (MF) membrane

Source	Sum of squares	df	Mean squares
Regression	20623.128	3	6874.376
Residual	216.527	21	10.311
Uncorrected total	20839.654	24	
Corrected total	1447.900[a]	23	
Dependent variable: J			

[a] R squared = 1 − (Residual Sum of Squares)/(Corrected Sum of Squares) = 0.850

Table 3. Parameter estimates for the hydrodynamic parameters (for the 1.4 µm membrane)

Parameter	Estimate	Std. error	95% Confidence interval	
			Lower bound	Upper bound
J_0	207.064	1.360	204.236	209.893
J_{SS}	69.683	0.670	68.290	71.077
K	3.971	0.088	3.789	4.154

Table 4. ANOVA for the 1.4 µm microfiltration (MF) membrane

Source	Sum of squares	df	Mean squares
Regression	300727.694	3	100242.565
Residual	52.906	21	2.519
Uncorrected total	300780.600	24	
Corrected total	34413.660[a]	23	
Dependent variable: J			

[a] R squared = 1 − (Residual Sum of Squares)/(Corrected Sum of Squares) = 0.998

The regression for the 5 µm membrane also showed an extremely high R^2 of 0.992, indicating excellent model fit. The ANOVA results showed a significant regression ($F(3.3) = 770.6$ $P < 0.001$) (Tables 5 and 6). These results demonstrate that membrane pore size significantly influences performance, particularly in maintaining flux over time.

3.3. Estimation of protein concentration

The initial total protein content of the Auchan and Riska UHT milk was found at 43.5 $g \cdot L^{-1}$ and 31.0 $g \cdot L^{-1}$. In Fig. 5 the total protein content ($g \cdot L^{-1}$) measured in the feed (UHT milk), permeate, and retentate fractions with the three different pore sized membranes (0.8, 1.4, and 5 µm) can

Table 5. Parameter estimates for the hydrodynamic parameters (for the 5 µm membrane)

Parameter	Estimate	Std. error	95% Confidence interval	
			Lower bound	Upper bound
J_{SS}	12728.285	1180.716	8970.721	16485.849
J_0	51500.775	2960.665	42078.616	60922.933
K	699.735	107.623	357.230	1042.240

Table 6. ANOVA for the 5 µm microfiltration (MF) membrane

Source	Sum of squares	df	Mean squares
Regression	$3.65 \cdot 10^9$	3	$1.21 \cdot 10^{10}$
Residual	$4.74 \cdot 10^7$	3	$1.58 \cdot 10^7$
Uncorrected total	$3.65 \cdot 10^{10}$	6	
Corrected total	$5.75 \cdot 10^{8a}$	5	
Dependent variable: J			

[a] R squared = 1 − (Residual Sum of Squares)/(Corrected Sum of Squares) = 0.992

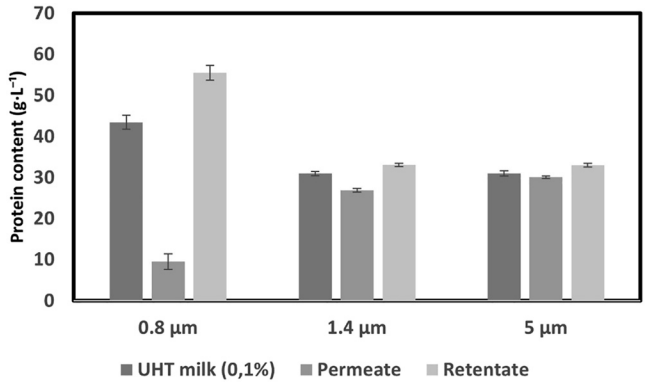

Fig. 5. Concentration of protein in the feed, permeate and retentate side of the microfiltration (MF) membranes

be observed. The total protein content in the feed, permeate, and retentate of the 1.4 and 5 μm MF membranes has only a little difference, indicating that the measured proteins were mostly able to pass through the membrane. Concentration of protein in the retentate and permeate were not changed significantly (26.9 and 33.09 $g \cdot L^{-1}$) during the filtration with the 1.4 μm membrane which refers to an overall protein retention (R%) of 13.2%. In the case of the 5 μm membrane, the permeate (30.09 $g \cdot L^{-1}$) and retentate (33.04 $g \cdot L^{-1}$) protein contents are very similar to the feed (31.0 $g \cdot L^{-1}$), indicating only negligible protein separation. On the contrary, in the case of the 0.8 μm membrane, a significant reduction in protein content is observed in the permeate (9.5 $g \cdot L^{-1}$), indicating a high retention efficiency ($R\% = 69.4$) and due to this fact the retentate had a high protein concentration (55.5 $g \cdot L^{-1}$).

These results indicating the threshold of ceramic microfilters for dairy protein retention. Due to its relatively large pore size, the 5 μm membrane practically showed zero protein retention, however, the protein concentration in the feed still increased with approximately 10%. This fact can be explained by the original low fat content of the milk (0.1%), which also contains some proteins, but due to molecular interactions between the fat globules and the surface of the membrane, the fat and a small amount of casein micelles can stick on the membrane. Panopoulos et al. (2020) demonstrated that microfiltration of skim milk using a 1.4 μm ceramic membrane results in limited casein retention while allowing most whey proteins to permeate. This highlights that, although such membranes are less effective for fractionating high-molecular-weight proteins, they provide high permeate flux and preserve overall protein transmission. For the 1.4 μm membrane, the pore size is more suitable to retain fat globules by the sieve effect compared to the 5 μm membrane, since the size range of the fat droplets are reported in the range of 0.2–2 μm. Because of the increased retention of fats and also some micellar proteins on the surface of the membrane even the number of fat–protein and protein–protein molecular bindings might increase and this phenomena can be the reason for the slight decrease of protein content in its permeate.

3.4. Estimation of molecular weight profile by SDS-PAGE method

The feed and samples of the 0.8 and 1.4 μm membrane separation treated with two different enzyme dose (0.016 $g \cdot 200\ mL^{-1}$ and 0.024 $g \cdot 200\ mL^{-1}$) have been analysed via gel electrophoresis. It can be seen that the higher concentration of papain hydrolyses the milk proteins to a greater extent, primarily the casein fractions (lane 5 and lane 8) (Fig. 6).

In Fig. 6, it can be observed that the 0.8 μm membrane separates the milk proteins with higher efficiency than the membrane with 1.4 μm pore size, which is consistent with the results of the total protein content determination. In the enzymatically treated permeate fractions of the 0.8 μm membrane, casein and whey proteins can only be detected in traces at both lower and higher papain concentrations (lane 15 and lane 17). In the case of other samples the proteolysis could reduce the molecular size, which can be observed in lanes 5 and 8, when comparing them to lane 4 (UHT milk feed). The colour intensity at the low molecular band ranges is increasing, showing the generation of peptides. However, the original bands for casein, α-lactalbumin and β-lactoglobulin are still significant. Enzymatic hydrolysis with papain further differentiates the functional characteristics of the fractions. The gel electrophoresis results show that higher papain concentrations enhance the cleavage of casein and other high-molecular-weight proteins, generating low-molecular-weight peptides, while whey proteins in the permeate of the 0.8 μm

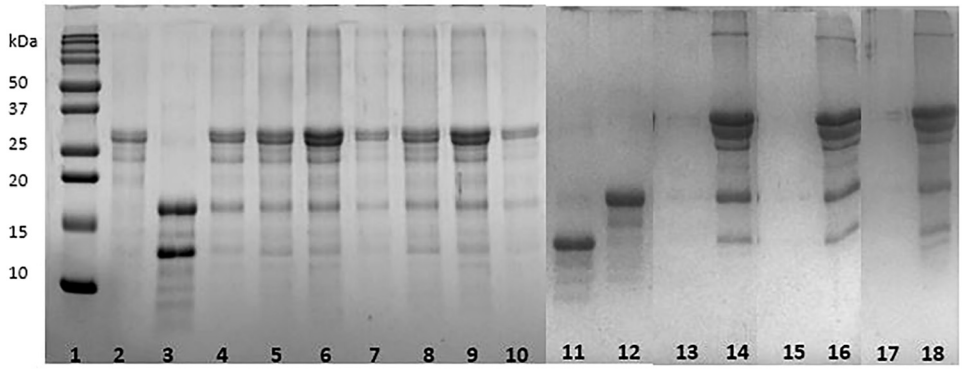

Fig. 6. SDS-PAGE patterns of the membrane separated and enzymatic treated milk proteins; (lane 1: molecular weight marker, lane 2: casein, lane 3: α-lactalbumin + β-lactoglobulin, lane 4: UHT milk, lane 5: UHT milk hydrolyzed by papain /0.016 g·200 mL^{-1}/, lane 6: retentate /1.4 μm/ hydrolyzed by papain /0.016 g·200 mL^{-1}/, lane 7: permeate /1.4 μm/ hydrolyzed by papain /0.016 g·200 mL^{-1}/, lane 8: UHT milk hydrolyzed by papain /0.024 g·200 mL^{-1}/, lane 9: retentate /1.4 μm/ hydrolyzed by papain /0.024 g·200 mL^{-1}/, lane 10: permeate /1.4 μm/ hydrolyzed by papain /0.024 g·200 mL^{-1}/, lane 11: α-lactalbumin, lane 12: β-lactoglobulin, lane 13: permeate /0.8 μm/, lane 14: retentate /0.8 μm/, lane 15: permeate /0.8 μm/ hydrolyzed by papain /0.016 g·200 mL^{-1}/, lane 16: retentate /1.4 μm/ hydrolyzed by papain /0.016 g·200 mL^{-1}/, lane 17: permeate /1.4 μm/ hydrolyzed by papain /0.024 g·200 mL^{-1}/, lane 18: retentate /1.4 μm/ hydrolyzed by papain /0.024 g·200 mL^{-1})

membrane are largely hydrolyzed to trace levels. This observation is consistent with the quantitative protein measurements and aligns with previous reports on the specificity of papain towards casein fractions (Alpay and Uygun, 2014; Kaur et al., 2024).

4. CONCLUSION

The main goal of this study was to evaluate ceramic microfiltration membranes of different pore sizes (0.8, 1.4, and 5 μm) for protein fractionation and their suitability for subsequent enzymatic hydrolysis to produce low-allergenicity biopeptides from fat-free UHT milk. The analysis of the microfiltration of fat free UHT milk with ceramic membranes suggested pore size 0.8 μm in order to fractionate the proteins of the feed with permeate fluxes ranging from 52 to 21 L m^{-2}·h^{-1} at VCF 2. Membrane filtration with larger pore sized membranes (1.4 and 5 μm) show only minimal or negligible protein fractionation enabling their application in milk cold sterilisation or safety filtration applications (Hu and Wu, 2023). The enzymatic treatment of the raw material and the separated streams were found to be outstandingly effective, decreasing the protein concentration from 43.5 to 10 g·L^{-1} on the permeate samples of the 0.8 μm membrane. This membrane could reduce the permeate's protein content below 10 g·L^{-1} ($R_{protein}$% = 69.4), which after the protein hydrolysis by papain had high effectiveness regardless the enzyme concentration. So, low-molecular-weight biopeptides (0.5–7 kDa), including ACE-inhibitory and antioxidant peptide fractions, were generated (Aguilar-Toalá et al., 2016). In case of the feed and the retentate of the 0.8 μm membrane as well in all samples of the 1.4 μm membrane, the enzyme treatment could not

hydrolyse the total amount of proteins with the applied parameters. Therefore, the deliberated peptides are mixed with large molecules in the broth, which has to be considered in future applications. In cases where hypoallergenicity is required in the final product, an additional downstream separation step (e.g., MF/UF) is necessary to remove the low-molecular-weight peptides from the hydrolysates. Or, on the other hand, simultaneous application of other proteases might be also a solution in order to fully digest the remaining large proteins and prevent consumers from dairy allergy, when supplying health promoting bioactive peptides to them.

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