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BIOACTIVE PEPTIDES FROM LIQUID MILK PROTEIN CONCENTRATE BY TRYPTIC AND MICROBIAL HYDROLYSIS

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Az iskolavezető jóváhagyása A témavezető jóváhagyása

1. Introduction and objectives

Improper nutrition, stress and fast lifestyles are responsible of several metabolic disorders. Such metabolic disorders include obesity, hypertension and cardiovascular diseases. Formation of free radicals and activation of pro-inflammatory cytokines are cause of a number of negative consequences, such as the development of metabolic disorders. Furthermore, circulatory hormone angiotensin, an important effector of the angiotensin system has a role on metabolic abnormality, closely associated with oxidative stress and inflammation. Bacterial infection has role on oxidative stress. Milk proteins offer numerous biological activities to neonate to aged individuals. Contradictorily, due to the presence of Immunoglobulin E- and Immunoglobulin G- binding epitopes, milk proteins are listed among the "big 8" allergens.

In 21st century, the food industry has undergone many significant changes. At the same time, due to revolution of industrial and manufacturing technologies, food products have also upgraded. Today's consumers are paying more attention to consume healthy foods. It has been proven that bioactive peptides have a great role to modulate metabolic disorder by versatile metabolic pathways. They can be produced by enzymatic and microbial hydrolysis of food proteins. Bioactive peptides with unique functional activities have been catapulted to wide range consumers around the globe because they confer anti-oxidant, anti-inflammatory, anti-angiotensin, anti-tumor/ cancer, gut homeostasis, anti-obesity, anti-diabetic, immunostimulatory, muscle tissue biosynthesis and osteoprotective activities. One of the important sources of bioactive peptides is milk proteins. Therefore, the production of hypoallergenic bioactive peptides open up a new market segment in society. However, several physical treatments have been used to produce bioactive peptides from milk proteins, biochemical route is considered as safe. In my research work, my goal is to produce bioactive peptides with antioxidant capacity, angiotensin-converting enzyme (ACE) inhibitory activity, antimicrobial activity and hypoallergenic properties.

Objectives

Objective of my research work is to produce bioactive peptides from cow's milk using membrane filtration, tryptic and microbial (lactic acid bacteria) hydrolysis of milk proteins.

For the production of bioactive peptides, I set the following goals in my research:

1. Development of liquid milk protein concentrate by de-watering process. Membrane

filtration was adopted for that purpose. Determination of optimal membrane filtration parameters by 2p factorial design to achieve high concentration of protein, permeate flux value and low energy consumption.

2. Proteolysis of liquid milk protein concentrate by different concentrations of trypsin.

3. Proteolysis of tryptic hydrolysate of milk protein concentrate by *lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus* starter culture with/ without addition of glucose.

4. Understand the molecular weight distribution of peptides, produced by sequential tryptic and microbial hydrolysis of milk protein concentrate.

5. Understand the antioxidant capacity, angiotensin converting enzyme inhibitory activity and anti-bacterial activity of protein hydrolysate.

6. Understand the allergenic activity of milk protein hydrolyzate.

2. Material and methods

Peptides with antioxidant capacity, angiotensin-converting enzyme inhibitory activity, antibacterial activity and hypoallergenic properties were prepared from skimmed UHT cow's milk by combination of membrane filtration, tryptic and microbial hydrolysis. Schematic diagram of work flow is represented in Figure 1.

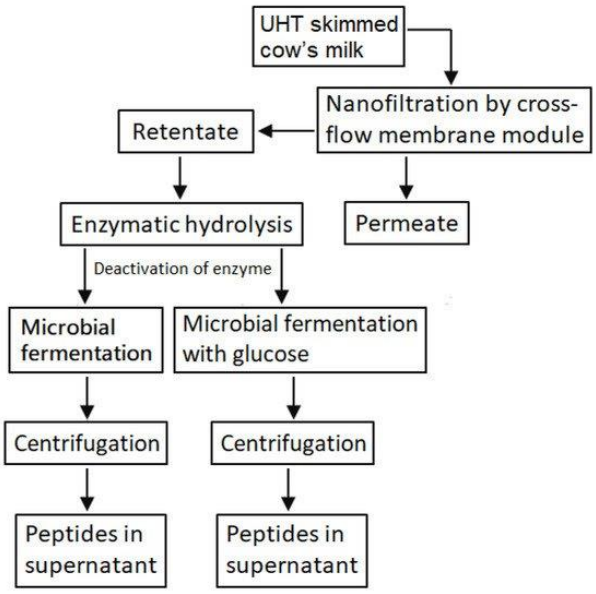

Figure 1. Experimental scheme to prepare peptides with antioxidant capacity, angiotensin-converting enzyme inhibitory activity, antibacterial activity and hypoallergenic properties from skimmed UHT cow's milk.

2.1 Ultra-Heat-Treated (UHT) Skimmed Cow's Milk

UHT skimmed cow's milk, with concentrations of protein, lactose, and fat of 31 ± 0,1 g/L, 47 ± 0,84 g/L and 1 ± 0,12 g/L, respectively, was procured from local supermarkets in and around Budapest, Hungary. The pH of the milk was 6.8 at room temperature (~25 °C).

2.2 Production of liquid milk protein concentrate (LMPC)

Liquid milk protein concentrate (LMPC) was prepared by a cross-flow membrane filtration process. In the cross-current membrane filtration process, a feed tank was attached with cross-flow membrane module. Transmembrane pressures (TMPs) of membrane modules were monitored by pressure gauges and flow control valves, fitted at the two opposite ends of the membrane modules.

Feed flow rates were controlled using hydraulic diaphragm pumps (Hydra-Cell G03; Verder Hungary Kft., Budapest, Hungary) and the inlet and outlet flow valves. A rotameter at the retentate end and a bypass valve were also used for controlling the flow rate in the membrane module. De-watering (remove of milk serum) of ultra-heat-treated skimmed milk was performed by a tubular nanofiltration membrane with active filtration area 5×10^{-3} m^2 and pore size 5 nm (Pall Corporation, Crailsheim, Germany), placed in a stainless steel-made cross-flow membrane module. The active layer, support layer, length, inner diameter, and outer diameter of the membrane were titanium oxide, aluminum oxide, 250 mm, 7 mm, and 10 mm, respectively. A commercially available static turbulent promoter was inserted on membrane tube. The promoter had an aspect ratio O_{tp}=2, diameter d_{tp}=6.5 mm, total length l_{tp}=241 mm, pitch length l_e=13.2 mm, number of mixing elements N_{TP}=36 and thickness δ_{TP}=1.2 mm. During the filtration, the volume concentration ratio (VCR) was maintained 2. Membrane filtration was carried out in batch operation mode. In my experiments, I also examined the energy consumption of membrane filtration. The transmembrane pressure (TMP) was between 2 - 3 bar, the recirculation flow rate (RFR) is changed between 100 and 200 L/h. Effect of static turbulence promoter in filtration process was studied. Retentate with membrane filtration process is used in subsequent experiment.

2.3 Hydrolysis of liquid milk protein concentrate by trypsin

Prior to tryptic hydrolysis of milk protein, milk with concentrated proteins was pre-incubated until the temperature reached 40 ^oC in a laboratory-scale well-controlled jacketed bioreactor (Solida Biotech, München, Germany). After pre-incubation of milk, tryptic hydrolysis of milk protein was performed. To prepare the trypsin stock solution, 0.009 g of trypsin was dissolved in 1 mL of distilled water, pH 7. The enzyme activity of the trypsin stock solution was 15.6 AU /(s×μg). Subsequently, 450 μL, 900 μL and 1.8 mL of trypsin solution was inoculated to 500 mL of preincubated milk protein concentrate in bioreactor. For hydrolysis of milk proteins with trypsin, the enzyme activity values associated with each enzyme concentration were as follows: 0.008 g/L (0.014 AU /(s×μg)), 0.016 g/L (0.028 AU /(s×μg)), 0.032 g/L (0.056 AU /(s×μg)). Tryptic hydrolysis of the protein concentrate was carried out at 40 °C for 10 minutes, while inactivation of trypsin was carried out at 70 °C for 30 minutes.

2.4 Microbial Hydrolysis

Microbial hydrolysis was carried out with *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus* microbial strains. After deactivating the trypsin, the temperature of the milk protein concentrate was reduced to 45 °C .

Microbial hydrolysis of tryptic hydrolyzed milk protein concentrate was performed in 50 mL of falcon tube. In every falcon tube, 45 mL of trypsin-treated milk protein concentrate was used. From the glucose stock solution (40% sterile glucose (w/v)), 3.6 mL of glucose solution was added to 45 mL of trypsin treated liquid milk protein concentrate to investigate the effect of glucose on microbial growth and proteolysis of trypsin-treated milk protein concentrate. Lactic acid bacteria (Thermophilic YoFlex® Mild 1.0, Chr. Hansen, Nienburg, Germany) were used for the microbial hydrolysis of the trypsin-treated milk protein concentrate. 30 µL of the inoculum from the stock culture was inoculated into each individual sample. After inoculation, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were 5.5×10^{6} CFU·mL^{-1} and 1.5×10^{7} CFU·mL^{-1}, respectively, in the fermentation medium. The samples were fermented in an incubator (HACH, Düsseldorf, Germany) at a temperature of 45 ^{o}C for 6 hours.

2.5 Analytical measurments

2.5.1 Understanding the Molecular Weight Distribution of Proteins and Peptides

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used I used to understand the molecular weight distribution of proteins and peptides. A vertical gel electrophoresis system (Bio-Rad Mini Protean 3 Cell, Bio-Rad Hungary Ltd) was adopted for this purpose. The molecule standard (BioRad, Precision Plus Protein TM Dual Color Standards, 1610374, USA), casein (20-30 kDa) and whey proteins (α-lactalbumin - 14.4 kDa; (β-lactoglobulin - 18 kDa) were used in gel. In BioRad power PAC1000 voltage transmitter, the parameters set were: U = 200 V constant; I = 54 mA, P = 11W, run time = 60 minutes. In the vertical electrophoresis system, the concentrations of running gel and stacking gel were 15% and 6%, respectively. 10 uL of sample was used in each channel of gel. The gel was evaluated using BIO-RAD Gel Doc 2000.

2.5.2 Determination of antioxidant capacity

2.5.2.1 Ferric Reducing Ability of Plasma (FRAP) assay

Prior to assay, milk protein concentrate, enzyme-treated milk protein concentrate, and corresponding fermentation broth prepared by with or without glucose were centrifuged by a temperature-controlled laboratory centrifuge (Z206A; Wehingen, Germany). Centrifugation was performed with 10,000 rpm for 20 min at temperature 4 ^{o}C and antioxidant capacity was measured in supernatants. Appropriate diluted 100 µL of supernatants were mixed with 2.9 mL of reagent and incubated at temperature ~35 °C for 30 min in an incubator (HACH, Düsseldorf, Germany). The absorbance was measured with wavelength 517 nm in UV-Vis spectrophotometer (EvolutionTM 300; Thermo ScientificTM, Waltham MA, USA) in room temperature (~25 °C). A blank sample was prepared with 100 µL of DI water and 2.9 mL of reagent for spectrophotometric analysis.

2.5.2.2 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical-Scavenging assay

Similar like before, milk protein concentrate, enzyme-treated milk protein concentrate, and corresponding fermentation broth prepared with or without glucose were centrifuged by a laboratory centrifuge. Assay was performed with 100 µL of supernatant and 3.9 mL of 6×10^{-5} M of DPPH-methanol solution. Control sample was prepared with 100 µL of methanol and 3.9 mL of 6×10^{-5} M of DPPH-methanol. The mixtures were incubated at room temperature (~25 ^{o}C) with dark condition for 30 min. Subsequently, absorbance was measured at 517 nm with a UV-Vis spectrophotometer (EvolutionTM 300; Thermo ScientificTM, Waltham MA, USA).

2.5.3 Estimation of angiotensin-converting-enzyme inhibitory activity

Supernatant of milk protein concentrate, enzyme-treated milk protein concentrate, and corresponding fermentation broth prepared with or without glucose were prepared by a laboratory centrifuge and used in assay. 100 mM of TRIS HCl (pH 7), 50 mM of sodium chloride, 10 µM of zinc chloride, 15 µM of substrate Abz-FRK(Dnp)-P (Sigma-Aldrich, Schnelldorf, Germany), recombinant angiotensin converting enzyme (amount of the recombinant angiotensin converting enzyme was chosen to result in about 10-fold activity than that in human serum), and supernatants of protein concentrate and corresponding fermentation broth (in a dilution range of 10-fold to 10^{6}-fold) was used to determine IC_{50} value of samples. Activity of angiotensin-converting enzyme in the absence of the

supernatants of samples was used to define uninhibited activity. Reaction was initiated by the addition of substrate.

The level of inhibition was calculated as % of uninhibited activity in each plate. The values of K_M and V_{max} were determined by measuring the activity of 160 – 4860-fold diluted samples in the presence of 15 μM, 7.5 μM, 3.75 μM and 1.625 μM of substrate. Non-linear fitting of data points and the Levenberg–Marquardt algorithm were considered to evaluate K_M and V_{max} values. Measurements were performed in a fluorescent plate reader (BMG Novostar, BMG Labtech, Germany) at temperature 37 ^{o}C. Changes in optical density were measured with excitation wavelength of 340 nm and emission wavelength of 405 nm for at least 20 cycles.

2.5.4 Estimation of trypsin enzyme activity

During the determination of trypsin activity, 10 μL of trypsin stock solution (trypsin concentration 0,009 g/mL) was prepared. The reaction mixture was prepared by 10 μL trypsin stock solution, 10 μL TRIS buffer solution and 80 μL substrate solution. I pippeted the 100 μL sample into a plate reader, and then I made the change in fluorescent signal intensity in a fluorescent disk reader for 30 minutes at a temperature of 37 ^{o}C. The excitation wavelength, similar to the ACE inhibition test (chapter 2.5.3), was λ = 340 nm with an emission wavelength of λ = 405 nm. I used 10 μL distilled water and 90 μL substrate to make blank samples.

2.5.5 Estimation of microbiological activity

Antibacterial activity of the supernatant of liquid milk protein concentrate, enzyme-treated liquid milk protein concentrate and corresponding fermentation broth against *Bacillus cereus, Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* CCM 4699. Operating condition of centrifugation is mentioned in earlier. Overnight grown (freshly prepared) each culture from soybean casein digest agar plate was diluted with maximum recovery diluent to reach the bacterial concentration 10^6 CFU/mL in individual tube. Freshly prepared bacterial cultures with $8 \cdot 10^6$ CFU/mL were spread on soybean casein digestive agar medium and agar wells with diameter 5 mm were prepared. Agar wells were filled with 100 μL of supernatants. Microbial plates were incubated at temperature 37 °C for 48 h in an incubator (HACH, Düsseldorf, Germany). After 24 h of incubator, diameter of zone of inhibitions in plates were measured by excluding the diameter of wells (5 mm) using a digital Vernier caliper (Uematsu shokai co., LTD., Sendai, Japan).

2.5.6 Determination of protein concentration

Supernatant of milk protein concentrate, tryptic hydrolysed milk protein concentrate and and corresponding fermentation broth were centrifuged by a laboratory centrifuge with previously mentioned operating parameters. Appropriate diluted 100 µL of supernatant and 3 mL of Bradford reagent were mixed. Subsequently, they are incubated at room temperature (~25 °C) for 30 min in an incubator (HACH, Düsseldorf, Germany). Spectrophotometry measurement was carried out at a wavelength of 595 nm with a spectrophotometer (Thermo ScientificTM, Waltham, MA, USA). In the study, bovine serum albumin was used as standard.

2.5.7 Immunoblotting of enzyme-treated concentrated milk protein and fermentation medium

Proteins from SDS-PAGE gel were transferred onto a 0.45 µm of polyvinylidene difluoride (PVDF) membrane (Merck Millipore, MA, USA) by a trans blot semi-dry transfer cell (Bio-Rad, CA, USA). It was operated with 0.25 V and 0.08 mA/cm2 for 60 min. Immune-reactive proteins were identified with clinically proved milk positive pooled human serum and peroxidase-labelled anti-human Immunoglobulin E. The binding patterns were visualised using a substrate solution containing 4-chloronaphtol, hydrogen peroxide and ethanol in phosphate buffered saline solution. Image analysis of blots was carried out with Gel Doc 2000 system (Bio-Rad, CA, USA)

2.5.8 Statistical Analysis

All experiments were performed in 3 times (technical triplicate). The mean value with standard deviation was calculated by a Microsoft Excel spread sheet (Microsoft Corporation, Washington WA, USA). Significant differences ($P < 0.05$) between different groups were determined by the one-way analysis of variance method, followed by the Tukey's post hoc test. SPSS 15.0 (IBM, Armonk NY, USA) was used for statistical analysis.

3. Result and Discussion

3.1 Production of liquid milk protein concentrate using membrane filtration

I examined the effect of the transmembrane pressure (TMP) and recirculation-flowrate (RFR) on the permeate flux value. The use of a static mixer during membrane filtration had a positive effect on the value of flux. Without static mixer at TMP = 3 bar and RFR = 100 L/h, maximum flux value was 13.45 L/m^{2}h, whereas, with static mixer at TMP = 3 bar and RFR = 100 L/h, maximum flux value was 34.22 L/m^{2}h. TMP had a positive influence on permeate flux. As example, with static mixer with TMP = 2 bar and RFR = 100 L/h, maximum flux value was 15.58 L/m^{2}h, whereas, with static mixer at TMP = 3 bar and RFR = 100 L/h, maximum flux value was 34.22 L/m^{2}h. Under same TMP, permeate flux is not statistically influenced by RFR. Permeate flux was reduced with time progress, due to formation of concentration polarization on membrane surface.

I carried out the experiment design with the Statistica program (Version 8.0) during my doctoral work. During the evaluation of the experiment plan, I considered the summary table of the 2^P factor plan. I set a linear model, where the value of initial flux was used for different TMP and RFR. In my studies, I used a 90% confidence level. In case of static mixer, after transformation of the statistical indicators, the equation is given below.

$$J = 24{,}426 + 9{,}402 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) + 0{,}0825 \cdot \left(\frac{RFR - 150}{50}\right) + 0{,}0825 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) \cdot \left(\frac{RFR - 150}{50}\right)$$

In case of without static mixer, after transformation of the statistical indicators, the equation became as below:

$$J = 12{,}242 + 3{,}797 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) + 1{,}172 \cdot \left(\frac{RFR - 150}{50}\right) + 1{,}102 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) \cdot \left(\frac{RFR - 150}{50}\right)$$

The test model is valid within a specified range: TMP = 2 - 3 bar, RFR = 100 - 200 L/h

By increasing the transmembrane pressure from 2 bar to 3 bar, the value of protein concentrations in the retentate improves at the end of the membrane filtration process (VCR = 2). Protein concentration, measured at TMP 2 bar and 3 bar with RFR of 100 L/h were 37.5 g/L to 60.5 g/L. Based on the measurement data, it can be said that the highest protein concentration was 62.83 g/L at TMP 3 bar and RFR 200 L/h.

3.2 Molecular weight of different proteins in concentrated milk and their enzymatic hydrolysis

I used polyacrylamide gel electrophoresis to understand the molecular weight distribution of peptides and proteins. It was shown that molecular weight of casein was incresed due to conjugation of casein with whey proteins by heat treatment during UHT processing. The efficiency of tryptic hydrolysis was improved by increasing the concentration of trypsin. Without any contradiction, it was found that the numbers of peptide bands were increased due to tryptic digestion of milk proteins and subsequently microbial hydrolysis process. Effect of glucose on microbial hydrolysis of milk protein was investigated but it was shown that glucose did not have any effect.

Hypoallergenic property

Presence of the main allergens in milk, such as caseins, α-lactalbumin and β-lactoglobulin were measured in each sample useing cow milk sensetive human pool blood serum. Reduction of allergenecity was trypsin dose dependent. Allergenecity of α-lactalbumin was not determined. No allergenicity was observed when milk protein concentration was treated with 0.032 g/L of trypsin. However, the remaining allergen was noted in caseins after treatment with 0,016 g/L of trypsin, it was completely removed after microbial hydrolysis.

Antioxidant capacity

The antioxidant capacity samples were measured by two separate analytical methods such as FRAP assay and DPPH assay. I measured the antioxidant capacity in the protein concentrate as well as the enzyme-treated, fermented samples with and without glucose. The results showed that the antioxidant capacity of the nanofiltrated protein concentrate was increased after tryptic hydrolysis of milk protein concentrate. Antioxidant capacity was reduced by microbial fermentation of tryptic hydrolysed milk protein concentrate. It was found that antioxidant capacity was not significantly changed due to addition of glucose in microbial fermentation process.

Angiotensin-converting-enzyme inhibitory activity

Angiotensin-converting-enzyme was not inhibited by the milk protein concentrate. It was increased when milk protein concentrate was treated with trypsin, however, 100% inhibition was not found. Inhibition of angiotensin-converting-enzyme was represented by IC_{50} value. Reduction of IC_{50} value was trypsin dose-dependent. After microbial fermentation of tryptic hydrolysed milk protein concentrate, inhibition was more. It was found that angiotensin-converting-enzyme activity was not significantly changed due to addition of glucose in microbial fermentation process. Furthermore, it was found that inhibition of angiotensin-converting-enzyme was non-competitive type.

Antimicrobiological activity

Tryptic hydrolysed milk proteins showed antibacterial activity against the strains *Bacillus cereus* and *Staphylococcus aureus*. By increasing the concentration of trypsin from 0.008 g/L to 0.032 g/L, the diameter of zone of inhibition was increased for all microbes. In the case of *Bacillus cereus*, the diameter of zone of inhibition was 2.5 mm and 4.3 mm for concentration of trypsin 0.008 g/L and 0.032 g/L, respectively. For *Staphylococcus aureus*, the diameter of zone of inhibition was 2.3 mm and 3.6 mm for concentration of trypsin 0.008 g/L and 0.032 g/L, respectively. Microbial fermentation of tryptic hydrolysed milk protein concentrate offered antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*. Addition of glucose did not have influence on antimicrobial activity.

4. Conclusion and Recommendations

During of my research, I managed to produce dairy-based peptides with antioxidant capacity, anti-angiotensin activity, anti-bacterial activity and hypoallergenic property. UHT milk was used to prepare such kind of peptides. In the first step of my experiments, milk protein concentrate was prepared by the membrane filtration. In this investigation, positive effect of static mixture was proven. Permeate flux was increased at TMP 3 bar, compare to TMP 2 bar, however change of permeate flux was insignificant when RFR was increased from 100 L/h to 200 L/h. Permeate flux was decreased with time filtration time progress due to formation of concentration polarization on membrane surface. Concentrated milk protein was hydrolysed by different concentrations of trypsin. Subsequently, tryptic hydrolysed milk protein concentrate was fermented with lactic acid bacteria. According to SDS-PAGE gel electrophoresis, hydrolysis of milk protein was increased with increase of the concentration of trypsin and subsequent microbial hydrolysis. Anti-oxidant capacity, anti-ACE activity and antibacterial activity were increased after tryptic and microbial hydrolysis of protein. Inhibition of angiotensin converting enzyme was non-competitive type. However, tryptic hydrolysed milk protein had antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus,* microbial fermentation of tryptic hydrolysed milk protein concentrate had antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*. Elimination of milk protein allergenic was increased with increase of the concentration of trypsin. There was no allergenicity when milk protein concentration was treated with 0.032 g/L of trypsin. However, the allergen was present in caseins, after treatment with 0.016 g/L of trypsin, it was completely removed after microbial hydrolysis. It was found that glucose did not have significant effect on microbial hydrolysis of proteins, antioxidant capacity, anti-angiotensin activity, anti-bacterial activity and hypoallergenic property.

In my studies I used trypsin enzyme, however, in later studies it would be worth to use other enzymes, to examine the efficiency of hydrolysis, biological activities and presence of allergen. Furthermore, antigenic activity of synthesized peptides will be performed by protein specific rabbit serum.

5. New scientific results

1. Using a statistical method, I have set up a model to estimate/calculate the initial flux of membrane filtration (NF, 5 nm, see 3.2) using a spiral static mixer (see subsection 3.2 Membrane Filtration Equipment):

Using a static mixer –

$$J = 24{,}426 + 9{,}402 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) + 0{,}0825 \cdot \left(\frac{RFR - 150}{50}\right) + 0{,}0825 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) \cdot \left(\frac{RFR - 150}{50}\right)$$

Without static mixer –

$$J = 12{,}242 + 3{,}797 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) + 1{,}172 \cdot \left(\frac{RFR - 150}{50}\right) + 1{,}102 \cdot \left(\frac{TMP - 2{,}5}{0{,}5}\right) \cdot \left(\frac{RFR - 150}{50}\right)$$

I prepared a model to understand the effect of flowrate, pressure, retention and flux in the constant temperature (25°C) range from 2-3 bar TMP to 100-200 L/h RFR.

2. During my studies, I managed to maximize the flux, and find the lowest energy consumption cost of membrane filtration in the operating parameters used (TMP = 3 bar and RFR = 100 L/h).

   During laboratory membrane filtration, I used a static mixer (see subsection 3.2 Membrane filtration equipment). I did my experiments at a constant 25°C. In addition to the membrane filtration parameters used, flux was 34.22±1.08 L/m^{2}h, while the energy consumption was 4.87 kWh/m^3.

   (During membrane filtration, as a raw material, I used homogenized 1.5% cow's milk produced using UHT technology, which I bought from a local supermarket. The protein, lactose and fat concentrations of the milk used are on average 31 ± 0,1 g/L, 47 ± 0,84 g/L and 1 ± 0,12 g/L

3. The optimal operating parameters were TMP = 3 bar and RFR = 100 L/h, at these values the highest protein concentration in the retentate can be ensured (59,21 g/L). At constant pressure (TMP = 3 bar) and changed recirculation-flowrate (RFR = 100 - 200 L/h), there is no significant difference in the protein content of the retentate, but

with a higher RFR, the specific energy consumption of membrane filtration was higher (35 kWh/m^3), so from an economic point of view I used RFR = 100 L/h (see subsection c. to produce milk protein concentrate using membrane filtration).

4. I have confirmed by measurements that after enzymatic hydrolysis of the NF protein concentrate, the antioxidant capacity increases significantly in the studied samples by doubling the enzyme concentration.

   My analytical experiments were based on the protein concentrate obtained during membrane filtration, which I hydrolyzed at various enzyme concentrations. During hydrolysis with trypsin, the enzyme activity values associated with each enzyme concentration were as follows: 0,008 g/L (AU 0,014/(s•μg), N2=0 ,016 g/L (AU 0,028/(s•μg), N3=0,032 g/L (AU 0,056/(s•μg).

   Enzymatic hydrolysis of the protein concentrate was carried out at 40 °C for 10 minutes and inactivation at 70 °C for 30 minutes.

   For DPPH, the increase was more than three times higher, while the increase in FRAP was measured by 20 to 50 % compared to the milk protein concentrate obtained during membrane filtration. In addition to the enzyme concentration N3, the DPPH method had an antioxidant content of 62.04 ± 2.74% during the 0.032 g/L enzymatic treatment. The same upward trend was observed in the FRAP method, where N3 enzyme concentrations were 202.65 ± 2.25 mg/L. For both factors (treatments, groups) ANOVA showed a significant effect on both variables (FRAP, DPPH) FRAP: $F(2.24) = 44.39$; ($p<0,01$; DPPH: $F(2,24) = 60,31$; $p<0,01$

   The antioxidant content of the samples shows a decreasing tendency under the influence of fermentation and added glucose. DPPH method 53,4 ± 1,17 %, which is 15 %, while for FRAP it is 128,3 ± 32,04 mg/L, representing a 15 % reduction in the antioxidant content.

5. At the highest enzyme concentration (0.032 g/L) I used, all proteins that triggered significant allergenic reactions (α lactalbumin, β lactoglobulin, caseins: α- β and κ, immunoglobulins, lactoferrins, lactoperoxidases) were hydrolyzed.
   The allergenic effect can be further reduced if the enzymatic hydrolyzed samples are fermented with lactic acid bacteria (N1, N1Y and N1Y +G). In total, at a concentration of 0.032 g/L of trypsin, no immune reactivity to the serum is observed.
   In the immunoblot studies, I found that by increasing enzyme concentrations (0.008 to

0.032 g/L), the activity of allergenic epitopes can be reduced, with a direct effect on reducing the allergenic effect of proteins. During the enzyme concentrations used, at the concentration of 0.032 g/L trypsin, the allergen effect of proteins is eliminated, enzymatic hydrolysis eliminated immunoreactive proteins. In addition to enzyme concentrations of 0.008 and 0.016 g/L, casein proteins showed immunoreactivity and some proteins in the upper molecular band.

6. ACE inhibition in fermented samples increases significantly by increasing enzyme concentration (0.008 to 0.032 g/L) (ANOVA - F (2.18) = 213.08; F (2,18) = 63,05, $p<0,01$).

   The reduction is between 20% and 40%, and the combination of fermentation and enzymatic hydrolysis promotes the degree of inhibition. The lower the IC_{50} value, the higher the ACE inhibition rate in the samples. Fermentation and added glucose had a positive effect on ACE inhibition, as in these cases the IC_{50} value continued to decrease. With enzyme concentrations and fermentation of 0.032 g/L, the 5.8 mg/mL value was reduced to 3 (excluding added glucose) and 2,5 mg/mL (with added glucose).

7. In my studies, I found that the peptides released during trypsin hydrolysis of milk proteins have a significant antibacterial effect against *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes* (ANOVA -*Bacillus cereus*: F(3,16) = 47,52, Staphylococcus aureus: F(3,16) = 24,39, *Listeria monocytogenes*: F(3,16) = 104,99, in all three cases $p <0,001$). By gradually increasing enzyme concentrations (from 0,008 g/L to 0,032 g/L), the inhibition zone increased (from 2,5 mm to 4,3 mm to 72 %) in *bacillus cereus*, while at the same concentrations, the inhibition zone for the *Staphylococcus aureus* microbial strain also increased, which varied from 2.3 mm to 3.6 mm to 56 %.

   The diameter of the inhibition zones continued to increase due to fermentation (10-20 %), which showed a further slight improvement (5 to 10 %) as the added glucose promoted the growth/metabolism of the starter culture. During fermentation, *Bacillus cereus* had an inhibition zone of 4.76 mm (excluding added glucose) and 5 mm (added glucose). A similar change was observed in *Staphylococcus aureus*, where the inhibition zone was 3.97 mm (excluding added glucose) and 4.17 mm (added glucose) during fermentation. In fermented samples, I also carried out the tests on *listeria monocytogenes* strain, where the diameter of the inhibition zone also increased to 3.4 mm under the influence of N1 = 0.008 g/L enzymatic treatment.

List of publications in the field of studies

IF articles

- Nath, A., **Csighy, A.,** Eren, B. A., Tjandra Nugraha, D., Pásztorné-Huszár, K., Tóth, A., Takács, K., Szerdahelyi, E., Kiskó, G., Kovács, Z., Koris, A., & Vatai, G. (2021). Bioactive Peptides from Liquid Milk Protein Concentrate by Sequential Tryptic and Microbial Hydrolysis. Processes. 9(10), 1688. **IF: 2,847** https://doi.org/10.3390/pr9101688
- **Csighy. A.**, Nath, A., Vozáry, E., Koris, A., Vatai, G., 2020. Investigating the Texture and Antioxidant Capacity of Papain and Trans-glutaminase Enzyme-treated Yogurt with Different Carbohydrates – Glucose, Sucrose and Maltodextrin. Periodica Polytechnica Chemical Engineering. Online session. **IF:1.248** https://doi.org/10.3311/PPch.15041
- Nath, A., Eren, B. A., **Csighy, A.,** Pásztorné-Huszár, K., Kiskó, G., Abrankó, L., Tóth, A., Szerdahelyi, E., Kovács, Z., Koris, A., & Vatai, G. (2020). Production of Liquid Milk Protein Concentrate with Antioxidant Capacity, Angiotensin Converting Enzyme Inhibitory Activity, Antibacterial Activity, and Hypoallergenic Property by Membrane Filtration and Enzymatic Modification of Proteins. Processes, 8(7), 871. **IF: 2,847** https://doi.org/10.3390/pr8070871

1.1 Conference papers, abstract

- Csighy, A., Nath, A., Vozáry, E., Galambos, I., Koris, A., Vatai, G., 2019. Reduction of allergenicity of cow milk and it's fermented product by combination of membrane filtration, enzymatic hydrolysis and microbial fermentation process. PERMEA 2019 - Membrane Conference of Visegrad Countries. Eötvös Lorand University. Budapest
- **Csighy, A**., Vatai, G., Pásztorné Huszár, K., Vozáry, E., Kiskó, G., Koris, A., Nath, A., 2019. Enzyme-treated milk yoghurt – Process development and biochemical characteristics, in: Zsomné, M.V., Márki, E., Baranyai, L. (Eds.), BiosysFoodEng 2019 - Proceedings. Szent István University, Budapest, p. 1.
- **Csighy, A**., Nath, A., Vozáry, E., Koris, A., Vatai, G., 2018. Investigating the texture and antioxidant capacity of papain and trans-glutaminase enzyme-treated yogurt with different substrate – glucose and sucrose and maltodextrin. The 9th International Congress of Food Technologist, Biotechnologist and Nutritionists. Zagreb, Croatia.

- Nath, A., Eren, B.A., **Csighy, A.**, Tóth, A., Némethné, S.E., Kiskó, G., Friedrich, L.F., Pásztorné Huszár, K., Koris, A., Vatai, G., 2018b. Biologically Active Peptides from Ultra-heat Treated Milk by Membrane- and Enzymatic Routes, in: Third International Conference on Food Science and Technology. Szent István Egyetem, Élelmiszertudományi Kar, Budapest, p. 57.
- **Csighy, A.**, Nath, A., Vozáry, E., Koris, A., Vatai, G., 2018. Investigating the texture and antioxidant capacity of papain enzyme-treated yogurt with different substrate – glucose and sucrose, in: Zsomné Muha, V. (Ed.), 2nd International Conference on Biosystems and Food Engineering in Memory of Professor András Fekete. Szent István Egyetem, Élelmiszertudományi Kar, Budapest.
- Pásztorné Huszár, K., Nath. A., **Csighy, A.**, Friedrich, L.F., Vatai, G., Koris, A., 2017. Enzyme-mediated Hypoallergenic Milk and Dairy Products - Present Scenario and Future Prospects, in: Sucur, Z., Spiwok, V., Paulova, L., Matatkova, O., Kas, J. (Eds.), Book of Abstracts. University of Chemistry and Technology (UCT), Prague, pp. 82–83.
- **Csighy, A.**, Koris, A., Nath, A., Vatai, G., 2016. Diafiltration based cow's milk partial demineralization - laboratory membrane filtration experiments, in: Szent, I.E.É.K. (Ed.), Proceedings of 1st International Conference on Biosystems and Food Engineering. Szent István Egyetem, Élelmiszertudományi Kar, Budapest, pp. 1–7.