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The calcium solubilization ability and anti-inflammatory effects of hydrolyzed casein protein

Abstract (within 250 words)

This study performed to evaluate the applicability of functional dairy food materials by comparing the calcium solubilization ability and anti-inflammatory effects of hydrolyzed casein protein.

Commercial enzyme was added to the 10% casein solution to prepare the casein hydrolysates. Samples obtained every hour (1:200(w/v)). According to results of measuring the degree of hydrolysis (DH), all of four enzymatic hydrolysates increased rapidly from 30 to 40 minutes, and after 150 minutes, there were no change. Protamex® and Neutrase® had the highest DH compared to others enzymatic hydrolysates. After that, peptides obtained throughout a preparative liquid chromatography system. In the calcium solubility experiments, Neutrase Fraction(NF)4 and NF7 showed similar activities with CPP(casein phosphopeptide). *In vitro* cell experiments showed that no cytotoxicity except for NF6. Also, the production of nitric oxide (NO) inhibited as the concentration of fraction samples increased. The cytokine (IL-1α, IL-6, TNF-α) production was lower than lipopolysaccharide (+) group significantly. Therefore, the possibility of anti-inflammatory activity found in the hydrolyzed samples. According to the above experiments, NF3 and Protamex Fraction(PF)3 selected. Amino acids selected throughout an AccQ-Tag system. As a result, 17 species of amino acids and several species of unknown amino acids identified. Both fractions had the highest content of Phenylalanine.

This study identified the potential of biologically active and functional peptides derived from casein that affect the food and dairy industry.

34 Keywords: (3-5 keywords) Milk protein, Casein, Calcium solubilization ability, Anti-
35 inflammatory effects, Amino acids

36

Introduction

Milk is made of milk fats, proteins, carbohydrates, water-soluble vitamins, minerals, and water. The whey protein and a casein exists as protein components. Casein is major component of milk proteins and it is widely studied because it has high availability and nutritional importance. It is about 3% in milk and it is about 80% of all protein in milk. Also, casein protein consisted of beta-casein fragments and molecular weight is about 75,000 ~ 375,000 kDa (Lucey et ala., 2011).

In recent years, calcium intake has increased, but its utilization in the body is low, leading to various deficiency diseases. Lack of calcium is the cause of various diseases such as poor bone growth, fracture, osteoporosis, hypercholesterolosis, arteriosclerosis, hypertension, and hyperlipidemia (lawrence GR, Smith JA., 1989; Yoon JH et al., 1996). In order to increase calcium intake, the use of calcium in the body should be higher than that of eating large amounts (Greger JL., 1988).

It has been reported that casein itself affects calcium absorption (Bronner, 1987), affecting immune system, so, it affects final immune response and cellular function (Coste and Tom, 1991; Kayser and Meisel, 1996).

Casein protein is characterized with a type of amino acid constituents. The use of casein hydrolysates that retain the bioactive amino acid domain or sequence may be a better alternative to prevent rancidity in foods without affecting food quality (Diaz and Decker, 2004). Also, the enzymatic hydrolysis of casein may act in a body as regulatory components with a hormone activity which can modulate specific physiological functions (Meisel and FitzGerald, 2003).

In vitro and *in vivo* enzymatic proteolysis of casein proteins produce bioactive peptides (Jelen and Lutz, 1998), which have specific biological functions throughout their ability to affect cellular function (Meisel, 1997). Because CPP(casein phosphopeptide) have a

potential effects to deliver calcium as a functional ingredient. (Kitts et al., 1992). It is important to examine their immunological activity further. In response to this, 32 articles examining the role of hydrolyzed infant formulas in the prevention of allergies have been investigated (Hays, 2005).

Through this, if casein is hydrolyzed using proteolytic enzyme to develop a specific peptide similar with CPP, superior calcium absorption and anti-inflammatory effects will be expected.

In addition, researchers at the Department of Pediatric Gastrointestinal and Nutrition at Johns Hopkins of Medicine reported that infants fed with totally hydrolyzed casein and infants had a reduced incidence of atopic disease during the five years of life. Also, casein hydrolysates have been reported that it has a biological function on cellular function (Meisel, 1997), and it affects immune system cells (Coste and Tom, 1991; Kayser and Meisel, 1996).

Analysis of bioactive peptides has been made in several studies. These peptide sequences, encrypted within proteins, are liberated *in vivo* during gastrointestinal digestion or *in vitro* by fermentation with proteolytic starter cultures or using proteases. BAP generally comprises 2–20 amino acid residues (Poosapati et al., 2018).

In this regard, the objective of this study is development of a specific peptide which have a similar effect with CPP and researchinga of peptide which has excellent physiological properties and promotes calcium absorption.

Materials and Methods

Preparation of casein hydrolysates

Commercial casein (95% protein) obtained from Meggle food Ingredients (Wasserburg, Germany).

A 10% casein solution prepared by mixing 10% casein and 90% 0.1 M Potassium phosphate buffer (pH 7.4). After a sterilization for 10 minutes at 90 ℃, a pH is adjusted to 7.0 which is optimum active pH of the enzyme with 1 N NaOH (Sigma, St. Louise, MO, USA).

Commercial enzymes used with Alcalase® 2.4 L FG (Protease from *Bacillus licheniformis*, 5 U/g), Neutrase® 0.8 L (Protease from *Bacillus amyloliquefaciens*, 0.8 U/g), Protamex® (Protease from *Bacillus sp*., 1.5 U/g) and Flavourzyme® (Protease from *Aspergillus oryzae*, 500 U/g). All commercial enzymes purchased from Novozymes™ (Denmark).

The enzymes added at a ratio of 1:200 (w/v) based on the substrate, and shaken at 120 rpm in a shaking water bath at 50 ℃. The samples taken at 30 minutes and inactivate the enzymes at 90 ℃ for 10 minutes. It preceded to 240 minutes. After a cooling at 20 ℃, the supernatant collected by centrifugation for 20 minutes under conditions of 4,000 g and 4 ℃. The supernatant was filtered with PVDF 0.22 Syringe membrane filter (Futecs co. Ltd., Korea). Samples stored at -20 ℃ and used for each experiment.

Degree of hydrolysis (DH) of casein hydrolysates

The hydrolysis degree of casein hydrolysates measured using the Lowry method (Lowry, 1951). The Lowry solution A and B prepared for the degree of hydrolysis. To

prepare solution A, 1% Cupric sulfate (Sigma, St. Louis, MO, USA) and 2% Sodium potassium tartrate (Sigma, St. Louis, MO, USA) mixed at a 1:1 ratio (v/v), and 1.0 mL of the mixed solution and 50 mL of 2% Na_2CO_3 (DaeJung Chemical & Metals Co., Ltd., Korea) were mixed. Solution B used to prepare a 1 N Folin-Ciocalteau reagent (Sigma, St. Louis, MO, USA).

First, 1.0 mL of casein hydrolysates and the same amount of 20% TCA (Duksan Chemical Co., Ltd., Korea) mixed and maintained for 1 hour to precipitate 20% TCA soluble protein. It centrifuged at 10,000 rpm for 20 minutes. The supernatant and dissolved residue removed in 5.0 mL of 0.1 N NaOH (Sigma, St. Louis, MO, USA).

Thereafter, 1.0 mL of a casein hydrolysates and 5.0 mL of solution A mixed and reacted at room temperature for 10 minutes. After that, 0.5 mL of solution B added and the contents mixed immediately with vortex mixer. After reacting for 30 minutes, an absorbance measured at 600 nm. An equivalent amount of protein calculated from standard curve prepared using Bovine serum albumin (Sigma, St. Louis, MO, USA). A bovine serum albumin measured in the range of 20 to 200 μg/mL.

The amount of TCA soluble protein measured and calculated by the following equation.

$$\text{Degree of Hydrolysis (\%)} = \frac{\text{Soluble protein from 10\% TCA}}{\text{Total protein}} \times 100$$

Purification of casein hydrolysates

In order to obtain peptide fractions from casein hydrolysates, gel filtration throughout a preparative chromatography system performed (Waters Corp, USA).

Peptides that identified in SDS-PAGE separated on a preparative scale for further study. The preparative pump (Waters Corp, USA) and Preparative liquid

chromatography W600 (Waters Corp, USA) used with a Dual λ Absorbance detector W2487 (Waters Corp, USA) and W717 Autosampler (Waters Corp, USA).

The casein hydrolysates dissolved in 5 mM sodium phosphate buffer with 0.15 M NaCl (Sigma, St. Louis, MO, USA), pH 7.0. It filtered with a PVDF 0.45 μm sterile syringe membrane filter (Futecs co. Ltd., Korea). After that, casein hydrolysates is loaded 3.0 mL throughout a Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences, USA) and eluted at flow rate of 1.0 mL/min. A detector was set at 280 nm.

All fractions obtained throughout a gel filtration with Hiprep 16/60 Sephacryl S-100 HR column on Preparative chromatography system collected and fractions stored at -20 ℃.

Table. 1. Condition of preparative liquid chromatography system

Instrument	Condition
Column	Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences, U.S.A)
Mobile phase	5 mM Sodium phosphate buffer (0.15 M NaCl, pH 7.0)
Detector (Detection)	Waters Dual λ Absorbance Detector W2487 (280 nm)
Flow rate	1 mL/min
Injection volume	3 mL

Calcium solubilization ability

In the experiment of promoting calcium absorption, the method of Natio (1986) and Yamamoto et al., (1994) was slightly modified to measure the effect on precipitation in solution after calcium phosphate formation.

The 10 mM Calcium chloride (Sigma, St. Louis, MO, USA) and 20 mM Sodium phosphate buffer were prepared. After that, 0.5 mL of 10 mM Calcium chloride and 0.5

mL of casein hydrolysate fraction samples mixed. And a 1.0 mL of 20 mM Sodium phosphate buffer added. The mixed solution incubated at 37 ℃ for 2 hours and centrifuged at 2,000 g for 30 minutes at 25 ℃.

Calcium solubility measured using Calcium colorimetric kit (Gene tex, Inc., USA) for the whole solution and the supernatant collected after centrifugation.

The whole solution and supernatant samples dispensed into each 10 μL 96 well plate, followed by mixing of Chromogenic reagent to 90 μL and Calcium assay buffer to 60 μL. After reacting for 5 minutes in dark room at room temperature, an absorbance measured at 570 nm.

Calcium concentration calculated according to the first equation below, and calcium solubility calculated according to the second equation below. All protein concentration of casein hydrolysate fraction samples were 200 μg/mL.

$$\text{Calcium concentration (mg/mL)} = \frac{\text{Sa}}{\text{Sv}}$$

* Sa = Sample amount from standard curve

* Sv = Sample volume

$$\text{Calcium solubility (\%)} = \frac{\text{Calcium concentration in supernatant}}{\text{Calcium concentration in whole solution}} \times 100$$

Cytotoxicity of casein hydrolysate fractions (MTT assay)

RAW 264.7 cell is a macrophage cell of mouse. RAW 264.7 cells obtained from the Dankook University (Cheonan, Korea). Cells grown in Dulbecco's Modified Eagle Medium (DMEM) including 10% heat-inactivated fetal bovine serum (FBS). The incubator temperature adjusted to 37 ℃ and maintained at 5% CO_2.

RAW 264.7 cells seeded in 96-well cell culture plates at a density of 5×10^4 cells/well and incubated for 16-18 hours. After removing the medium, RAW 264.7 cells treated with various concentrations of fraction samples for 20-22 hours.

After that, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (Sigma, St. Louis, MO, USA) solution added to each well at a final concentration of 5 mg/mL and incubated (37 ℃, 5% CO_2). After 2-3 hours, culture supernatants removed, and 100 uL of Dimethyl sulfoxide (Sigma, St. Louis, MO, USA) added to each well to completely and dissolve formazan crystals. The absorbance measured at 540 nm (Fotakis et al., 2006).

Measurements of nitric oxide (NO assay)

RAW 264.7 cells seeded in 96-well cell culture plates at a density of 5×10^4 cells/well and incubated for 16-18 hours. The amount of nitric oxide (NO) calculated by measuring the amount of nitrite, an oxidized product, in the cell culture supernatants as previously explained. After removing the medium, RAW 264.7 cells treated with various concentrations of fraction samples in medium for 2-3 hours.

After that, Lipopolysaccharide (LPS) with final concentration of 100 ng/mL treated and stimulated at the same volume in medium for 20-22 hours. A Griess reagent added to the supernatant in a ratio of 1:1 (v/v). The absorbance at 540 nm measured in a Microplate reader after 15 min at room temperature (Lim et al., 2010).

Measurement of Cytokine (ELISA)

RAW 264.7 cells seeded in 96-well plates at a concentration of 5×10^4 cells/mL for 24 hours, preprocessed cell free extracts were prepared. After that, the supernatant taken and the cytokine content measured by a Mouse IL-1α, IL-6 and Mouse TNF-α ELISA

kit (Komabiotech Inc., Korea) using an enzyme-linked immunosorbent assay (ELISA).

The 100 μL of fraction samples added to each well coated with specific antibodies against cytokines (IL-1α, IL-6 and TNF-α), reacted at room temperature for 2 hours. And the supernatant removed and washed five times with washing buffer. Thereafter, detection antibody added to react with antibody. Also, streptavidin-Horseradish peroxidase (HRP) conjugated with avidin added and reacted at room temperature for 30 minutes. After that, it washed for 4 times. A 100 μL of Tetramethylbenzidine (TMB) added to each well as a substrate. After that, it incubates at the room temperature as a proper color development. A stop solution added to each well and absorbance measured at 450 nm (Eckel et al., 2011).

Profiling of Amino acids (AccQ-Tag system)

Amino acid profiling preceded according to Waters amino acid analysis AccQ-Tag manual. A waters AccQ-Tag·Fluor reagent kit used for a derivatization of the sample and standard. An amino acid standard (Sigma, St. Louise, MO, USA) used as a standard. The range of sample amount is 0.02-0.08 μg (20-1,000 pmol). Others system condition shown in Table. 2 which showed in below.

Table. 2. Profiling of amino acids (AccQ-Tag system)

Instrument	Condition
Column	Waters AccQ-Tag (Waters, U.S.A)
Mobile phase	Waters AccQ-Tag eluent A, 60% Acetonitrile (pH 5.02)
Detector (Detection)	Waters Dual λ Absorbance Detector W2487 (280 nm)
Flow rate	1 mL/min
Injection volume	10 μL

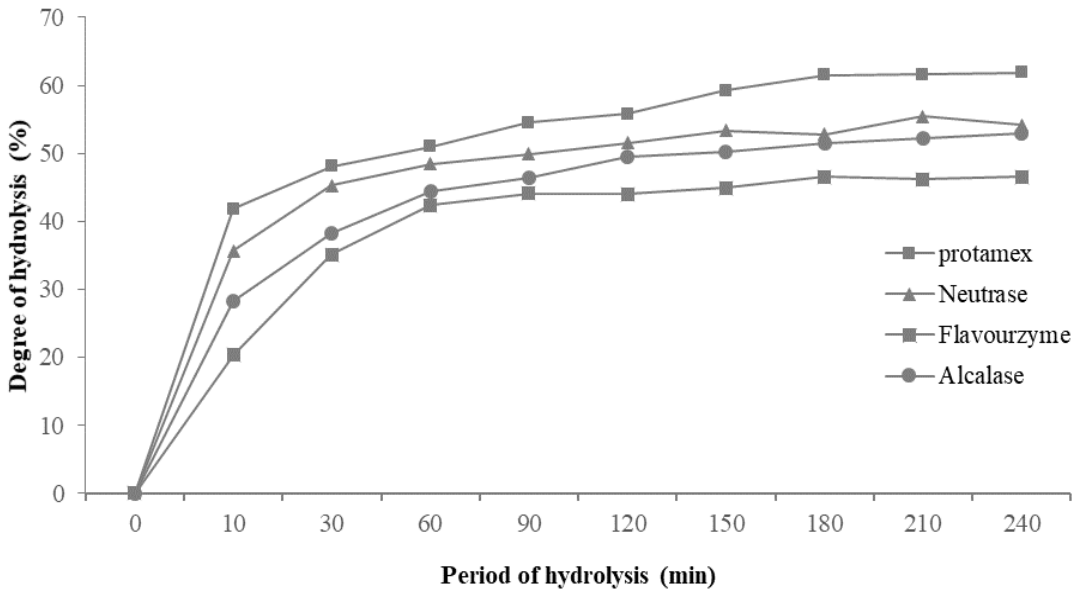
225 # Results

226

227 **Degree of hydrolysis (DH) of casein hydrolysates**

228 The degree of casein hydrolysis defined as the percentage of total number of peptide
229 bonds in a protein that has been cleaved during hydrolysis (Adler-Nissen, 1986). The
230 degree of hydrolysis of the four enzymes tended to increase into a similar pattern. There
231 was no significant difference from 150 minutes and after increasing from 120 minutes
232 to 150 minutes. Among them, the highest degree of hydrolysis of the casein hydrolyzed
233 by Protamex® measured, and the degree of hydrolysis of the casein hydrolyzed by
234 Neutrase® and Flavourzyme® are similar.

235



236

237 Figure. 1. Degree of hydrolysis (DH) of casein hydrolysates

238 *Reaction were carried out in a water bath (50 ℃ , 120 rpm)

239 *All enzymes were applied at concentration 1:200 (w/v) to the casein solution

240 *Alcalase® 2.4 L; Neutrase® 0.8 L; Flavourzyme®; Protamex® (Novozymes™,

241 Denmark)

242

243 **Purification of casein hydrolysates**

244 Neutrase® and Protamex® selected throughout the results of degree of hydrolysis
245 (DH). Peptide fractions of casein hydrolysates isolated using Hiprep 16/60 Sephacryl S-
246 100 HR column in Preparative liquid chromatography system. Each fraction separated
247 by molecular weight. 10 peptide fractions obtained totally. Each fraction compared to
248 the original casein hydrolysates via SDS-PAGE for a molecular weight (Data are not
249 shown).

250

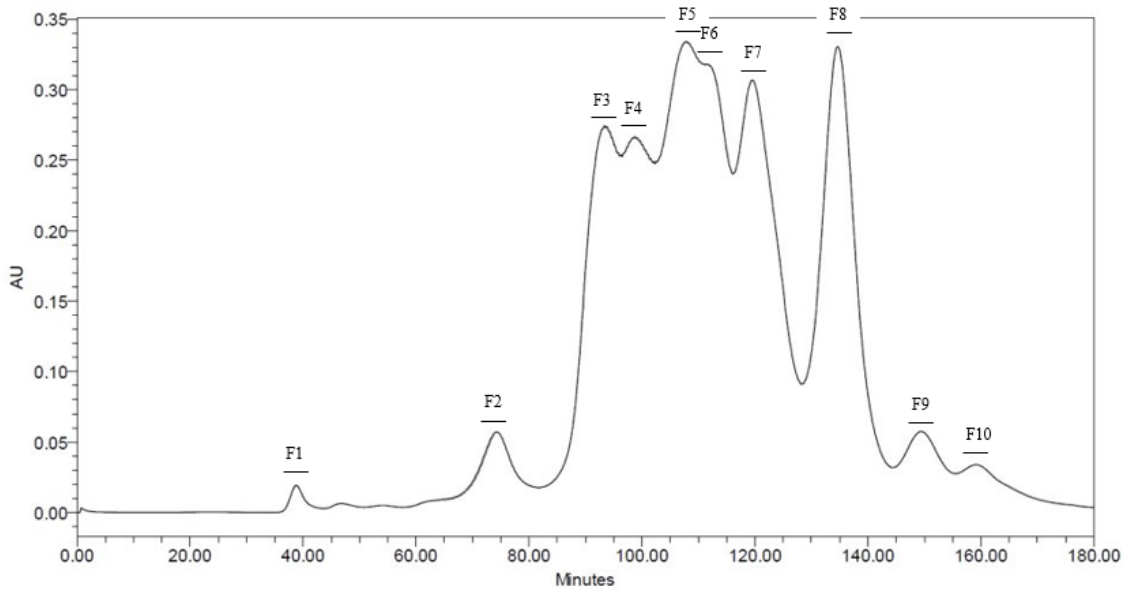
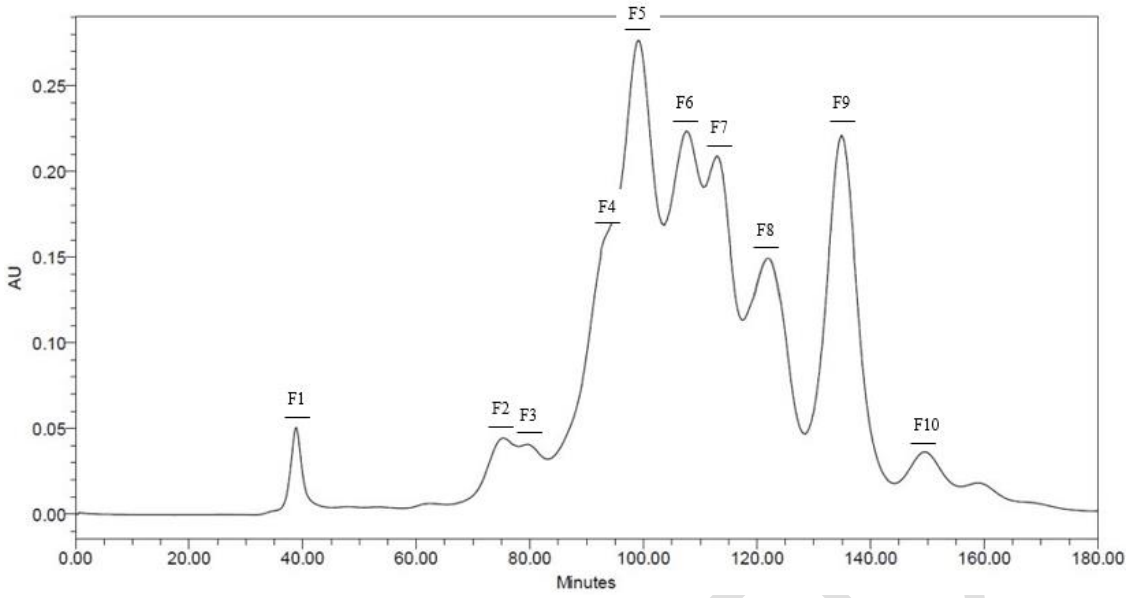


Figure. 2. Separations of casein hydrolysates using Neutrase® in preparative LC

*F# : Fraction number in prep LC system



255

256 Figure. 3. Separations of casein hydrolysates using Protamex® in preparative LC system

257 *F# : Fraction number in prep LC system

258

Calcium solubilization ability

CPP(casein phosphopeptide) contains phosphoserine and solubilizes calcium, which can promote calcium solubilization ability. In this study, calcium solubility measured to determine whether each fraction promote calcium solubilization ability similar with CPP.

According to experimental results, CPP showed high calcium solubility with 93.67%. Phosphoserine contained in CPP solubilizes calcium, resulting in high calcium solubility.

Both Neutrase® and Protamex® hydrolysate fractions showed lower calcium solubility than CPP. Also, F4 and F7 in the Neutrase® fractions showed lower than CPP but about 80% calcium solubility. The F6 have a similar level with 79.84%. The Protamex® fractions showed lower calcium solubility than CPP, but F3, F4, F5, F6, and F7 showed calcium solubility of about 80% or more.

This may show the potential of calcium solubilization ability. Based on the results of the above experiments, fractions that showed good activity selected.

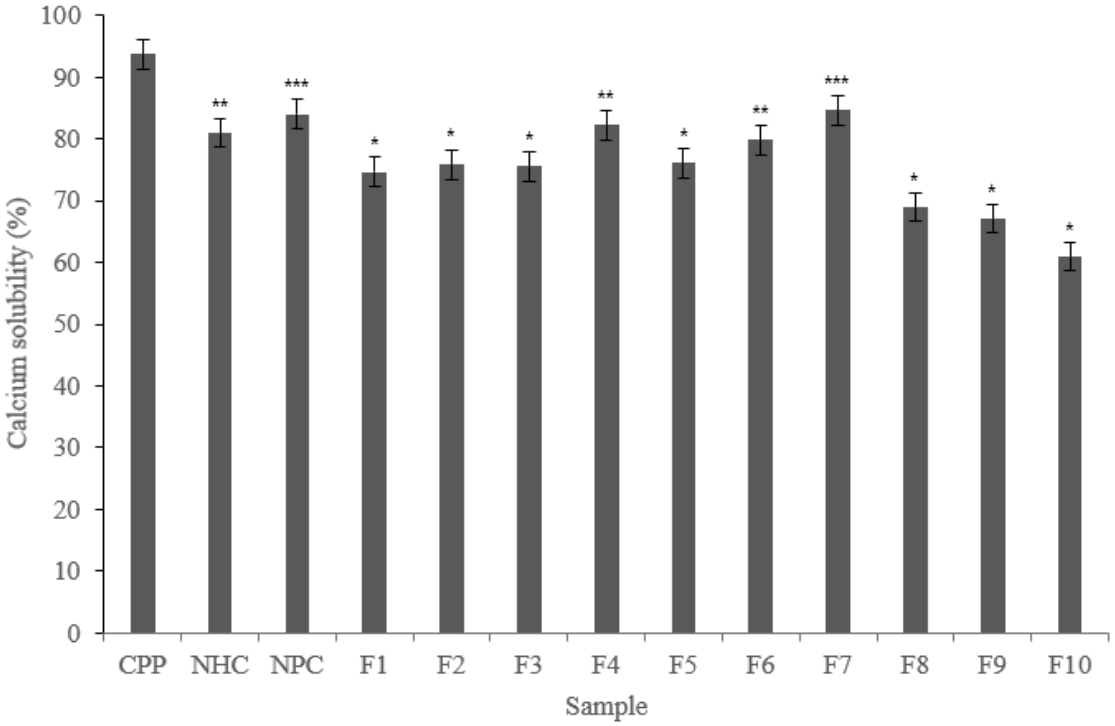


Figure. 4. Calcium solubility of casein hydrolysate fractions using Neutrase®

*CPP : casein phosphopeptide; NHC : Non-hydrolyzed casein; NPC : Non-purified casein

*F# : Fraction number in Prep LC system

*All samples protein concentration were 200 μg/mL

*All values were mean±SD of triplicates

*Statistical difference *p<0.05; **p<0.01;***p<0.001; CPP vs Sample

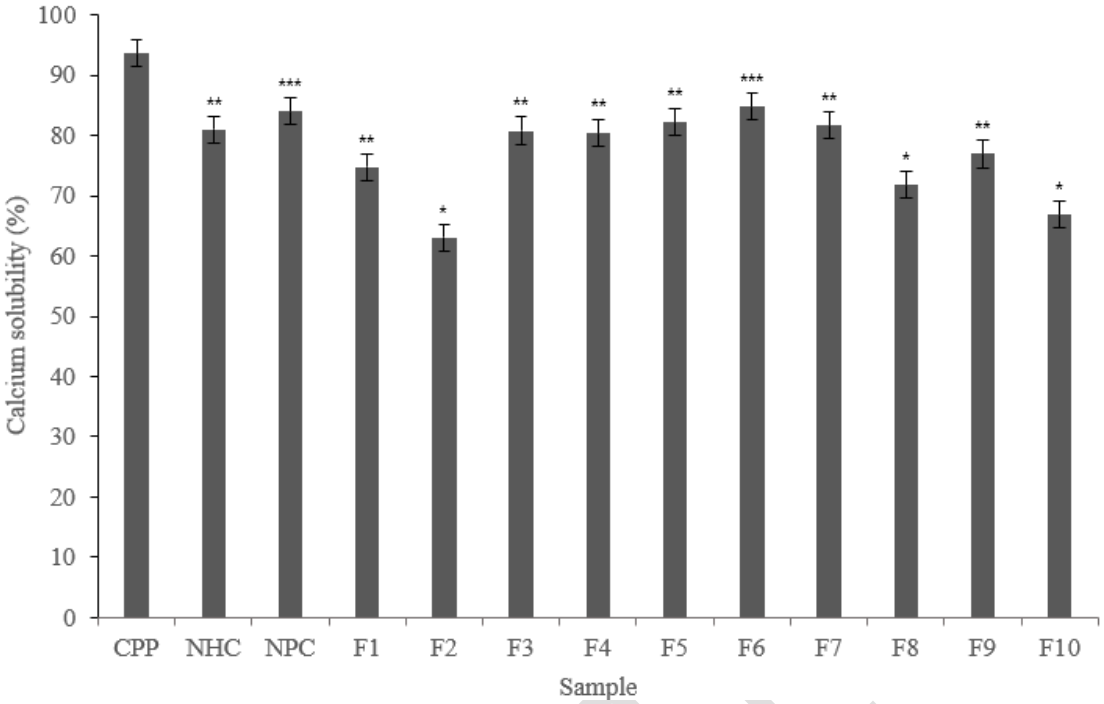


Figure. 5. Calcium solubility of casein hydrolysate fractions using Protamex®

*CPP : casein phosphopeptide; NHC : Non-hydrolyzed casein; NPC : Non-purified casein

*F# : Fraction number in Prep LC system

*All samples protein concentration were 200 μg/mL

*All values were mean±SD of triplicates

*Statistical difference *p<0.05; **p<0.01;***p<0.001; CPP vs Sample

Cytotoxicity of casein hydrolysate fractions

Cytotoxicity test via the MTT assay is used *in vitro* toxicology experiment widely. According to experimental results, despite the increase in concentration, there were no significant differences by concentration. Except for a NF6, there were no significance on concentration differences and cell death. In addition, it showed cell viability of 80% or more.

Therefore, it confirmed that there are no cytotoxicity of fraction samples on macrophage cells.

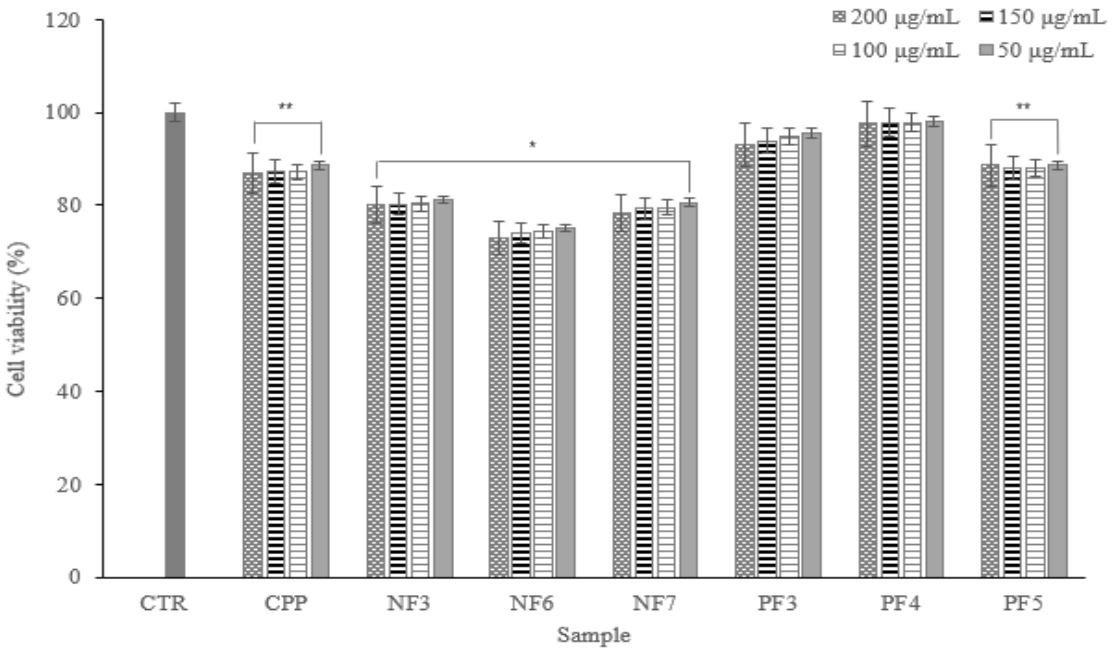


Figure. 6. Effects of hydrolysate fractions on cell viability in RAW 264.7 cells

*CPP : casein phosphopeptide

*NF# : Neutrase® hydrolysate fraction number in Prep LC system; PF# : Protamex® hydrolysate fraction number in Prep LC system

*200, 150, 100, 50 μg/mL : Samples protein concentration

*All values were mean±SD of triplicates

*Statistical difference : *$p<0.05$; **$p<0.01$; CTR vs NF# or PF#

Measurement of nitric oxide

The nitric oxide (NO) effect an important role in a blood coagulation, blood pressure regulation and immune function against cancer cells. However, it oxidized like reactive oxygen species (ROS) and converted into active NO. It produces oxidants that cause cytotoxicity. The NO produced by cells exposed to inflammatory mediators increased in tissue damage or various inflammatory diseases.

According to the results of the study, as the concentration of fraction samples increased, NO production inhibited. The MTT experiments showed that inhibition of NO production not caused by cytotoxicity.

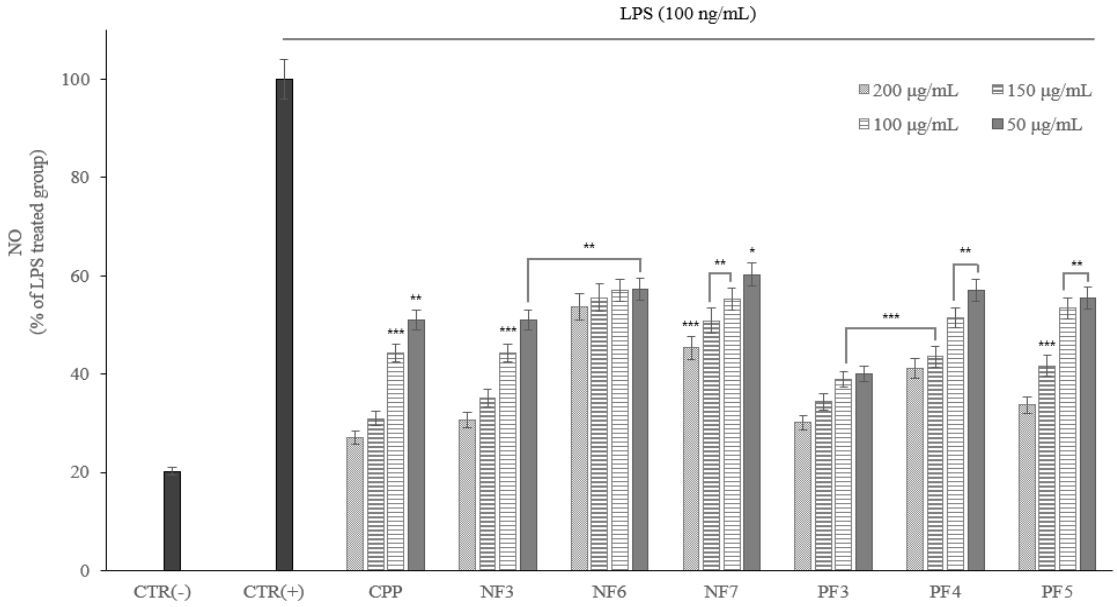


Figure. 7. Effects of hydrolysate fractions on NO in LPS-stimulated RAW 264.7 cell

*CPP : casein phosphopeptide

*NF# : Neutrase® hydrolysate fraction number in Prep LC system; PF# : Protamex® hydrolysate fraction number in Prep LC system

*200, 150, 100, 50 μg/mL : Samples protein concentration

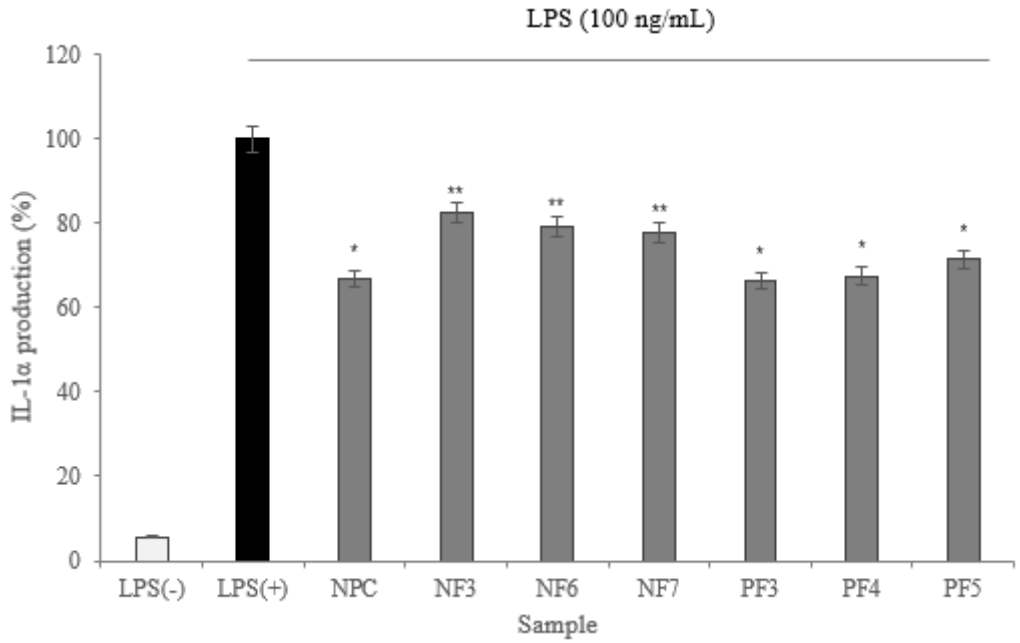
*All values were mean±SD of triplicates

*Statistical difference : *p<0.05, **p<0.01, ***p<0.001; CTR(-) vs NF# or PF#

Measurement of cytokine

To maintain immune balance, they must be direct or indirect interactions of immune cells. The cytokines can induce proliferation, differentiation, changes in function and activity of various immune cells. A disease is mostly associated with inflammation, and inflammatory cells secrete inflammatory cytokines that induce inflammation (Barland et al., 2004). In this study, the expression levels of IL-1α, IL-6 and TNF-α measured.

According to the results of cytokine measuring using ELISA, three cytokine (IL-1α, IL-6, TNF-α) production was significantly lower than LPS(+) group (*$p<0.05$). Based on results, the possibility of anti-inflammatory activity found in the hydrolysate fractions. In addition, the low expression level of TNF-α from macrophage effect by samples prevents the activation of proinflammatory cytokines which is produced by the expression of TNF-α. It reduces the inflammatory response.



342

343 Figure. 8. Comparison of IL-1α production in LPS-stimulated RAW 264.7 cells

344 *CPP : casein phosphopeptide

345 *NF# : Neutrase® hydrolysate fraction number in Prep LC system; PF# : Protamex®

346 hydrolysate fraction number in Prep LC system

347 *All values were mean±SD of triplicates

348 *Statistical difference : *$p<0.05$; **$p<0.01$; LPS(+) vs NF# or PF#

349

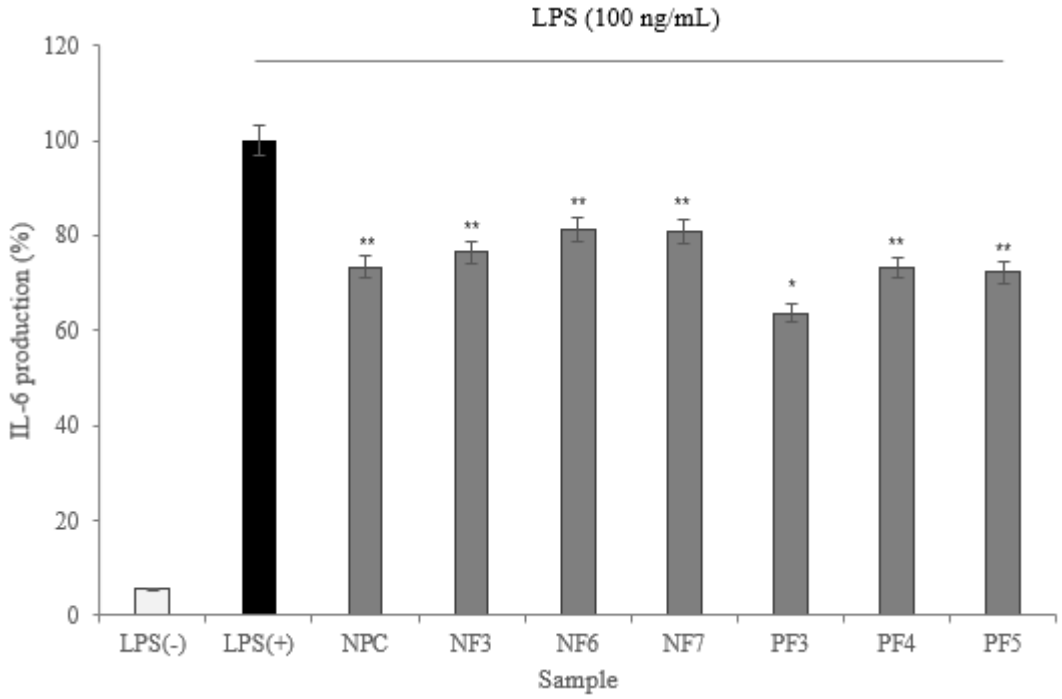


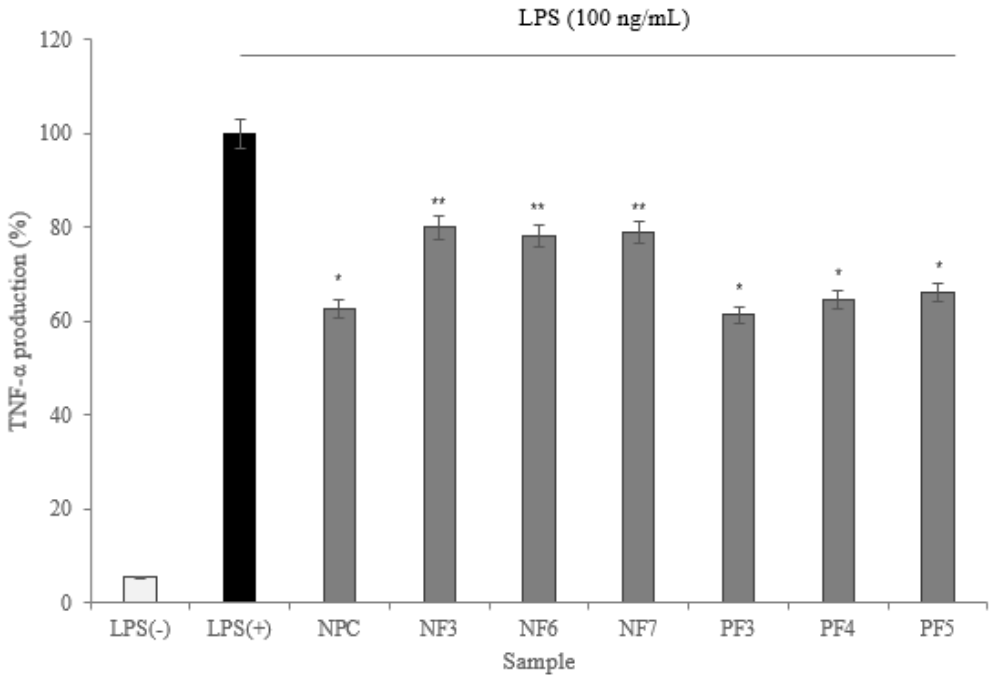
Figure. 9. Comparison of IL-6 production in LPS-stimulated RAW 264.7 cells

*CPP : casein phosphopeptide

*NF# : Neutrase® hydrolysate fraction number in Prep LC system; PF# : Protamex® hydrolysate fraction number in Prep LC system

*All values were mean±SD of triplicates

*Statistical difference : *p<0.05; **p<0.01; LPS(+) vs NF# or PF#



358

359 Figure. 10. Comparison of TNF-α production in LPS-stimulated RAW 264.7 cells

360 *CPP : casein phosphopeptide

361 *NF# : Neutrase® hydrolysate fraction number in Prep LC system; PF# : Protamex®

362 hydrolysate fraction number in Prep LC system

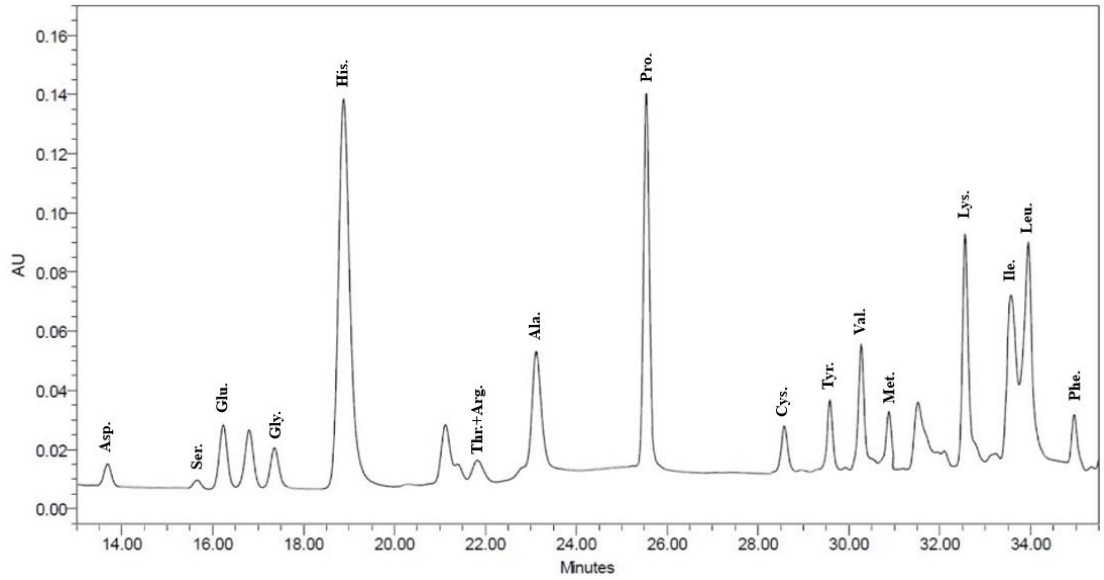
363 *All values were mean±SD of triplicates

364 *Statistical difference : *$p<0.05$; **$p<0.01$; LPS(+) vs NF# or PF#

365

Profiling of amino acids

Based on the above experiment results, NF3 and PF3 selected. According to results of confirming Amino acid sequence of the selected fractions throughout the AccQ-Tag system, 17 species of amino acids and several species of unknown amino acids identified (Fig. 11, 12, Table. 3). Both fractions had the highest content of Phenylalanine and the lowest content of Threonine and Arginine.

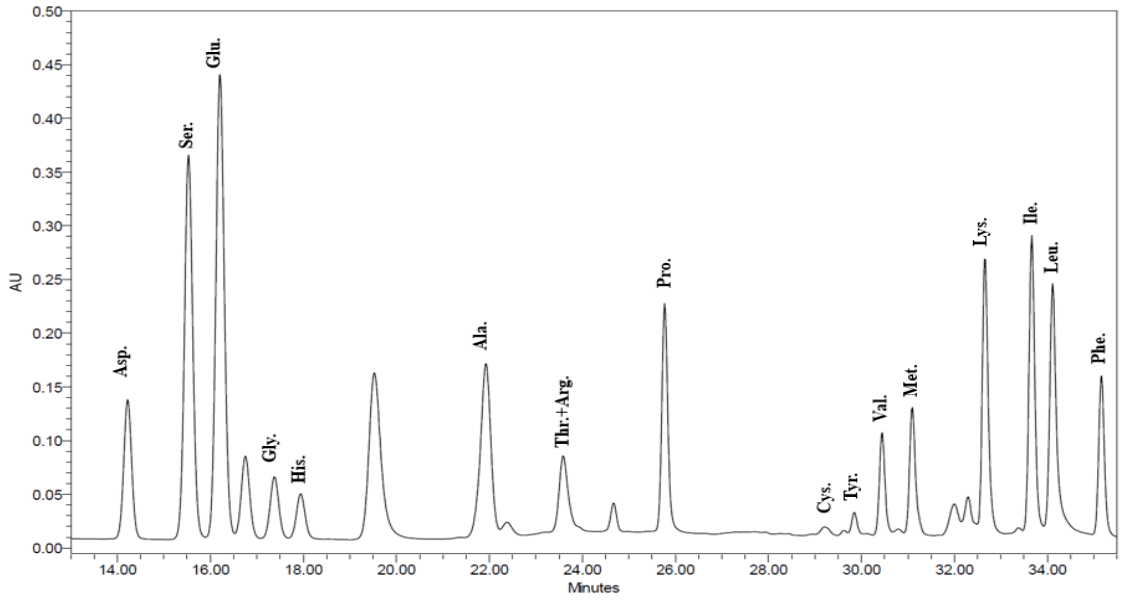


373

374 Figure. 11. HPLC chromatogram of amino acids in selected Neutrase® hydrolysate

375 fraction

376



377

378 Figure. 12. HPLC chromatogram of amino acids in selected Protamex® hydrolysate

379 fraction

380

381 Table. 3. Amino acids concentration in selected fraction

AAs	RT[a]		Area (%)[b]	
	NF3	PF3	NF3	PF3
Asp.	11.284	14.221	2.504	2.649
Ser.	13.688	15.530	3.038	2.892833
Glu.	16.232	16.206	3.602	3.018754
Gly.	17.361	17.378	3.853	3.237067
His.	18.882	17.941	4.190	3.341939
Arg.	10.913	10.963	2.422	2.04212
Thr.	10.913	10.963	2.422	2.04212
Ala.	23.120	23.587	5.131	4.393641
Pro.	25.542	25.769	5.669	4.800091
Cys	28.573	29.207	6.341	5.4405
Tyr.	29.582	29.848	6.565	5.559902
Val.	30.270	30.445	6.718	5.671107
Met.	30.882	31.095	6.854	5.792185
Lys.	32.557	32.658	7.225	6.083331
Ile	33.566	33.664	7.449	6.270723
Leu	33.946	34.113	7.534	6.35436
Phe	34.956	35.161	7.758	6.549575
Unknown AA	48.325	128.095	10.725	23.86075
Total	450.592	536.844	100	

382

383 * [a] Retention time; [b] Amount of total amino acids, %

384 * NF3 : Neutrase® hydrolysate fraction; PF3 : Protamex® hydrolysate fraction

385

Discussion

In this study, proteolytic enzymes used to focus on the function of specific peptides derived from the hydrolysis of a casein.

According to results, the degree of hydrolysis (DH) of the four enzymes tended to increase in a similar pattern. There was no significant difference after increasing from 120 minutes to 150 minutes. DH of Protamex® found to increase most appropriately. All enzymatic hydrolysates maintained from 30 to 40 minutes and gradually increased after 50-60 minutes. This is similar to the previous report (Jinshui et al., 2013). In this study, the casein contained 95% of protein, and the retention time of hydrolysis was about 10 minutes.

In this study, fractions separated by molecular weight throughout the Hiprep 16/60 Sephacryl S-100 HR column. In another study, FPLC used either size exclusion or ion exchange chromatography (Kim et al., 2001). Chromatographic methods for peptide separation are very diverse, but the purpose of this study was to obtain peptides similar to CPP, so fractions separated using the column separated by molecular weight.

In the case of calcium solubility experiments, no fractions with calcium solubilization higher than CPP found. However, it has a similar level of calcium solubilization, which has confirmed the potential to the CPP. The calcium is solubilized by gastric acid, and most of it is absorbed in the small intestine. If excess phosphate ions present in the small intestine before absorption, calcium reacts to form of calcium phosphate and it is released into the body. Therefore, there is a need to have material capable of preventing such as a precipitation reaction, amino acids and peptides have been reported to have such properties from before (Naito et al., 1969).

Except for the NF6, there were no significance on concentration differences and cell

411 death. In addition, it showed with cell viability of 80% or more.

412 According to results of the study, as the concentration of fraction samples increased,
413 NO production inhibited. The MTT experiments showed that inhibition of NO and NO
414 production was not caused by cytotoxicity. In previous study, there were results that
415 hydrolyzed proteins significantly inhibited NO production in NO assay experiments
416 comparing non-hydrolyzed protein and hydrolyzed protein (Hoon et al., 2017).

417 In addition, studies on the inhibitory ability of inflammatory cytokines of casein
418 hydrolysates reported that it have high activities of Alcalase® hydrolysates (Mao et al.,
419 2012).

420 According to results of confirming Amino acid sequence of selected fractions
421 throughout the AccQ-Tag system, 17 species of amino acids and several species of
422 unknown amino acids identified. Both fractions had the highest content of
423 Phenylalanine. Except for the αs_1-casein, there are common sequence feature and it is
424 defined a N-terminal tyrosine residue. Also, it is absolutely essential for activities.
425 Typically, a second aromaticamino acid residue, such as phenylalanine, is also present
426 in the third or fourth position (Clare et al., 2000).

427 Therefore, these protein fractions must be obtained and produced active peptides
428 from them for use as dietary supplements and milk based nutraceuticals. This study
429 identified the potential of new biologically active peptides derived from milk proteins
430 that affect the food and healthcare industry.

431

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576 Tables and Figures