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Effects of the Whey Protein Hydrolysates of Various Protein Enzymes on the Proliferation and Differentiation of 3T3-E1 Osteoblasts

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ABSTRACT: In this study, we used various proteinases to investigate the effect of whey protein hydrolysates on proliferation and differentiation of MC3T3-E1 osteoblasts. To confirm hydrolysis of the whey protein hydrolysates, the yield and α-amino acid content were determined. Since osteogenic cell activity is an important factor in osteogenesis, we evaluated the proliferation of osteogenic cells by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and alkaline phosphatase (ALP) activity. To analyze bone matrix formation, we identified calcium deposition by staining with Alizaline red-S. The free amino acid content was significantly higher in the whey protein hydrolysates prepared using Protamex, Flavourzyme, and Alcalase than in the control. When cells were treated with 500 μg/mL of whey protein hydrolysates prepared using Protamex and Alcalase, cell proliferation increased by 120% and 130%, respectively, compared with the control group. In addition, ALP activity was significantly higher following treatment with 500 μg/mL of whey protein hydrolysates prepared using Protamex and Alcalase (142.61% and 135.06%, respectively; $P<0.05$). Furthermore, when treated with 125 μg/mL of the same hydrolysates, the rate of calcium deposition increased significantly to 157.56% compared with the control group ($P<0.05$). Therefore, our results suggest that whey protein hydrolysates prepared using Protamex and Alcalase may have more beneficial effects on osteoblast proliferation and bone health than those prepared using other proteolytic enzymes.

Keywords: alkaline phosphatase, calcium deposition, MC3T3-E1, proliferation, whey protein hydrolysates

INTRODUCTION

Cheese whey is a liquid by-product separated during the production of cheese or casein, and contains proteins, lactose, minerals, and vitamins. Whey is used as both a nutritional food and as a food additive due to its nutritional value and physical functional characteristics, such as its ability to improve physical properties and increase flavor (Ahmad et al., 2019). Whey proteins have nutritional and physiological activities in addition to a wide range of functions in foods. Since dairy products can be used in a variety of functional foods, baking products, syrups, dairy products, and fermented products, they have been well studied (Sharma, 2018). Whey protein is a collection of proteins with different properties. The main proteins in whey protein are α-lactalbumin and β-lactoglobulin, which account for 70~80% of total whey protein; other protein components include bovine serum albumin, lactoferrin, lactoperoxidase, immunoglobulin, and growth factors, which each have various physiological activities (Walzem et al., 2002). Recently, developing new protein resources and effectively utilizing existing protein sources have become an important issue; for example, utilizing whey protein as a food material by treating hydrolysates with proteolytic enzymes. Since protein hydrolysates have excellent physical properties as a food material, many studies have attempted to solubilize whey proteins using degradative enzymes to increase its utilization in food.

Enzymatic hydrolysis of milk protein causes whey proteins to be hydrolyzed into trypsin, papain, and iron-binding peptides (Kim et al., 2007). Hydrolysis of β-lactoglobulin into pepsin has been previously shown to alter the antigenicity of IgE-binding peptides (Chicón et al., 2008). Peptides with excellent physiological activities have been identified via the enzymatic hydrolysis of whey proteins, such as opioid peptides (Meisel and FitzGerald, 2000), angiotensin I-converting enzyme inhibitory peptides (Gobbetti et al., 2004), antithrombotic peptides (Chabance et

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al., 1995), immunoregulatory peptides (Mercier et al., 2004), and mineral transport peptides (Kim and Lim, 2004). Furthermore, Ashmead (1993) reported that calcium and amino acid-chelated calcium salts are solubilized in the small intestine and exhibit better absorption than inorganic calcium. Kato et al. (2000) showed that milk is a good source of calcium but this calcium has a low absorption. Moreover, milk whey protein has been shown to inhibit osteoclast formation and bone resorption, promote the proliferation and differentiation of osteoblastic MC3T3-E1 cells, and increase bone strength in ovariectomized rats. MC3T3-E1 osteoblasts are derived from the skulls of mice, and their proliferation, differentiation, and calcification during bone formation have metabolic characteristics similar to those of osteoblasts (Sudo et al., 1983).

In this study we investigated the effects of whey protein hydrolysates prepared using various proteases on osteoblast proliferation and differentiation by measuring alkaline phosphatase (ALP) activity of and calcium deposition in MC3T3-E1 cells.

MATERIALS AND METHODS

Materials and enzymes

The whey proteins used in this experiment were supplied by Neo Crema (Seoul, Korea), the proteases Alcalase, Flavourzyme, and Protamex were purchased from Novozymes (Basel, Switzerland), and Ficin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Manufacture of whey protein hydrolysates

Distilled water (100 mL) was added to whey protein (10 g) and dissolved with 0.1 N NaOH to adjust the pH to 8.0～8.5. Alcalase was added to the substrate at a concentration of 1% (w/w) and hydrolyzed at 50°C for 4 h in a shaking water bath. After Alcalase hydrolysis, 1% (w/w) of Alcalase, Flavourzyme, Collupulin, Ficin, or Protamex was added to the substrate, and hydrolysis was carried out for 14 h under the optimal pH and temperature conditions of each enzyme (Table 1). The hydrolyzed samples were centrifuged (4°C, 3,000 *g*, 10 min) and the hydrolysates were obtained by freeze-drying.

Measurement of α-amino nitrogen content by 2,4,6-trinitrobenzenesulfonic acid (TNBS)

The TNBS method was modified from that described by Adler-Nissen (1979) as follows. Whey protein hydrolysate (25 μL), 0.2 mL of phosphate buffer (0.2 M, pH 8.2), and 0.2 mL of 0.1% TNBS were mixed, and a color reaction was carried out for 1 h in a dark water bath at 50°C. To quench the reaction, 0.4 mL of 0.1 M HCl was added and the mixture was allowed to stand at 25°C for 20 min. Distilled water (1.6 mL) was then added and the absorbance was measured at 340 nm using a spectrophotometer. The α-amino acid content of each sample was quantified using the standard curve of L-leucine.

Measurement of degree of hydrolysis (DH)

The DH is defined as the proportion of cleaved peptide bonds and is approximated by measuring free amino groups using TNBS (Adler-Nissen, 1979). The total number of amino groups were measured by TNBS after acid hydrolysis at 110°C for 12 h (10 mg of whey protein in 6 N HCl).

Measurement of surface hydrophobicity

The surface hydrophobicity of the hydrolysate was measured using 1-amino-8-naphthalene sulfonate (ANS) (Hayakawa and Nakai, 1985). The hydrolysate was serially diluted to 0.0015～0.015% with 10 mM phosphate buffer (pH 7.0); then, 10 μL of ANS (8 mM in 10 mM phosphate buffer) was added to the hydrolysate solution (2 mL). The fluorescence intensity of the ANS-hydrolysate conjugate was measured using a spectrophotometer (Kontron Model SFM23/B, Kontron S&T AG, Augsburg, Germany) at excitation/emission 390/470 nm.

Evaluation of MC3T3-E1 cell proliferation

MC3T3-E1 cells were cultured using α-minimum essential medium (α-MEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% antibiotic (Penicillin G sodium 100 units/mL and streptomycin sulfate 100 μg/mL, Gibco BRL) at a temperature of 37°C and 100% humidity. CO_2 (5%) was continuously supplied, and the culture medium was changed every 2 days until sufficient cell proliferation.

Table 1. Source and optimum conditions of various proteases

Enzyme	Source	Optimum reaction condition	
		Temperature (°C)	pH
Alcalase (P1)	*Bacillus licheniformis* (Endo)	30～65	7.0～9.0
Flavourzyme (P2)	*Aspergilus oryzae* (Endo/Exo)	50～55	5.5～7.5
Collupulin (P3)	*Carica papaya* (Endo)	50～70	5.0～7.5
Ficin (P4)	*Ficus carica* (Endo)	45～55	6.5～8.5
Protamex (P5)	*Bacillus* spp. (Endo)	50	7.0～8.0

MC3T3-E1 cells were seeded (2×10^4 cells/well) onto a 96-well plate and cultured with α-MEM containing 10% FBS. When the cells in each well had grown to 80% confluency, the culture medium was removed and the normal group and the experimental group were divided. In the normal group, 200 μL of α-MEM containing 10% FBS was added, and in the experimental group, 200 μL of whey protein and hydrolyzed whey proteins were added at concentrations of 62.5～500 μg/mL. Cells were then cultured under the same conditions, and after two days 200 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to each well and cells were incubated at 37°C for 3 h. Formazan crystals formed in the cells were dissolved by adding 50 μL dimethyl sulfoxide, and the absorbance was measured at 540 nm using a spectrophotometer (Mosmann, 1983).

Evaluation of ALP activity in MC3T3-E1 cells

MC3T3-E1 cells were seeded (2×10^4 cells/well) in a 6-well plate and cultured for 2 days with α-MEM supplemented with 10% FBS. After 2 days, the control group was replaced with differentiation medium (α-MEM supplemented with 10% FBS, 1% antibiotic, 10 mM sodium β-glycerophosphate, and 50 μg/mL ascorbic acid) treated with unhydrolyzed whey protein (Fatokun et al., 2006). In the experimental group, the whey protein hydrolysates prepared using various proteases (62.5～500 μg/mL) were added to the same medium and cells were cultured for 15 days. The cells were then centrifuged (14,000 rpm, 5 min) to remove the supernatant, transferred onto ice, and 0.1% Triton X-100 in phosphate buffered saline was added to disrupt the cell membranes. After centrifugation (4°C, 8,000 rpm, 10 min), the supernatant was collected and used to measure ALP activity. ALP activity and protein concentration were determined enzymatically using an alkaline phosphatase assay kit (ab83369, Abcam, Cambridge, UK) and a bicinchoninic acid-protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA), respectively. ALP activity was calculated by dividing the ALP concentration by the protein concentration. Each measurement was converted to a percentage of the normal group and statistically analyzed.

Evaluation of calcium deposition in MC3T3-E1 cells

Alizaline red-S staining was carried out to determine the effects of whey protein hydrolysates on the formation of calcified nodules in MC3T3-E1 cells. The differentiated MC3T3-E1 cells were first treated with whey protein or whey protein hydrolysate. Cells were washed with saline, fixed with 70% ethyl alcohol, and stained with 40 mM (pH 4.2) Alizarine red-S (Sigma-Aldrich Co.) solution for 10 min. The area of calcified nodules was measured using an Image Analyzer (Media Cybernetics, Inc., Rockville, MD, USA) and expressed as a percentage compared with the normal group (Kawazoe et al., 2004).

Statistical analyses

All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA). Differences between the groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. All data are reported as the mean±standard deviation (SD).

RESULTS AND DISCUSSION

Whey protein hydrolysates prepared by various proteases

α-Amino nitrogen content was measured using the TNBS method to assess the DH. Treatment with Alcalase for 4 h increased the amount of α-amino nitrogen (Fig. 1A). Protamex hydrolysates had the highest α-amino nitrogen content (26.11±0.10 mg/mL), whereas no significant difference was observed between the α-amino nitrogen content of the Flavourzyme hydrolysate and the control. The DH measures amino nitrogen change (Fig. 1B); therefore, the DH results were similar to those for the amino nitrogen content.

The dry weight of the whey proteins hydrolyzed by Flavourzyme after Alcalase treatment was 79.37±2.66 mg/mL, but there was no significant difference between the dry weights of the hydrolysates (Fig. 2A). The surface hydrophobicity of the hydrolysates increased after hydrolysis and was highest for the Ficin hydrolysate (Fig. 2B). As shown in Fig. 2B, surface hydrophobicity increases after hydrolysis as hydrophobic residues in the protein are exposed. Hydrolysis of the peptide bonds in proteins can increase the number of hydrophobic sites by exposing the interior of the proteins, affecting surface hydrophobicity and bitterness (Ludescher, 1996).

During hydrolysis, enzymatic degradation of proteins involves significant structural changes as the protein is converted into smaller peptide units (Kristinsson and Rasco, 2000), which in turn increases the α-amino nitrogen content. This change in α-amino nitrogen content occurs because the enzyme exhibits different specificities for different protein substrates. The substrate specificity of an enzyme has been reported to affect both its molecular size (Adler-Nissen, 1979; Kristinsson and Rasco, 2000) and different hydrolysate activities (Suetsuna, 2000; Jun et al., 2004).

Protamex is a *Bacillus* protease complex, whereas Flavourzyme is an enzyme produced by *Aspergillus oryzae* that has a mixture of endoproteinase and exoprotease activities. Flavourzyme eliminates hydrophobic residues and does not have the bitter taste of other enzymes, while Protamex degrades proteins using other proteases in the food (Pommer, 1995).

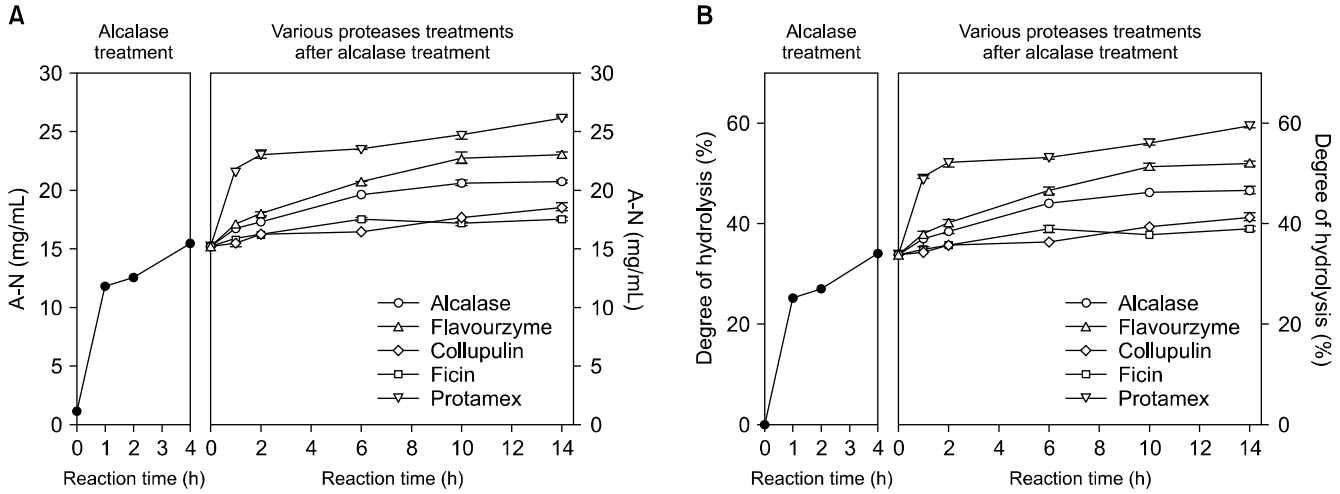

Fig. 1. Changes in amino nitrogen content and degree of hydrolysis during hydrolysis with various proteases. After hydrolysis with Alcalase for 4 h, whey proteins were hydrolyzed with various proteases for 14 h. Data show mean±SD.

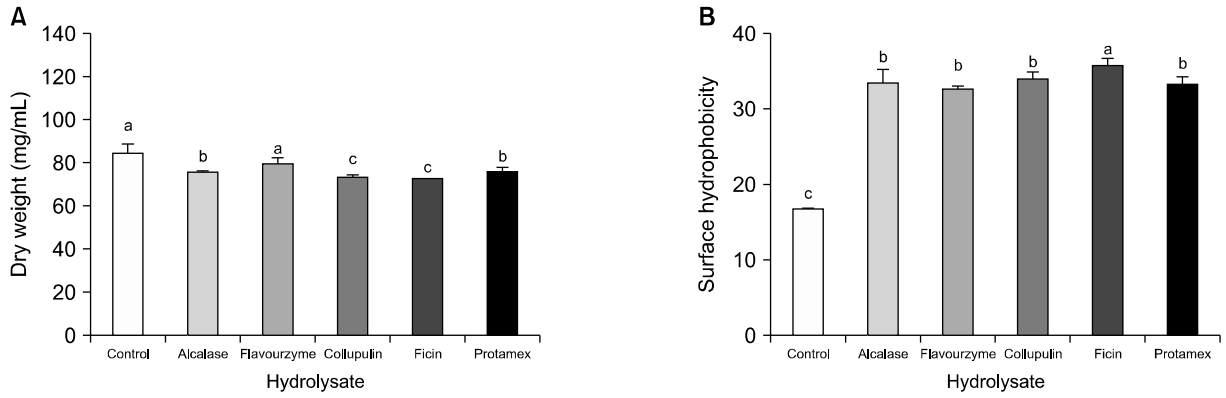

Fig. 2. Dry weight and surface hydrophobicity of whey protein hydrolysates. After hydrolysis with Alcalase for 4 h, whey proteins were hydrolyzed with various proteases for 14 h. Data show mean±SD. Different letters (a–c) indicate statistically significant differences (P<0.05).

Effects of whey protein hydrolysates on MC3T3-E1 cell proliferation

After Alcalase hydrolysis for 4 h, Alcalase, Flavourzyme, Collupulin, Ficin, and Protamex were used to hydrolyze the whey protein for a further 14 h. To determine the effects of the whey protein hydrolysates on the proliferation of osteogenic precursor cells, the absorbance was measured using MTT assays. The whey protein hydrolysates tended to increase proliferation at all concentrations (62.5～500 μg/mL) compared with the normal (NOR) and whey protein-treated groups (CON) (Fig. 3). In particular, cell proliferation increased by 120% and 130%, respectively, when treated with 500 μg/mL of P5 (P< 0.05).

MTT assays are widely used to assess the proliferation and cytotoxicity of living cells. MTT assays measure the activity of metabolically active intracellular mitochondria, allowing the amount of viable cells to be quantified. Living cells reduce MTT reagent to purple water-insoluble MTT formazan via dehydrogenase activity of their mitochondria. Since the absorbance of MTT formazan is characterized by a maximum wavelength of 540 nm, measuring the absorbance at this wavelength reflects the concentration of metabolically active cells. Therefore, the absorbance indirectly represents increases or decreases in cell number (Mosmann, 1983). In this study, we investigated the effects of whey protein hydrolysates prepared using various proteases on the proliferation of MC3T3-E1 osteoblasts. When treated with hydrolysate concentrations of 62.5～500 μg/mL, the absorbance was similar to, or higher than, NOR, suggesting that the whey protein hydrolysates increased cell proliferation in a concentration-dependent manner.

Effects of whey protein hydrolysates on MC3T3-E1 cell ALP activity

We then measured the effects of whey protein hydrolysates treated with various proteases on the ALP activity of MC3T3-E1 cells. High concentrations (250 and 500 μg/mL) of Flavourzyme-treated hydrolysates significantly increased ALP activity compared with NOR, which exhibited a higher ALP activity than the control group treated

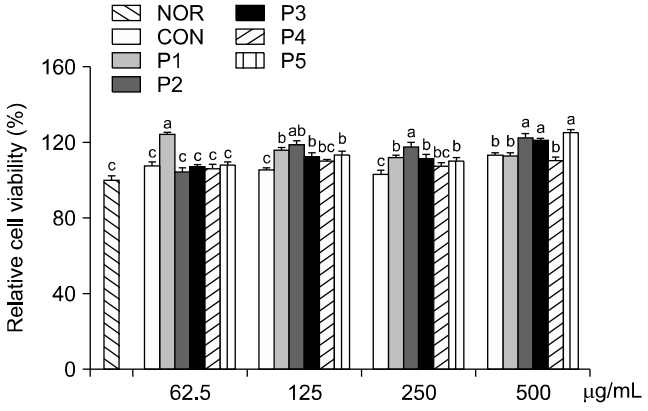

Fig. 3. Effects of whey protein hydrolysates on MC3T3-E1 cell proliferation. P1: 18 h hydrolysis with Alcalase. P2~5: After hydrolysis with Flavourzyme for 4 h (2), whey proteins were hydrolyzed with Collupulin (3), Ficin (4), and Protamex (5) for 14 h. Data show mean±SD. Different letters (a-c) indicate statistically significant differences between the groups (P<0.05; Tukey's test). NOR, normal group; CON, whey protein-treated group.

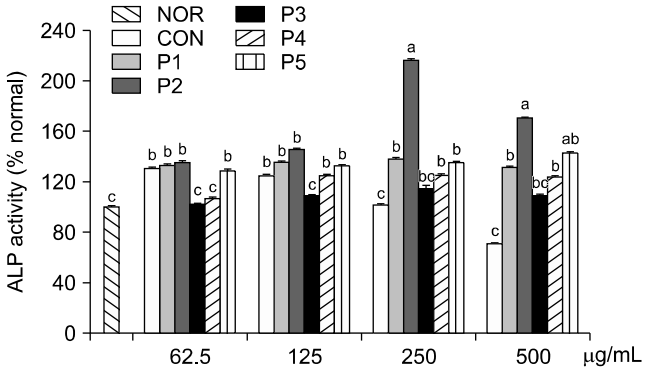

Fig. 4. Effects of whey protein hydrolysates on MC3T3-E1 cell alkaline phosphatase (ALP) activity. P1: 18 h hydrolysis with Alcalase. P2~5: After hydrolysis with Flavourzyme for 4 h (2), whey proteins were hydrolyzed with Collupulin (3), Ficin (4), and Protamex (5) for 14 h. Data show mean±SD. Different letters (a-c) indicate statistically significant differences between the groups (P<0.05; Tukey's test). NOR, normal group; CON, whey protein-treated group.

with CON (P<0.05; Fig. 4). In addition, ALP activity was significantly increased (135.06%) by the P5-treated hydrolysates at high concentrations (500 μg/mL) which increased approximately 2-fold compared with CON (P< 0.05; Fig. 4).

ALP activity was measured because ALP catalyzes the cleavage of *p*-nitrophenyl phosphate (*p*-NPP) into *p*-nitrophenol and phosphate; therefore, measuring ALP activity allows the amount of *p*-nitrophenol to be quantified through measuring the absorbance. Since differences in cell number can affect ALP activity, the ALP activity per unit cell count can be calculated by dividing the ALP concentration by the total protein amount.

Bones and cartilage are types of connective tissue. Cartilage is secreted around the bone and exhibits intercellular (mainly length) and parallel (mainly width) growth. ALP activity is a phenotype of osteoblasts and ALP is essential for mineralization (Bellows et al., 1991). According to Kodama et al. (1981), MC3T3-E1 cells are osteoblasts isolated from the skulls of mice which exhibit a fibroblast-like morphology and have high ALP activity during long-term culture. Stein et al. (2005) reported that basal phosphatase activity can be measured to determine the degree of bone cell differentiation since high ALP activity is a marker of bone cell differentiation. The samples hydrolyzed with Flavourzyme and Protamex increased ALP activity and affected osteoclast differentiation; therefore, we investigated the effects of the whey protein hydrolysates on calcified nodule formation.

Effects of whey protein hydrolysate on calcium deposition in MC3T3-E1 cells

The osteoblast phenotype requires two steps: firstly, a specific protein associated with the bone cell phenotype, such as ALP, is detected during the matrix maturation stage; secondly, the substrate is mineralized by calcium. As a result, cancellous bone is formed around the existing cartilage, and the space between the bone and cartilage is filled with bone matrix during development to form dense bone (Logan, 1942). Based on the ALP activity results, we investigated the effects of whey protein hydrolysates on the intracellular calcium content of osteoblasts for 15 days. The resulting nodules were circular or elliptical, with densely packed cells; the central part of the nodule was deep red, while the surrounding area was light red. The area of the calcified nodules formed was expressed a percentage based on NOR. The whey protein hydrolyzed using Flavourzyme, Collupulin, and Protamex significantly increased calcified nodule formation by 156.07, 159.17, and 161.39%, respectively, compared with NOR (P<0.05; Fig. 5). Furthermore, when treated with 125 μg/mL of Protamex, calcified nodule formation was significantly increased to 157.56% compared with the normal group; therefore, the effect of Protamex-treated hydrolysates on calcified nodule formation was greater than that of the other enzyme hydrolysates (P<0.05; Fig. 5).

Whey protein hydrolysates contain a number of therapeutic substances in addition to their high nutritional value, some of which have already been produced on an industrial scale for food and medical applications. Whey protein hydrolysates are therefore a source of desirable peptides and amino acids and can be used to promote growth and bone health. The whey protein hydrolysate produced using Protamex and Alcalase performed better than those prepared using the other enzymes, exhibited high levels of hydrolysis, and increased MC3T3-E1 cell proliferation, ALP activity, and calcium deposition. Further research is required to identify the active peptides in whey protein hydrolysates and to assess their effects on

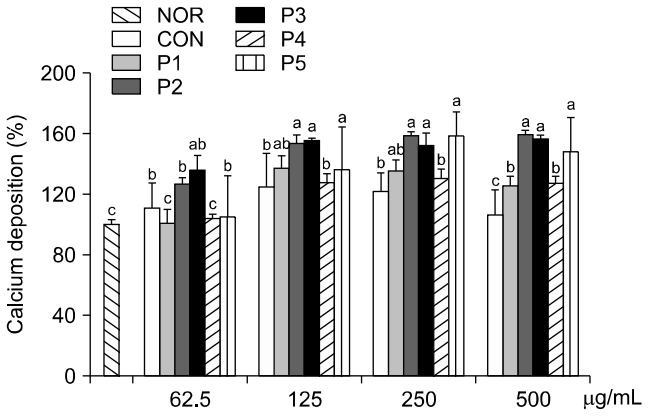

Fig. 5. Effects of whey protein hydrolysates on 3T3-E1 cell calcified nodule formation. P1: 18 h hydrolysis with Alcalase. P2~5: After hydrolysis with Flavourzyme for 4 h (2), whey proteins were hydrolyzed with Collupulin (3), Ficin (4), and Protamex (5) for 14 h. Data show mean±SD. Different letters (a-c) indicate statistically significant differences between the groups (P<0.05; Tukey's test). NOR, normal group; CON, whey protein-treated group.

cell proliferation and bone formation. Furthermore, continued investigation into the biological functions of whey protein hydrolysates could help identify biologically active compounds with specific health benefits and potential roles in the prevention and treatment of diseases.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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