ANTIMICROBIAL EFFECT AGAINST ORAL BACTERIA ON BIOACTIVE COMPOUNDS IN A HIGH-PRESSURE ENZYMATIC *Prunus mume* EXTRACT

EFEITO ANTIMICROBIANO CONTRA BACTÉRIAS ORAIS SOBRE COMPOSTOS BIOATIVOS EM UM EXTRATO ENZIMÁTICO DE Prunus mume *DE ALTA PRESSÃO*

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ABSTRACT: To evaluate high-pressure processing combined with enzymatic treatment for extraction of *Prunus mume* by determining the optimum extraction conditions for the antimicrobial activity against oral bacteria. High-pressure enzymatic extraction was used to isolate biologically active components from *P. mume*. The effects of process variables such as enzyme type (Pectinex Ultra SP-L, Novozym 33095 and Viscozyme L), enzyme concentration, incubation time/temperature, pH, and ratio of enzymes antimicrobial activity against oral pathogens related to dental caries and periodontal diseases were determined by disk diffusion assay. The optimal conditions for enzymatic extraction from *P. mume* were pH 6.0, 45°C, 20 h, and 5 v/v% with Pectinex Ultra SP-L. The maximum antimicrobial activity of *P. mume* extract obtained using Novozym 33095 was at pH 7.0, 45°C, 20 h, and 5 v/v%. The Viscozyme L extract showed the maximum inhibitory effect at pH 6.0, 45°C, 20 h, and 5 v/v%. Use of combinations of enzymes did not result in significantly different antimicrobial activity ($p < 0.05$) compared with each enzyme alone. Minimum inhibitory concentration values were 3.125 to 12.50%. These results indicated that high-pressure enzymatic extraction yielded *P. mume* extract with antimicrobial activity which has the potential for improving oral environment.

KEYWORDS: Antimicrobial activity. High-pressure enzymatic extract. Minimum inhibitory concentration. *Prunus mume*

INTRODUCTION

The most common oral diseases aredental cavities and periodontal diseases, which are infectious diseases. Dental caries **is** the medical term for tooth decay or cavities. Gram-positive bacteria are commonly found in the oral cavity and are significant contributors to tooth decay. The most important pathogens of dental caries are mutans streptococci, such as *Streptococcus mutans* and *Streptococcus sobrinus* (VAN HOUTE, 1994). Periodontal disease, including gingivitis and periodontitis, is an inflammatory disorder that can destroy periodontal tissue and causes loss of teeth. *Aggregatibacter actinomycetemcomitans* is the most common pathogen associated with periodontitis, and *Actinomyces* species are associated with gingivitis (SOCRANSKY; HAFFAJEE, 1992).

Antimicrobial agents for the prevention and treatment of oral disease as alternatives to conventional oral antimicrobial agents, such as chlorhexidine, have been investigated (WALSH et al., 2015). Numerous natural compounds have been reported to be effective in preventing and treating oral infections, and in maintaining good oral health (LIEN et al., 2014). Among them, *Prunus mume*, known as Japanese apricot, is used as a traditional medicinal food in Korea, China, and Japan. Today, it is cultivated globally for commercial purposes in food and drink because of its health benefits, including anticancer, antioxidant, and anti-inflammatory properties (BOUAYED et al., 2009; KAWAHARA et al., 2009). Moreover, *P. mume* has potential as an oral antimicrobial agent to prevent and treat dental diseases (SENEVIRATNE et al., 2011; JANG et al., 2014). However, the common methods of *P. mume* extraction, including solvent or aqueous thermal-based extraction, result in low yields, long extraction times, and traces of organic solvents (CHO et al., 2013).

Numerous methods have been investigated to enhance plant extraction procedures, such as microwave-assisted extraction, supercritical fluid extraction, ultrahigh-pressure extraction, and enzymatic extraction (JUN et al., 2011). Enzymatic extraction of bioactive compounds from plants is a potential alternative to conventional solvent or aqueous thermal-based extraction. Enzymatic treatment is used in the food industry due to its low operating temperature and ability to increase extraction yields. The enzymes break down plant cell walls, resulting in release of bioactive cell

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contents, including pectic substances, cellulose, hemicelluloses, and lignin (PINELO et al., 2006). High-pressure processing is a novel technology that has shown potential for extraction of bioactive components. High-pressure processing induces structural changes that damage the plant cell wall. Therefore, the bioactive components are more easily washed out, such as flavonoids, alkaloids, vitamins, saponins and pigments. The high-pressure extraction technique is associated with a short extraction time, high extraction yield and lower levels of impurities.

In this study, we evaluated high-pressure processing combined with enzymatic treatment for extraction of *P. mume* and evaluated the antimicrobial activity of the extract against oral bacteria. Several variables–extraction pressure, extraction pH, extraction temperature, extraction time, enzyme concentration, and enzyme ratio–were altered to determine the optimum extraction conditions. In this study, Pectinex Ultra SP-L, Novozym 33095 and Viscozyme L enzymes were used in these extractions. In addition, high-pressure processing was conducted at 100 MPa.

MATERIAL AND METHODS

Materials and reagents

Prunus mume was obtained from Gwangyang-si, Korea. Pectinex Ultra SP-L, Novozym 33095, and Viscozyme L were purchased from Novozyms Co. (Bagsvaerd, Denmark) and were used to disrupt cell walls. The characteristics of these commercial enzymes are summarized in Table 1.

Table 1. Characteristics of the enzymes used in this study.

Commercial name	Enzyme	Source	Activity
Pectinex Ultra SP-L	Polygalacturonase	*Aspergillus aculeatus*	3,800 PGNU/mL
Novozym 33095	Pectin lyase, Polygalacturonase	*Aspergillus aculeatus* *Aspergillus niger*	10,000 PECTU/mL
Viscozyme L	Multi-enzyme complex containing arabanase, cellulase, hemicellulase, and xylanase	*Aspergillus aculeatus*	100 FBGU/g

PGNU: polygalacturonase unit; PECTU: pectinase unit; FBGU: fungal beta-glucanase unit.

Bacterial strains and growth conditions

Nine oral bacterial strains were purchased from the Korean Collection for Type Cultures (KCTC, Korea). *Staphylococcus aureus* ATCC6538P, *Streptococcus sobrinus* ATCC27607, *Streptococcus mutans* ATCC25175, *Streptococcus ratti* ATCC19645, and *Streptococcus sanguinis* ATCC10556 were grown in Brain-Heart Infusion (BHI; Difco, Detroit, MI, USA) broth. Trypticase Soy Broth (TSB; BD Co., USA) was used for the growth of *Streptococcus anginosus* ATCC12395, *Actinomyces viscosus* KCTC5531, *Actinomyces naeslundii* KCTC5525, and *Aggregatibacter actinomycetemcomitans* ATCC33384. The bacteria were incubated under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) at 37°C for 24 h. The bacterial cell density was adjusted to 0.5 McFarland Standard (1.5×10^8 CFU/mL).

High-pressure enzymatic extraction of *P. mume*

A reactor (ITC21, Toyokoatsu, Japan) was used for high-pressure enzymatic extraction of *P. mume*. *P. mume* was extracted using Pectinex Ultra SP-L, Novozym 33095, or Viscozyme L, individually and in combinations. Fifty grams of *P. mume* without seed were added to 150 mL of buffer (pH 4 - 8) with 0 - 10 v/v% enzyme. The mixture was placed in a plastic bag, and transferred to a 2.0-L stainless steel vessel. High-pressure processing was conducted at 100 MPa for 10 - 40 h at 35 - 65°C. After incubation, *P. mume* extracts were filtered through filter paper (ADVANTEC No. 2, Advantec MFS, Inc., Tokyo, Japan), concentrated in a rotary evaporator (EYELA A-1000S, Tokyo Rikakikai Co., Japan) and stored at -20°C. The high-pressure enzyme-aided extraction procedure is described in Figure 1, and the variables are presented in Table 2.

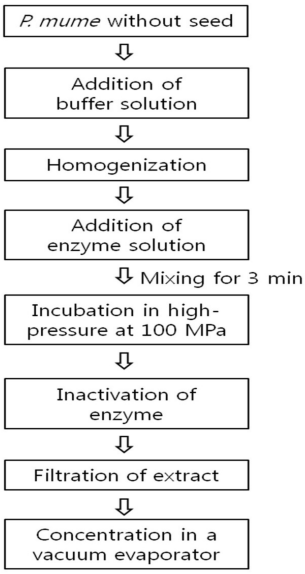

Figure 1. Flow diagram of high-pressure enzyme extraction from *P. mume.*

Table 2. Extraction variables.

Influence of enzyme concentration				
Changing variables				
Enzyme concentration	0 v/v%	1 v/v%	5 v/v%	10 v/v%
Constant variables				
pH	6.0/7.0			
Temperature	45°C			
Incubation time	20 h			
Pressure	100 MPa			
Influence of pH				
Changing variables				
pH	5.0	6.0	7.0	8.0
Constant variables				
Temperature	45°C			
Incubation time	20 h			
Enzyme concentration	10 v/v%			
Pressure	100 MPa			
Influence of temperature				
Changing variables				
Temperature	35°C	45°C	55°C	65°C
Constant variables				
pH	6.0/7.0			
Incubation time	20 h			
Enzyme concentration	10 v/v%			
Pressure	100 MPa			
Influence of incubation time				
Changing variables				
Incubation time	10 h	20 h	30 h	40 h
Constant variables				
pH	6.0/7.0			
Temperature	45°C			
Enzyme concentration	10 v/v%			
Pressure	100 MPa			

Antimicrobial activity against oral bacterial strains

The antimicrobial activity of *P. mume* extract against nine oral bacterial strains was evaluated by the disc diffusion method (PIDDOCK, 1990). Bacterial suspensions were spread on agar plates using cotton swabs, and sterile filter paper discs (6 mm diameter, Advantec, Japan) soaked in 20 μL extract were placed on the preloaded plates. The plates were incubated anaerobically at 37°C for 12 h, and then the diameter of the inhibition zones around the discs was measured in millimeters.

Determination of MIC against oral bacterial strains

The minimum inhibitory concentration (MIC) of *P. mume* extract was determined using the NCCLS reference broth dilution method with slight modifications (SWENSON et al., 1982). This test was performed in sterile, flat-bottom 96-well microtiter plates (F-Type, SPL-Lifesciences Co. Ltd, Korea). *P. mume* extract was serially diluted twofold to final concentrations of 25 to 0.0997% of the original extract, and then added to diluted bacterial suspensions (1.0×10^5 CFU/mL) at a 1:1 ratio. After incubation at 37°C for 12 h, bacterial growth was measured by determining the absorbance of the suspensions at 600 nm against a culture medium blank.

Statistical analysis

All measurements were performed in triplicate and data are presented as means ± standard deviation of triplicate experiments. Statistical analysis was carried out using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between values was determined by ANOVA/Duncan's multiple range tests, using a 95% criterion for a significant difference ($p < 0.05$).

RESULTS AND DISCUSSION

Optimum high-pressure enzymatic extraction conditions

Enzymatic extraction was performed using Pectinex Ultra SP-L, Novozym 33095, and Viscozyme L. To determine the optimum enzyme concentration, *P. mume* was extracted at pH 7.0, 45°C and 100 MPa for 20 h using enzyme concentrations of 0 to 10 v/v% according to a previous report (CHO et al., 2013). Then, the antimicrobial activity of the *P. mume* extracts was determined by the disc diffusion method. As shown in Table 3, the optimum concentration of all of Pectinex Ultra SP-L, Novozym 33095, and Viscozyme L treatment was 5 v/v%. Antimicrobial activity increased with increasing enzyme concentration up to 5 v/v%. All Pectinex Ultra SP-L treatment increased the antimicrobial activity from 103 to 168% compared to non-enzymatic extraction. Viscozyme L treatment increased antimicrobial activity was 102 to 165%. Novozym 33095 treatment resulted in significant increases in antimicrobial activity of 114 to 231%.

The effect of incubation time (10 - 40 h) in the presence of 5 v/v% Ultra SP-L, Novozym 33095 and Viscozyme L was next determined. As shown in Table 4, the antimicrobial activities of the extracts increased up to 20 h and decreased thereafter; however, none of the differences was significant.

Next, the effect of temperature (35 - 65°C) in the presence of 5 v/v% Pectinex Ultra SP-L, Novozym 33095 and Viscozyme L at 100 MPa for 20 h on antimicrobial activity was evaluated. The extraction was performed at the optimum pH value of each enzyme (pH 6.0 for Ultra SP-L and Viscozyme L, pH 7.0 for Novozym 33095). As shown in Table 5, 45°C was the optimum temperature, irrespective of the enzyme used.

Finally, the optimum pH value was determined. *P. mume* was extracted using different pH values (pH 5.0 to 8.0 for Novozym 33095 and Viscozyme L, and pH 4.0 to 7.0 for Pectinex Ultra SP-L), while temperature (45°C), enzyme concentration (5 v/v%), pressure (100 MPa) were kept constant for 20 h. As shown in Table 6, *P. mume* extract showed similar antimicrobial activity against all eight bacterial strains. However, the highest antimicrobial activity of the Novozym 33095 extract was at pH 7.0, which resulted in an inhibition zone of 13.76 to 16.53 mm. This extract exhibited the strongest antimicrobial activity against *A. naeslundii* and the weakest against *S. anginosus*. Pectinex Ultra SP-L and Viscozyme L resulted in zones of inhibition of up to 15.58 and 16.14 mm, respectively, against *A. actinomycetemcomitans* at pH 6.0.

Additionally, compared to Novozym 33095, the effects of combinations of enzymes against *A. naeslundii* and *A. actinomycetemcomitans* were investigated. As shown in Figure 2, no synergistic effect of enzyme combinations was detected. The combination of Novozym 33095 and Pectinex Ultra SP-L produced an inhibition effect similar to that of each enzyme alone. However, use of Viscozyme L in combination with either of the other two enzymes resulted in a reduced inhibitory effect.

Table 3. Antimicrobial activities of *P. mume* extract according to enzyme concentration.

	Diameter of the zone of inhibition (mm, Means ± SD)											
	Pectinex Ultra SP-L				Novozym 33095				Viscozyme L			
Microorganisms	0%	1%	5%	10%	0%	1%	5%	10%	0%	1%	5%	10%
Staphylococcus sobrinus	8.29±0.33^{b}	8.95±0.21^{b}	10.46±0.27^{a}	9.08±0.76^{b}	6.07±0.07^{c}	9.28±0.76^{b}	11.78±1.68^{a}	11.95±0.16^{a}	9.23±0.97^{b}	9.77±0.80^{ab}	11.88±1.33^{a}	11.26±1.89^{ab}
Streptococcus aureus	7.96±0.21^{c}	8.95±0.37^{b}	10.46±0.55^{a}	9.08±0.07^{b}	6.02±0.01^{c}	7.86±0.40^{b}	11.48±0.81^{a}	11.13±0.90^{a}	8.29±0.67^{b}	8.99±0.99^{b}	12.16±0.38^{a}	11.82±0.51^{a}
Streptococcus mutans	7.32±0.31^{b}	7.99±0.25^{ab}	8.98±1.01^{a}	8.36±1.07^{ab}	6.08±0.09^{c}	8.87±0.26^{b}	11.10±0.75^{a}	11.46±0.92^{a}	8.75±1.92^{b}	10.58±0.97^{ab}	13.13±2.13^{a}	12.04±2.43^{ab}
Streptococcus ratti	7.55±0.56^{b}	8.77±0.85^{ab}	9.39±0.77^{a}	9.03±0.30^{a}	6.02±0.02^{c}	9.62±0.47^{b}	10.73±0.40^{a}	10.42±0.39^{a}	7.85±0.80^{b}	9.53±0.85^{a}	10.65±0.60^{a}	9.94±0.39^{a}
Streptococcus sanguinis	7.25±0.82^{a}	7.50±1.00^{a}	8.52±1.90^{a}	7.76±0.95^{a}	6.01±0.01^{b}	6.87±0.52^{b}	11.14±0.84^{a}	10.28±0.69^{a}	8.43±0.42^{c}	8.89±0.39^{bc}	10.60±0.63^{a}	9.95±1.04^{ab}
Actinomyces viscosus	7.73±1.69^{b}	12.69±1.85^{a}	13.00±1.61^{a}	12.44±0.36^{a}	6.76±0.54^{c}	12.32±1.11^{b}	14.00±0.70^{a}	12.96±0.50^{ab}	10.53±1.36^{b}	14.03±1.42^{a}	15.09±1.88^{a}	13.82±1.64^{a}
Actinomyces naeslundii	9.54±0.92^{b}	10.56±0.57^{ab}	11.42±1.00^{a}	11.77±0.41^{a}	6.21±0.25^{c}	9.35±0.68^{b}	14.16±1.10^{a}	13.50±0.49^{a}	9.68±0.22^{b}	13.04±0.50^{a}	13.15±0.88^{a}	12.06±0.58^{a}
Streptococcus anginosus	8.06±0.42^{b}	8.59±0.07^{b}	11.21±1.50^{a}	9.65±0.72^{ab}	6.01±0.01^{c}	9.50±0.88^{b}	13.88±0.36^{a}	13.00±0.63^{a}	9.61±0.22^{c}	9.76±0.50^{bc}	12.01±0.88^{a}	10.87±0.58^{b}
Aggregatibacter actinomycetemcomitans	8.51±1.27^{b}	10.09±0.48^{b}	12.89±0.78^{a}	12.12±1.30^{a}	6.27±0.05^{c}	10.77±1.39^{b}	13.13±0.48^{a}	12.16±1.35^{ab}	7.75±1.60^{b}	11.16±0.71^{a}	12.75±1.82^{a}	11.56±2.10^{a}

Values represent means ± standard deviation, n=3.
Different letters in rows indicate significant differences between mean values of treatments ($p < 0.05$).

Table 4. Antimicrobial activities of *P. mume* extract according to incubation time.

	Diameter of the zone of inhibition (mm, Means ± SD)											
	Pectinex Ultra SP-L				Novozym 33095				Viscozyme L			
Microorganisms	10 h	20 h	30 h	40 h	10 h	20 h	30 h	40 h	10 h	20 h	30 h	40 h
Staphylococcus sobrinus	13.89±1.29^{a}	14.49±1.28^{a}	13.9 ±1.00^{a}	11.21±0.89^{b}	11.46±0.95^{a}	13.62±2.40^{a}	12.38±0.57^{a}	13.09±0.47^{a}	10.55±0.29^{a}	12.35±1.53^{a}	11.75±0.85^{a}	10.52±0.79^{a}
Streptococcus aureus	12.68±1.55^{a}	14.33±1.83^{a}	13.76±2.20^{a}	12.58±1.74^{a}	11.42±1.07^{b}	14.13±0.31^{ab}	12.87±1.50^{ab}	14.72±2.10^{a}	13.90±0.86^{a}	14.95±0.99^{a}	14.03±1.95^{a}	13.45±1.16^{a}
Streptococcus mutans	12.65±0.94^{a}	13.13±0.98^{a}	13.19±1.09^{a}	12.58±1.28^{a}	11.00±0.87^{b}	14.34±1.06^{a}	12.57±1.77^{ab}	12.51±2.05^{ab}	11.24±0.77^{a}	12.68±0.99^{a}	12.63±1.86^{a}	11.71±1.16^{a}
Streptococcus ratti	13.05±2.37^{a}	12.91±1.22^{a}	13.17±1.40^{a}	12.13±0.99^{a}	10.94±1.16^{b}	13.60±0.18^{a}	12.52±0.9^{a}	12.45±0.52^{a}	11.82±2.14^{b}	15.69±1.18^{a}	14.47±1.89^{ab}	12.87±1.67^{ab}
Streptococcus sanguinis	12.37±0.96^{a}	12.04±0.90^{a}	11.02±1.06^{a}	10.85±0.52^{a}	10.09±0.61^{a}	12.21±1.25^{a}	11.89±0.89^{a}	12.03±2.01^{a}	11.41±0.11^{c}	14.00±0.57^{a}	13.02±0.86^{ab}	12.09±0.82^{bc}
Actinomyces viscosus	15.13±0.14^{a}	14.15±1.76^{a}	15.27±1.27^{a}	14.74±1.07^{a}	13.31±0.36^{a}	15.63±1.51^{a}	14.37±1.54^{a}	14.65±1.71^{a}	11.53±0.81^{b}	14.54±2.58^{a}	14.04±1.04^{ab}	12.43±0.42^{ab}
Actinomyces naeslundii	14.56±1.26^{a}	13.70±1.53^{a}	14.27±0.49^{a}	13.95±0.75^{a}	13.37±1.27^{a}	14.87±1.02^{a}	13.95±0.67^{a}	14.45±0.09^{a}	11.71±1.14^{a}	13.45±1.77^{a}	13.40±0.84^{a}	11.71±0.87^{a}
Streptococcus anginosus	14.16±1.53^{a}	14.18±2.10^{a}	13.96±0.80^{a}	13.74±0.78^{a}	12.38±0.50^{b}	14.33±0.74^{a}	13.04±0.87^{b}	12.62±0.09^{b}	11.35±1.39^{a}	13.47±1.24^{a}	12.98±1.69^{a}	11.48±0.77^{a}
Aggregatibacter actinomycetemcomitans	14.59±0.79^{a}	14.20±1.67^{a}	14.59±0.33^{a}	13.71±0.57^{a}	11.76±2.02^{b}	15.00±1.24^{a}	13.24±0.48^{ab}	13.67±0.98^{ab}	11.16±1.85^{a}	13.58±1.32^{a}	13.40±0.24^{a}	11.96±1.29^{a}

Values represent means ± standard deviation, n=3.
Different letters in rows indicate significant differences between mean values of treatments ($p < 0.05$).

Table 5. Antimicrobial activities of *P. mume* extract according to temperature.

	Diameter of the zone of inhibition (mm, Means ± SD)											
	Pectinex Ultra SP-L				Novozym 33095				Viscozyme L			
Microorganisms	35°C	45°C	55°C	65°C	35°C	45°C	55°C	65°C	35°C	45°C	55°C	65°C
Staphylococcus sobrinus	13.28±0.67^{a}	14.23±1.14^{a}	13.21±1.18^{a}	13.27±0.63^{a}	13.45±1.05^{a}	14.77±1.18^{a}	13.13±0.50^{a}	13.44±1.07^{a}	13.16±0.82^{ab}	14.50±0.40^{a}	13.10±0.83^{ab}	12.84±0.88^{b}
Streptococcus aureus	13.16±0.70^{ab}	14.14±0.47^{a}	12.99±1.01^{b}	13.32±0.37^{ab}	13.82±0.63^{a}	15.27±0.77^{a}	12.77±1.89^{a}	13.59±1.32^{a}	13.14±2.60^{a}	13.85±2.13^{a}	13.14±1.89^{a}	12.80±2.35^{a}
Streptococcus mutans	11.26±1.63^{a}	11.63±1.61^{a}	11.26±1.51^{b}	11.24±1.27^{a}	11.77±0.46^{ab}	12.90±0.46^{a}	10.80±1.62^{b}	11.84±0.36^{ab}	10.44±2.51^{a}	11.13±2.12^{a}	10.18±1.59^{a}	10.10±2.06^{a}
Streptococcus ratti	13.87±0.69^{a}	13.27±0.31^{ab}	12.34±0.03^{a}	13.12±0.72^{bc}	13.69±2.02^{ab}	15.51±0.53^{a}	13.00±0.32^{b}	12.89±0.91^{b}	11.88±0.66^{b}	14.17±1.06^{a}	12.75±0.63^{ab}	11.82±0.93^{b}
Streptococcus sanguinis	12.74±0.78^{ab}	13.17±0.74^{a}	11.98±1.19^{b}	13.01±0.57^{a}	13.08±1.24^{a}	14.26±1.50^{a}	13.30±1.63^{a}	13.27±0.57^{a}	13.41±0.51^{a}	15.13±0.98^{a}	13.55±1.00^{a}	13.16±1.41^{a}
Actinomyces viscosus	14.72±2.51^{a}	14.78±2.25^{a}	12.83±1.44^{a}	14.59±1.33^{a}	13.51±3.01^{a}	12.45±2.77^{a}	13.43±2.97^{a}	14.18±2.54^{a}	13.00±2.68^{a}	15.19±2.71^{a}	13.40±2.86^{a}	12.59±2.63^{a}
Actinomyces naeslundii	14.34±0.47^{a}	14.37±1.25^{ab}	13.64±0.87^{b}	13.29±0.13^{b}	13.86±0.16^{b}	15.21±0.40^{a}	13.22±1.04^{b}	13.34±0.72^{b}	12.57±0.80^{a}	13.86±0.93^{a}	12.39±1.11^{a}	12.11±0.91^{a}
Streptococcus anginosus	13.54±2.14^{a}	14.94±2.82^{a}	12.85±1.90^{a}	13.18±2.13^{a}	13.80±2.15^{a}	14.53±2.57^{a}	13.81±2.33^{a}	13.38±2.29^{a}	12.58±2.55^{a}	14.42±2.02^{a}	13.62±1.77^{a}	12.40±1.77^{a}
Aggregatibacter actinomycetemcomitans	14.34±0.93^{a}	14.72±0.89^{a}	13.21±0.91^{b}	14.26±0.48^{a}	14.92±0.66^{a}	15.48±0.53^{a}	13.80±1.87^{a}	14.10±0.50^{a}	12.86±0.83^{b}	15.22±0.71^{a}	13.97±0.94^{ab}	12.57±0.45^{b}

Values represent means ± standard deviation, n=3.
Different letters in rows indicate significant differences between mean values of treatments ($p < 0.05$).

Table 6. Antimicrobial activities of *P. mume* extract according to pH.

	Diameter of the zone of inhibition (mm, Means ± SD)											
	Pectinex Ultra SP-L				Novozym 33095				Viscozyme L			
Microorganisms	pH 4	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8
Staphylococcus sobrinus	11.88±2.25^{a}	13.76±1.30^{a}	14.76±1.69^{a}	13.80±2.01^{a}	14.58±2.80^{a}	14.62±1.81^{a}	16.19±2.99^{a}	14.56±3.36^{a}	14.29±2.90^{a}	15.14±4.03^{a}	14.80±3.82^{a}	14.21±4.62^{a}
Streptococcus aureus	12.09±1.37^{b}	13.63±0.99^{ab}	14.85±0.90^{a}	13.92±0.88^{ab}	14.31±2.17^{a}	14.09±1.36^{a}	15.16±0.91^{a}	14.04±1.49^{a}	13.06±2.11^{a}	14.12±1.70^{a}	12.60±1.99^{a}	11.96±2.73^{a}
Streptococcus mutans	10.20±1.05^{b}	12.32±1.43^{a}	12.39±0.32^{a}	11.19±0.58^{ab}	14.63±2.00^{a}	13.84±2.30^{a}	15.68±1.88^{a}	14.84±2.64^{a}	12.20±2.08^{a}	13.28±2.60^{a}	11.47±2.25^{a}	11.24±2.33^{a}
Streptococcus ratti	11.93±1.76^{a}	14.48±1.20^{a}	15.41±2.07^{a}	14.22±2.64^{a}	13.19±1.66^{a}	13.22±1.42^{a}	14.85±3.32^{a}	13.50±1.75^{a}	13.08±1.52^{a}	14.64±2.65^{a}	12.49±2.24^{a}	12.08±1.76^{a}
Streptococcus sanguinis	12.89±3.80^{a}	14.10±3.92^{a}	15.28±4.33^{a}	14.61±4.17^{a}	14.79±3.99^{a}	13.70±3.67^{a}	15.04±4.81^{a}	13.64±5.05^{a}	13.97±4.76^{a}	14.95±4.80^{a}	13.99±4.76^{a}	13.02±5.00^{a}
Actinomyces viscosus	10.07±0.19^{b}	12.22±1.10^{ab}	13.00±1.90^{a}	11.42±0.78^{ab}	12.92±1.53^{a}	12.98±1.28^{a}	15.46±1.37^{a}	13.52±1.68^{a}	13.06±2.28^{a}	14.28±2.31^{a}	12.08±1.34^{a}	11.59±2.28^{a}
Actinomyces naeslundii	11.89±2.60^{a}	13.81±2.61^{a}	15.41±1.49^{a}	13.94±2.15^{a}	14.65±1.97^{a}	14.73±1.82^{a}	16.53±1.66^{a}	15.32±2.40^{a}	15.52±2.10^{a}	15.97±2.63^{a}	14.66±2.72^{a}	14.04±2.31^{a}
Streptococcus anginosus	12.42±2.93^{a}	14.03±3.11^{a}	14.72±2.88^{a}	13.28±3.51^{a}	13.14±2.43^{a}	13.04±2.04^{a}	13.76±2.61^{a}	12.94±1.71^{a}	14.55±2.67^{a}	15.47±3.76^{a}	13.50±1.28^{a}	12.88±2.48^{a}
Aggregatibacter actinomycetemcomitans	12.13±2.43^{a}	13.78±3.01^{a}	15.58±1.34^{a}	13.68±2.02^{a}	14.17±3.30^{a}	15.40±2.31^{a}	15.85±2.41^{a}	15.05±2.51^{a}	15.14±3.65^{a}	16.14±3.76^{a}	14.12±4.07^{a}	13.07±3.33^{a}

Values represent means ± standard deviation, n=3.
Different letters in rows indicate significant differences between mean values of treatments ($p < 0.05$).

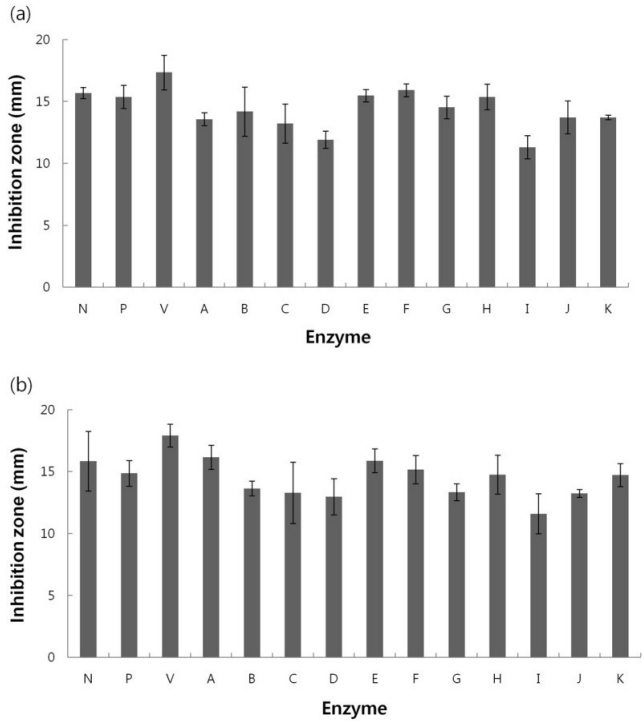

Figure 2. Antimicrobial activities of *P. mume* extracted using combinations of enzymes a) *Actinomyces naeslundii* and b) *Aggregatibacter actinomycetemcomitans.* Bars are means ± standard deviation, n=3. N: Novozym 33095; P: Pectinex Ultra SP-L; V: Viscozyme L; A: combination of Novozym and Viscozyme, ratio of Novozym and Viscozyme = 8:2; B: combination of Novozym and Viscozyme, ratio of Novozym to Viscozyme = 6:4; C: combination of Novozym and Viscozyme, ratio of Novozym to Viscozyme = 4:6; D: combination of Novozym and Viscozyme, ratio of Novozym to Viscozyme = 2:8; E: combination of Novozym and Pectinex, ratio of Novozym to Pectinex = 8:2; F: combination of Novozym and Pectinex, ratio of Novozym to Pectinex = 6:4; G: combination of Novozym and Pectinex, ratio of Novozym to Pectinex = 4:6; H: combination of Novozym and Pectinex, ratio of Novozym to Pectinex = 2:8; I: combination of Viscozyme, Pectinex and Novozym, ratio of Viscozyme, Pectinex and Novozym = 6:2:2; J: combination of Viscozyme, Pectinex and Novozym, ratio of Viscozyme, Pectinex and Novozym = 2:6:2; K: combination of Viscozyme, Pectinex and Novozym, ratio of Viscozyme, Pectinex and Novozym = 2:2:6

Determination of MIC

The optimal conditions for enzymatic extraction were pH 6.0, 45°C, and 20 h for Pectinex Ultra SP-L. The maximum antimicrobial activity of *P. mume* extract by Novozym 33095 was obtained at pH 7.0, 45°C, and 20 h. The Viscozyme L extract showed the greatest inhibitory effect at pH 6.0, 45°C, and 20 h. The MICs of *P. mume* extracts against oral bacterial strains were determined using a broth microdilution method. The MIC was defined as the concentration at which a sharp decline, followed by a constant absorbance value, occurred. The MICs of *P. mume* extracts under the optimum conditions are presented in Table 7. The concentration of extract was expressed as a percentage dilution of the original extract of *P. mume*. The extracts exhibited MIC values of 3.125 to 12.50% against the bacteria species. The Pectinex Ultra SP-L extract showed a MIC of 6.25% against *S. mutans, S. sanguinis, S. anginosus, A. viscosus,* and *A. naeslundii.* The Novozym 33095 extract exhibited an MIC of 6.25% against *S. sanguinis, A. viscosus, A. naeslundii,* and *A. actinomycetemcomitans*. The Viscozyme L extract exhibited an MIC of 6.25% against *S. sobrinus, S. mutans, S. anginosus, A. viscosus,* and *A. naeslundii*. *S. sanguinis* showed the greatest susceptibility (MIC, 3.125%). Indeed, all extracts showed higher antimicrobial activity against *S. sanguinis*, than the other bacterial strains. The MIC of enzyme combination extracts did not differ significantly compared with those of each enzyme alone.

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Table 7. Minimum inhibitory concentrations (% of dilution) of *P. mume* extracts.

	MIC (% of dilution)						
	Enzyme						
Microorganisms	N	P	V	C1	C2	C3	C4
S. aureus	12.50	12.50	6.25	12.50	12.50	12.50	12.50
Staphylococcus sobrinus	12.50	12.50	6.25	3.125	12.50	12.50	12.50
S. mutans	12.50	6.25	6.25	6.25	6.25	12.50	6.25
S. ratti	12.50	12.50	6.25	12.50	6.25	12.50	12.50
S. sanguinis	6.25	6.25	3.125	6.25	6.25	6.25	6.25
S. anginosus	12.50	6.25	6.25	12.50	12.50	12.50	12.50
A. viscosus	6.25	6.25	6.25	6.25	6.25	6.25	12.50
A. naeslundii	6.25	6.25	6.25	6.25	6.25	12.50	12.50
Aggregatibacter actinomycetemcomitans	6.25	12.50	12.50	6.25	6.25	12.50	12.50

Values represent the percentages of dilution on the basis of the original extract.
N: Novozym 33095; P: Pectinex Ultra SP-L; V: Viscozyme L; C1: combination of Novozym and Pectinex, ratio of Novozym to Pectinex = 8:2; C2: combination of Novozym and Viscozyme, ratio of Novozym to Viscozyme = 8:2; C3: combination of Pectinex and Novozym, ratio of Pectinex to Novozym = 8:2; C4: combination of Pectinex and Viscozyme, ratio of Pectinex to Viscozyme = 8:2.

Dental caries and periodontal disease are the most important infectious oral diseases. Oral diseases can be prevented using antimicrobial agents such as chlorhexidine that alter the oral microbiota and result in teeth staining (WALSH et al., 2015). Therefore, alternative natural products have been investigated, with a focus on and phytochemical antibacterial compounds (GONZALEZ et al., 2013). Previous observations have suggested that *P. mume* solvent extract exhibits antioxidant and antimicrobial activities (PINELO et al., 2006; XIA et al., 2011). Unfortunately, these methods suffer from low extraction yields, long extraction times and potential for trace organic solvents in the final products, which decreases the product quality (CHO et al., 2013).

High-pressure enzymatic extraction of phytochemical compounds is a potential alternative to solvent-based extraction. In this study, enzymatic extraction was conducted at 100 MPa, which does not affect the stability and functionality of most enzymes (MASSON et al., 2001; SUNWOO et al., 2013). The efficiency of enzymatic extraction is influenced by multiple variables, including enzyme concentration, incubation time, incubation temperature, pH and use of enzyme combinations (LANDBO; MEYER, 2001; PINELO et al., 2006). Accordingly, the high-pressure enzymatic extraction conditions should be optimized to maximize the antimicrobial activity against oral bacteria. We found that enzymatic treatment increased the inhibitory effects significantly in comparison to the control treatment. Briefly, the *P. mume* cell wall was disrupted by treatment with Pectinex Ultra SP-L, Novozym 33095 and Viscozyme L, which resulted in the release of biologically active components. With enzymatic treatment, the longer the incubation time, the greater the hydrolysis of cell wall components. However, a longer hydrolysis time will result in increased microbial growth.

The polyphenols of some edible plants have been reported as potential sources of agents capable of controlling the growth of oral bacteria (YOO et al., 2011). Temperature has the greatest influence on the release of phenols from phytochemical compounds (MEYER et al., 1998). With increasing temperature, antimicrobial activity increases due to enhanced solubility, enzyme activity, and diffusion coefficient. However, a temperature >45°C affects activity negatively because of the instability of the phenolic compounds and membrane denaturation (PINELO et al., 2006). A temperature higher or lower than 45°C partly inhibited enzyme activity as reported previously (LANDBO; MEYER, 2001). The maximum inhibition zone diameter was obtained at pH 6.0 or 7.0, depending on the enzyme. The pH value of the reaction solution plays an important role in cell wall hydrolysis and polyphenol extraction in plants (ZHENG et al., 2008). Use of combinations of enzymes did not significantly affect activity compared with each enzyme alone. Especially, used of Viscozyme L led to a decrease in the antimicrobial activities against *A. naeslundii* and *A. actinomycetemcomitans*. The *P. mume* extracts showed similar MIC values irrespective of the enzyme used. However, Viscozyme L showed lower MIC values than other enzymes for all oral bacteria tested.

The antimicrobial activities of *P. mume* extracts obtained by solvent extraction have been reported that they inhibited oral bacterial strains, such as *S. mutans*, *S. sobrinus*, *S. gingivalis,* and *A. actinomycetemcomitans* (SENEVIRATNE et al., 2011; JANG et al., 2014). However, it has not been investigated about the antimicrobial activities of *P. mume* extracts based on high-pressure enzymatic treatment against oral bacteria. In this study, *P. mume* extracts obtained by high-pressure enzymatic treatment exhibited antimicrobial activity superior to that reported in the above-mentioned study. Thus, high-pressure enzymatic extraction yields products with considerable antimicrobial activity.

CONCLUSIONS

High-pressure enzymatic treatment induces release of biologically active components from the cell wall matrix, which results in enhanced antimicrobial activity. We determined the optimum pH, temperature, incubation time, and enzyme concentration for the extraction of *P. mume* using three commercially available enzymes (Pectinex Ultra SP-L, Novozym 33095 and Viscozyme L) in a high-pressure (100 MPa) reactor.

The optimal conditions were 5 v/v% enzyme concentration at a temperature of 45°C, an incubation time of 20 h, at pH 6.0 or 7.0 (pH 7.0 with Novozym 33095, pH 6.0 with Pectinex Ultra SP-L or Viscozyme L).

The *P. mume* extracted by high-pressure enzymatic extraction have higher antimicrobial activity compared to that of conventional solvent extraction. Therefore, compared with a solvent extraction method using large quantity of organic solvent, the high-pressure enzymatic extraction is a novel and environmentally friendly approach for extraction of ingredients with antimicrobial activity against oral bacteria.

The pharmaceutical and food industry can be promoted by the use of high-pressure enzymatic extraction, which provides high extraction efficiency with short extraction time, fewer impurities, and non-solvent use.

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RESUMO: Avaliar o processamento de alta pressão combinado com o tratamento enzimático para a extração de *Prunus mume*, determinando as condições ótimas de extração para a atividade antimicrobiana contra as bactérias orais. A extração enzimática de alta pressão foi utilizada para isolar os componentes biologicamente ativos de *P. mume.* Os efeitos das variáveis do processo como o tipo enzimático (Pectinex Ultra SP-L, Novozym 33095 e Viscozyme L), a concentração de enzima, o tempo/temperatura de incubação, pH, e a relação da atividade antimicrobiana de enzimas contra os patógenos orais relacionados à cárie dentária e doenças periodontais foram determinados pelo ensaio de difusão em disco. As condições ótimas para a extração enzimática de *P. mume* foram pH 6.0, 45°C, 20 h, e 5 v/v% com Pectinex Ultra SP-L. A máxima atividade antimicrobiana do extrato de *P. mume* obtida usando Novozym 33095 foi em pH 7.0, 45°C, 20 h, e 5 v/v%. O extrato de Viscozyme L apresentou o efeito inibitório máximo em pH 6.0, 45°C, 20 h, e 5 v/v%. O uso de combinações de enzimas não resultou em uma atividade antimicrobiana significativamente diferente ($p < 0.05$) em comparação com cada enzima por separada. Os valores mínimos da concentração inibitória foram de 3.125 a 12.50%. Estes resultados indicaram que a extração enzimática de alta pressão produziu o extrato de *P. mume* com atividade antimicrobiana,o qual tem o potencial para melhorar o ambiente bucal.

PALAVRAS-CHAVE: Atividade antimicrobiana. Extrato enzimático de alta pressão. Concentração Inibitória Mínima. *Prunus mume*.

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