

PARTIAL PURIFICATION, CHARACTERIZATION, AND APPLICATION OF EXTRACELLULAR ASPARTIC PROTEASE FROM *Lactobacillus casei* WSP IN PRODUCING THE BIOACTIVE PEPTIDES WITH ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY

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Abstract

Lactobacillus casei WSP-derived an aspartic protease was sequentially purified by using chromatography gel filtration sephadex G-50. It resulted in a 22.81-fold increase of specific activity (51.5 U/mg) with a final yield of 1.9%. The estimated molecular weight of the purified enzyme was 37 kDa and showed gelatinolytic activity in zymogram assay. The enzyme exhibited optimum activity at 40°C and pH 6 with casein as the substrate. Enzyme activity was significantly inhibited by pepstatin A (0.5 mM and 1 mM), confirming that this enzyme is a group of aspartic proteases, while other inhibitors such as EDTA, PMSF and iodoacetic acid showed no inhibition effect on the activity of enzyme. The addition of metal ion to the enzyme decreased enzyme activity, indicating the proteolytic enzyme was metal ion-dependent. Denaturant such as DDT tended to increase caseinolytic activity. Furthermore, this enzyme was capable of generating the new peptides from skimmed milk with the size 8 kDa, 10 kDa and 15 kDa. These peptides have potential as antibacterial and antioxidant agents.

Keywords: Aspartic protease • Antibacterial • Antioxidant • Peptide

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Introduction

Protease is an enzyme that produced by various organisms such as viruses, bacterium, protist, fungus, plants, animals, and human. It has substrate specificity, catalytic mechanism, and active site. The general function of protease is to hydrolyze peptide bond in protein as a substrate. Proteases are generally classified as aspartic, cysteine, glutamic, serine, and threonine protease, it depends upon the amino acids that present in the active site, or as metalloproteases if a metal ion is required for catalytic activity (Sumantha *et al.*, 2006). Protease is an enzyme that widely used in detergent, food, leather, and pharmaceutical industry (Rao *et al.*, 1998). Recently, the

production of bacterial protease has extensively been studied by scientists and industries. Furthermore, bacteria are the potential microorganism to produce enzymes because it can quickly grow and be induced by environmental changes to obtain the desirable enzymes during incubation. Almost a more half of bacterial protease production in the world is a type of alkaline and neutral protease. It classified as cysteine, glutamic, serine, threonine, and metalloprotease. There are only a few reports related to the bacterial aspartic protease. It becomes the background of this study bacterial aspartic protease is needed to be explored more.

Aspartic protease is endopeptidase with the molecular weight in the range of 30-40 kDa, with the maximum activity at a low of pH and

it depends on aspartic acid residues for its catalytic activity (Vishwanatha *et al.*, 2009). Aspartic protease is also known as acidic protease, that has been isolated from diverse source (Hsiao *et al.*, 2014). The studies related to the aspartic protease isolated from viruses, protists, and fungi are known to be used as the agent to inhibit several diseases caused by HIV (Ghosh *et al.*, 2016), HCV (Hirano *et al.*, 2017), Plasmodium (Coombs *et al.*, 2001), and *Candida albicans* (Braga & Santos, 2011). Meanwhile, aspartic protease isolated from an animal is known as chymosin used as milk-coagulating enzymes for manufacturing cheese (Yegin *et al.*, 2011). Aspartic protease is also isolated from a plant such as potato that has the potential antimicrobial activity (Guevara *et al.*, 2004). Whereas there are still a few reports about aspartic protease isolated from bacteria and their application.

Aspartic protease is frequently used in the production of seasoning materials, such as soy sauce and protein hydrolysate, or used as digestive aids (Rao *et al.*, 1998). This enzyme is also widely used to improve the texture of flour paste, to clear beer and fruit juice. Because of their high activity and stability in acidic environments, aspartic proteases are useful reagents in the food processing industry (Hsiao *et al.*, 2014). Moreover, The aspartic protease is also used as additives to improve food flavor and texture. Most of the microbial aspartic proteases are produced from filamentous fungi. Aspartic protease from fungi has the ability to grow on a solid substrate or both and produces a wide range of extracellular enzyme, that is low cost, high productivity and fast in production (Yin *et al.*, 2013). It has the same potential with bacteria as other sources for a microbial aspartic protease. One of the bacteria that has a potential to produce aspartic protease is *Lactobacillus casei* (*L. casei*).

L. casei is Lactic Acid Bacteria known as Generally Recognize as Safe (GRAS) microbe that is applied to fermented product. *L. casei* in this research is isolated from goat milk and has been identified as *L. casei* WSP. There are several studies related to the protease from Lactic Acid Bacteria. Production of protease had been reported from *L. plantarum* SK5 and *L. plantarum* S31 that isolated from *bekasam* (traditional Indonesian food) (Kurniati *et al.*, 2015; Budiarto *et al.*, 2016). Another study has reported that *L. sanfrancisco* CB1 was able to

produce serine protease (Gobbetti *et al.*, 1996). There still has been no report about aspartic protease from *L. casei*.

On the other hand, the emergence of a new type of diseases due to fast food consumption increased consumer awareness of the importance of functional food. Functional food has an important component such as vitamin, fiber, mineral, unsaturated fatty acid, prebiotic, probiotic and bioactive peptide. Milk and dairy products, including skimmed milk are one of the functional food that has high protein content (Baht & Bhat, 2011). Recently, milk protein has been reaching an increased consideration, especially the bioactive peptides released from the parent protein by the digestive enzyme, proteolytic starter culture, and proteolytic enzyme (Ahmed *et al.*, 2015). Aspartic protease from *L. casei* WSP has a potential function to produce bioactive peptide from skimmed milk. The skimmed milk hydrolysis by aspartic protease *L. casei* WSP is able to be used as antibacterial and antioxidant. The present study focuses on the purification, characterization and application of aspartic enzyme *L. casei* WSP.

Materials and Methods

Substrate and chemical reagents

The culture of *L. casei* WSP was isolated from goat milk (Research Center for Biotechnology-Indonesian Institute for Science collection). Sephadex G-50 was from GE Heatcare Bio-Science, Uppsala, Sweden. Dialysis membrane (MWCO 12 kDa) was from Thermo Fisher Scientific, Massachusetts, USA. MRS (de Man, Rogosa, Sharpe) medium was from Himedia, Mumbai, India. Pierce bicinchonic acid (BCA) protein assay kit was from Thermo Scientific, USA. All other chemicals were purchased from Sigma Chemical Co.

Preparation of protease-containing culture supernatant

L. casei WSP was maintained on MRS broth medium. For the experimental purpose, 0.1% of *L. casei* WSP suspension was subcultured into new 5 mL MRS medium incubated at 37°C for overnight prior to use. After incubation, centrifugation used to separate the cell and the supernatant of culture. The supernatant was collected as crude extract.

The crude extract used to measure extracellular activity. This method following Espla et al. (1997) with some modification.

Protease activity assay

Proteolytic activity was measured using casein as a substrate. Based on the tyrosine formation during casein hydrolysis by the protease, this method evaluated the tyrosine released at wavelength 540 nm. Water was used as a control and Tris HCl buffer (pH 7.4, 25 mM) was used as a blank. The sample enzyme was firstly mixed with Tris HCl buffer (pH 7.4, 25 mM) at volume 6 μ L and 6 μ L respectively. Control, blank, and samples then added with 6 μ L 1% (w/v) casein solution. The reaction mixture was incubated at 37°C for 30 min. Before centrifugation at 13000 rpm for 10 min, the reaction was added with 12 μ L trichloroacetic acid, followed by adding 143 μ L of reagents C [a mixture of Na₂CO₃ solution and CuSO₄.5HO solution (5:1)] and 30 μ L of folin ciocalteau reagent, then measured at 540 nm. One unit of protease activity is defined as 1 μ mol of tyrosin released during enzymatic reaction per mL reaction mixture per minute under the experimental condition (Budiarto *et al.*, 2016).

Determination of cell growth curve and its protease activity

L. casei WSP was inoculated (0.1%) at 5 mL of fresh MRS medium and incubated at 37°C without shaking. Over night culture of *L. casei* WSP (200 μ L) then was transferred into 200 mL MRS medium incubated at 37°C without shaking. The sampling of culture was done by taking 1.5 mL of culture at interval 4 h of cultivation then the cell density (OD₆₀₀ nm), pH medium, and extracellular protease activity were measured. (Maier *et al.*, 2009; Budiarto *et al.*, 2016)

Purification of protease.

The supernatant (crude extract) of 24 h *L. casei* WSP liquid culture was subjected to the saturation using 80% of solid ammonium sulfate, then resuspended in 25 mM Tris HCl pH 7.4. The crude extract was then applied onto a dialysis membrane with MWCO 12 kDa for dialysis. The last step, the result of dialysis applied into chromatography gel filtration sephadex G-50, pre-equilibrated with 25 mM Tris HCl pH. 7.4. Crude protein-containing sephadex G-50 was then separated using the

same buffer. The flow rate of this process was fixed at 1 mL/min. The fraction with protease activity was used for the next analysis (Budiarto *et al.*, 2016).

Molecular weight determination and protein quantification

Protein molecular weight was determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and gelatin zymography method. For SDS-PAGE, the sample was run in 3.9% stacking and 12% separating gel and used the broad-range protein marker (5 kDa) as the standard of the protein size. The protein in the gel was stained by using Bio-safe Coomassie Blue (Bio-Red laboratories) or silver staining reagent (Thermo scientific) (Laemmi, 1970). Gelatin zymography method was used to detect not only molecular weight but also protease activity. The sample was run on 0.2% gelatine-containing gel electrophoresis. Then, the protein was reactivated with triton-X 100 for 40 min at 37°C. After reactivated, the sample incubated with buffer Tris HCl for overnight. Gel staining was performed in 0.05% coomassie blue solution for 2 h, while removing the excess coomassie blue used destaining solution until clear band appeared on the gel (Raser *et al.*, 1995; Kleiner & Stetler-Stevenson, 1994). Protein molecular weight was determined using Rf method, whereas protein concentration was quantified using bicinchoninic acid assay (BCA) kit with bovine serum albumin as standard.

Characteristic of Extraceluller Protease *L casei* WSP

The optimum pH for obtaining protease was determined by measuring the activity of enzyme at 37°C for 30 min. Tris HCl used as an assay buffer with different condition pH at range 3-7. The optimum temperature for protease activity was determined using temperature of 37-70°C for 30 min incubation.

Effect of chemical reagents and metal ions on enzyme activity

To determine the effects of protease inhibitor on the enzyme, purified enzyme was incubated, at 37°C for 30 min, followed by measuring its activity under the standard assay condition described above. The enzyme was assayed for inhibition by the serine protease inhibitors (PMSF : 0.5 mM and 5 mM), the

cysteine protease inhibitor (Iodoacetic acid : 0.5 mM and 5 mM), the metalloprotease inhibitor (EDTA : 0.5 mM and 5 mM) and the aspartic protease inhibitor pepstatin A (0.5 mM and 1 mM). For metal ions treatment, MgCl₂ and CaCl₂ (0.5 mM and 1 mM) were used. While dithiothreitol (0.5 mM and 5 mM) was used as the reducing agent. Other reagents, tween-20 (non ionic detergent) and sodium dodecyl sulphate (anionic detergent) at 2.5% and 5% respectively are used as surfactants. Relative activity was expressed as a percentage of untreated enzyme activity (Yadav *et al.*, 2012).

Skimmed milk hydrolysis using aspartic proteases *L. casei* WSP

Substrate skimmed milk 2.5% (w/v) was made by weighing 0.25 g skimmed milk in distilled water and the suspension was adjusted to pH 7. Protease *L. casei* WSP (51.50 U/mg) was added to the enzyme and substrate with ratio 1:100 (v/v). The hydrolysis was conducted by incubating sample at 37°C for several times (3, 6, 9, 12, 15, 18 h) on a shaker. After hydrolysis treatment, the sample was boiled at 100°C for 10 min and centrifuged at 13000 rpm, in the temperature 4°C for 15 min. The supernatant was then collected for the antibacterial and antioxidant assay (Pan *et al.*, 2004).

Antibacterial and antioxidant activity assay of hydrolysate skimmed milk

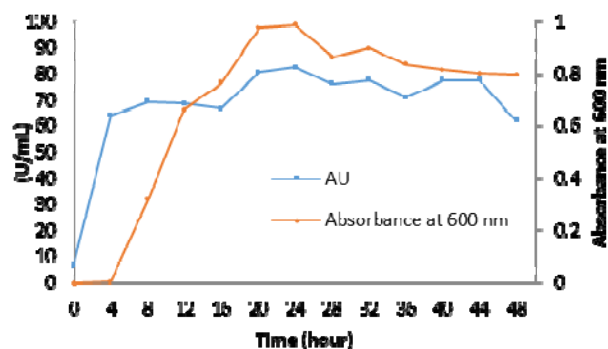
Antibacterial assay was conducted by using agar diffusion method (Kumar & Srivastava, 2010). EPEC K11 was used as pathogenic bacteria in this assay. The hydrolysate skimmed milk with the volume 30 µL was poured on a paper disk at agar medium that contains pathogenic bacteria. Then, the sample was incubated at 37°C for 18 h. The antibacterial activity was measured with Equation (1) to know the inhibition of sample toward EPEC K11 on agar medium. Antioxidant activity was measured by 2,2 diphenyl-1-picrylhydrazyl (DPPH) method used microplate 96 well. Ascorbic acid with different concentration used as a positive control. The final volume of each sample reaction was 200 µL, consisted of 180 µL DPPH in methanol solution and 20 µL sample. The absorbance was measured with a microplate reader, Thermo Scientific^R (Hasim *et al.*, 2017). Antioxidant activity was

determined as the percentage of inhibition with the following in Equation (2).

Results

L. casei WSP growth and its extracellular aspartic protease activity

The correlation of *L. casei* WSP growth with its extracellular aspartic protease activity is shown in Fig. 1a. In the preliminary experiment, during 48 h cultivation, pH medium decreased from 6.42 to 4.75 (data not shown). The initial aspartic protease activity (63.7 U/mL) was detected when the cell at 4 h incubation and then the aspartic protease activity steadily increased to its optimum value at 24 h incubation (84.3 U/mL). Then Aspartic protease reached a plateau after 24 h incubation.

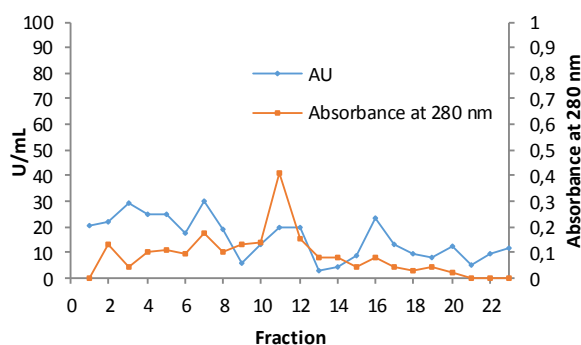


(a)

Purification of Extracellular aspartic protease *L. casei* WSP

Chromatogram pattern and its aspartic protease activity created several peaks (Fig. 1b). The peak from fraction 7 was chosen as it has the highest proteolytic activity and also showed gelatinolytic activity as confirmed by zymogram assay. Furthermore, a summary of purification steps for the crude extract aspartic protease is shown in Table 1. Compare to those proteolytic activities, aspartic protease in our study exhibited the lowest result and it might be the first report of producing aspartic protease by *Lactobacillus casei*. In addition, the final yield in this study (1.9%) was also still lower than other studies from fungi such as *Aspergillus oryzae* MTCC 5341, *Rhizopus*

oryzae and *Aspergillus oryzae* BCRC which were about at 15.1%, 29% and 58.8%, respectively (Vishwanatha *et al.*, 2009; Hsiao *et al.*, 2014; Yin *et al.*, 2013). It seems that the purification steps have a role to determine the final yield and specific activity of the enzyme. Furthermore, every source of aspartic protease needs an optimization study to get high yield and specific activity.



(b)

Fig. 1 Activity of aspartic protease *L. casei* WSP and chromatogram profile of filtration gel sephadex G-50. (a) Production of aspartic protease during *L. casei* WSP growth on MRS medium. (b) The sample loaded was result of chromatography filtration gel sephadex G-50.

Table 1. Purification of aspartic protease from *Lactobacillus casei* WSP

Purification Step	Total protein (mg)	Total activity (U/mL)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude extract	760.00	1757	2.31	1	100
80% Ammonium Sulphate	27.67	47.02	3.40	1.47	2.68
Dialysis	5.03	41.12	6.54	2.83	2.34
G-50 Sephadex	0.65	33.42	51.50	22.81	1.90

After ammonium sulfate precipitation, dialysis and chromatography purification steps, the degree of enzyme purity in each purification step was 1.47-fold, 2.83-fold and 22.81-fold from crude extract respectively. The final yield from this step purification was 1.9% with the observed specific activity of 51.50 U/mg. Aspartic protease purified from *Aspergillus oryzae* MTC5341, *Rhizopus oryzae* and *Aspergillus niger* BCRC 32720 showed the high proteolytic activity, specifically, about 43.6×10^3 U/mg, 57.5×10^3 U/mg and $23.3 \times$

10^3 U/mg, respectively (Vishwanatha *et al.*, 2009; Hsiao *et al.*, 2014; Yin *et al.*, 2013).

Fig. 2a shows several protein bands with the molecular weight of approximately 35 kDa, 37 kDa, and 60 kDa. This was later proven by zymogram assay and only one band appeared with molecular weight approximately of 37 kDa (Fig. 2b) and it corresponded to the extracellular of aspartic protease *L. casei* WSP.

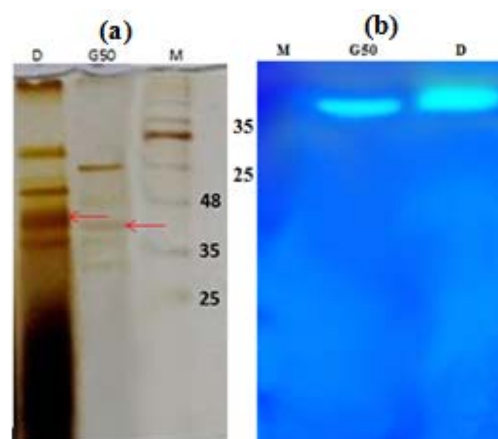


Fig. 2 SDS-PAGE and zymogram aspartic protease *L. casei* WSP. (a) SDS-PAGE analysis protein in dialysis (D) and fraction 7 of sephadex G-50 (G50). The red arrow indicates the protein corresponding to aspartic protease. (b) Zymogram result of dialysis (D) and fraction 7 of sephadex G-50 (G50). M was broad molecular weight marker (Bio-rad).

Characteristic of Extraceluller Protease *L. casei* WSP

Protease activity of the enzyme was evaluated using the pH that ranges from 3 to 7 in 1% casein as the substrate at the temperature of 37°C. The enzyme showed the optimum activity at pH 6. This result was similar to another report such as *Rhizopus oryzae* MTCC 3690 enzyme that exhibited the optimum activity at pH 5.5 (Kumar *et al.*, 2005). In contrast, the optimum pH of aspartic protease activity was at pH 3 and 3.4 have been reported (Hsiao *et al.*, 2014; Yin *et al.*, 2013).

The optimum temperature of *L. casei* WSP extracellular aspartic protease was at 40°C. This result was similar to the aspartic protease from *Aspergillus oryzae* BCRC 30118 and *Phycomyces blakesleeana* (Yin *et al.*, 2013; Vicente *et al.*, 1996).

Effect of inhibitors, surfactants and metal ions on enzyme activity

The aspartic protease activity was inhibited significantly by pepstatin A at 0.5 mM and 1 mM (39.14% and 30.43%). Pepstatin is a hexapeptide inhibitor that specifically inhibits acid protease by irreversibly binding to the active site of aspartate (Umezawa *et al.*, 1970). In contrast, PMSF, iodoacetic acid and EDTA showed no significant loss of aspartic protease activity.

Table 2. Characteristic of aspartic protease *L. casei* WSP

No	Reagent	Concentration	Relative activity (%) [*]
1	None	0	100±0
2	Tween-20	2.5%	73.36±3.71
		5%	76.53±1.81
3	SDS	2.5%	95.72±1.49
		5%	98.29±2.37
4	DDT	0.5 mM	128.17±1.24
		5 mM	133.71±4.80
5	PMSF	0.5 mM	91.17±4.15
		5 mM	82.47±4.29
6	Pepstatin A	0.5 mM	39.14±12.58
		1 mM	30.43±0.69
7	Iodoacetic acid	0.5 mM	32.80±1.49
		5 mM	40.92±8.34
8	EDTA	0.5 mM	83.85±3.95
		5 mM	94.93±3.48
9	CaCl ₂	0.5 mM	83.65±3.82
		1 mM	78.71±2.72
10	MgCl ₂	0.5 mM	79.30±0.59
		1 mM	77.72±2.99

^{*}Value are represented as mean±SD (n=3)

The antioxidant and antibacterial activity of Bioactive peptides from the hydrolysis product of skimmed milk

The purified aspartic protease was used to produce bioactive peptides from skimmed milk as the substrate. Fig. 5 shows the profile of SDS-PAGE of hydrolyzed-skimmed milk by the purified *L. casei* WSP aspartic protease. All proteins composing skimmed milk were degraded significantly after 9 h hydrolysis process. The hydrolysis process had been done at 37°C, pH 7 for 18 h of hydrolysis. There were new three bioactive peptides that were produced from skimmed milk (approximately 8 kDa, 10 kDa, and 15 kDa) at 9 h until 18 h incubation time (Fig. 5).

The molecular weight of the purified enzyme was lower than that of *Rhizopus oryzae* (39 kDa) (Hsiao *et al.*, 2014), but it was higher than that of results reported from *Rhizopus oryzae* MTCC 3690 (34 kDa),

Rhizopus oryzae NBRC 4749 (34 kDa) and *Rhizopus microsporus* (34.5 kDa) (Kumar *et al.*, 2005; Chen *et al.*, 2009; Schoen *et al.*, 2002). This result suggested that the purified aspartic protease from *L. casei* WSP in this study was a distinct form from any other aspartic proteases that have been previously reported.

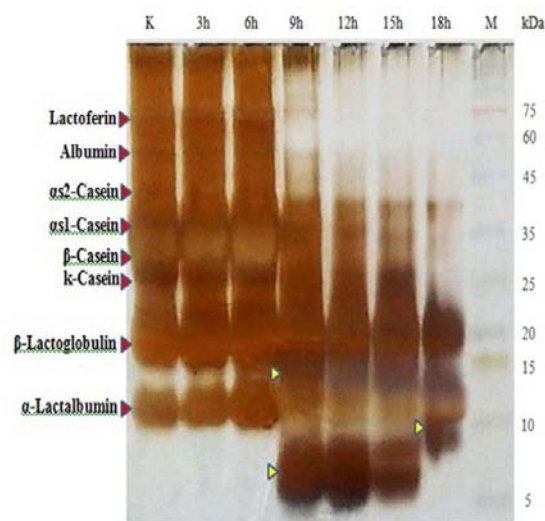


Fig. 5 SDS-PAGE of skimmed milk hydrolysis by aspartic protease *L. casei* WSP at 3, 6, 9, 12, 15 and 18 h time incubation. Lane K (control) : skimmed milk without protease treatment was adjusted at 0 h. Lane M : broad molecular weight markers (Bio-Rads). Yellow arrow head : new peptide band. The protein in gel stained with silver stain reagent.

The bioactive peptide of skimmed milk hydrolyzed by aspartic protease exhibited antibacterial activity toward EPEC K11, with the value of 1543.83±0.57 mm²/mL (Fig. 6). Whereas the control sample (skimmed milk without hydrolysis) and control antibiotic (kanamycin) obtained 1164.42±0.28 mm²/mL and 8190.17±1.15 mm²/mL. In this study, The hydrolysate skimmed milk sample showed low activity compared to the commercial control of antibiotic.

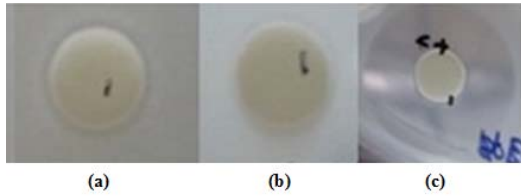


Fig. 6 Antibacterial activity toward EPEC K11. (a) Hydrolysate skimmed milk 25 mg/mL, (b) Control hydrolysate 25 mg/mL, (c) Kanamycin 5 mg/mL (positive control).

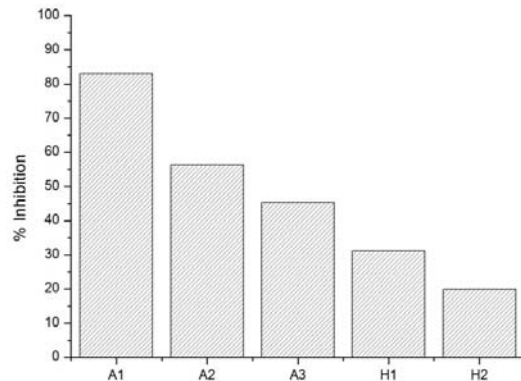


Fig. 7 Antioxidant activity of hydrolysis skimmed milk by aspartic protease *L. casei* WSP. A1 : Ascorbic acid 200 μ g/mL, A2 : Ascorbic acid 70 μ g/mL, A3 : Ascorbic acid 50 μ g/mL, H1 : Hydrolysate skimmed milk 25 mg/mL, H2 : Control hydrolysate skimmed milk 25 mg/mL.

Skimmed milk hydrolysis by aspartic protease *L. casei* WSP shows antioxidant activity toward scavenging DPPH. Ascorbic acid with several concentrations was used as positive control. The hydrolysate sample could inhibit free radical of DPPH approximately 31.22% with the concentration of 25 mg/ml (Fig. 7).

Discussion

This result showed that the highest production of aspartic protease occurred at the end of stationary phase of *L. casei* WSP growth. The dynamic of protease production is influenced mostly by the ratio of carbon to nitrogen source (C/N ratio) added in medium neutral or alkaline protease would be dominantly produced when the C/N ratio was at low level, while acidic protease being produced abundantly when C/N ratio at a high level (Yin *et al.*, 2013; Palsaniya *et al.*, 2012). The yield and pattern of protease enzyme may

be further engineered by modifying the nutritional composition of growth medium (Budiarto *et al.*, 2016). Incubation time and nutritional composition have an important role in producing the bacterial aspartic protease.

The pepstatin A concentration in our study was one thousand fold higher. While the similar concentration pepstatin (0,5 mM and 1 mM) could completely inhibit aspartic protease activity (Yin *et al.*, 2013). The concentration of pepstatin A applied was only 1 μ M to completely inhibit aspartic protease activity (Hsiao *et al.*, 2014). *L. casei* WSP aspartic protease is neither serine, cysteine nor metalloprotease. According to the data obtained, it could be considered to be an aspartic protease. Furthermore, there are other inhibitors that could be used to conclude whether the protease is a type of aspartic, that is diazoacetyl-noleucinemethyl ester and 1,2-epoxy-3-(p-nitrophenoxy) propane. Each of those reacted specifically with the side-chain carboxyl of two aspartic acid residue of the catalytic site (Szcecsi, 1992).

Hydrolysis of skimmed milk by *L. protease plantarum* S31 produced new two bioactive peptides with the molecular weight of 16 kDa and 21 kDa (Budiarto *et al.*, 2016). The profile of a new bioactive peptide will be different if the substrate and the enzyme that was used is also different. Hydrolysis of horse milk casein with chymosin produced three bioactive peptides (Egito *et al.*, 2001), while, human milk hydrolysate obtained one unique bioactive peptide from hydrolysis by the human gastrointestinal enzyme (Inglingstad *et al.*, 2010).

Currently, the multifunctional properties of biologically active milk peptide have been increasingly acknowledged (Mohanty *et al.*, 2016). It could show a positive impact on human physiology and metabolism either, directly or through enzymatic hydrolysis *in vivo* or *in vitro* (Kitts & Weiler, 2003). The antibacterial activity can be increased if the hydrolysate is purified and characterized. EPEC K11 was pathogenic bacteria as food contaminant and the causal of diarrheal diseases. This result was similar to hydrolysate skimmed milk with antibacterial activity toward *E. coli* and *L. monocytogenes* (Budiarto *et al.*, 2016). Another result has proven that hydrolysate of goat milk by protease *Bacillus sp.* E.13 exhibited antibacterial activity toward *S. aureus*, *L.*

monocytogenes, *E. coli* and *S. typhimurium* (Kusumaningtyas *et al.*, 2015). The antibacterial activity of bioactive peptide correlated with the net positive charge of the peptides. The cationic peptides kill sensitive bacteria by increasing the outer cell membrane permeability and resulting in the release of lipopolysaccharide (LPS) (Meisel & Bockelmann, 1999; Jenssen & Hancock, 2009).

Nowadays, there is an increasing interest in the use of natural antioxidant such as bioactive peptide for food preservation. It may occur because of this natural antioxidant help to avoiding the undesired health problems that may arise from the use of synthetic antioxidants which have a toxic effect (Romano *et al.*, 2009).

The antioxidant effect could be increased by adding the treatment dose. Moreover, further fractionation and characterization of hydrolysate was needed to elucidate which peptides that have high antioxidant activity. Skimmed milk hydrolysis by another enzyme such as papain also showed the antioxidant activity (Ha *et al.*, 2015). Furthermore, goat milk hydrolysis by protease *Bacillus sp.* E.13 also showed the antioxidant activity of scavenging DPPH (Kusumaningtyas *et al.*, 2015). The mechanism of a peptide to scavenging DPPH would be responsible for inhibiting electron migration and hydrogen atom transfer (Zou *et al.*, 2016). The antioxidant activity could be increased by disruption of its tertiary structure to increase the solvent accessibility of amino acid residues that could donate protons for free radicals (Ahmed *et al.*, 2015). According to this data, aspartic protease from *L. casei* WSP has a potential to produce bioactive peptides from skimmed milk with antibacterial and antioxidant property.

Conclusion

A partial purified aspartic protease was obtained from 24 h culture of *Lactobacillus casei* WSP and used as a proteolytic enzyme to generate bioactive peptides. The purified enzyme has molecular weight approximately of 37 kDa estimated by the SDS-PAGE assay. Furthermore, it showed the optimum activity at pH 6 and temperature of 40°C. The purified enzyme was an aspartic protease based on enzyme inhibition test in the presence of

Pepstatin A. Enzyme activity tended to be enhanced by the presence of DDT. Meanwhile, cofactor Mg²⁺ and Ca²⁺, inhibitor PMSF, Iodoacetic acid, EDTA and SDS tend to decrease enzyme activity insignificantly. Moreover, skimmed milk hydrolysate produced by aspartic protease *Lactobacillus casei* WSP showed the antibacterial activity toward *EPEC* K11 and DPPH free-radical scavenging activity.

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