

## Production of $\alpha$ -glucosidase inhibitory peptides during milk fermentation using indigenous lactic acid bacteria *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13

<sup>1,2</sup>Miftakhussolikhhah, <sup>1</sup>Utami, T., <sup>3</sup>Lisdiyanti, P. and <sup>1,\*</sup>Rahayu, E.R.

<sup>1</sup>Faculty of Agricultural Technology, Gadjah Mada University, Jl. Flora Bulaksumur no.1, Kocoran, Caturtunggal, Yogyakarta, Indonesia 55281

<sup>2</sup>Research Center for Food Technology and Processing (PRTTP), National Research and Innovation Agency (BRIN), Jl Jogja-Wonosari km 31,5 Gading, Playen, Gunungkidul, Yogyakarta, Indonesia 55861

<sup>3</sup>Research Center for Biosystematics and Evolution, National Research and Innovation Agency (BRIN), Jalan Raya Jakarta-Bogor Km. 46 Cibinong, Indonesia 16911

### Article history:

Received: 9 January 2024

Received in revised form: 11 August 2024

Accepted: 9 November 2024

Available Online: 9 December 2025

### Keywords:

$\alpha$ -glucosidase inhibitor,  
Bioactive peptides,  
Fermented milk,  
Lactic acid bacteria,  
*Lactiplantibacillus plantarum*  
Dad-13

### DOI:

[https://doi.org/10.26656/fr.2017.9\(6\).014](https://doi.org/10.26656/fr.2017.9(6).014)

### Abstract

$\alpha$ -glucosidase inhibitor (AGI) peptides from various food sources have been widely studied. This study aimed to determine the optimal incubation period for producing peptides with high AGI activity in fermented milk commonly used as a protein source. After sterilization, milk was inoculated with *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and incubated for 6, 12, 14, 16, 18, and 24 h. Various parameters such as proteolytic activity, peptide content, degree of hydrolysis, protein pattern, and  $\alpha$ -glucosidase inhibition were studied during incubation. Milk fermentation caused hydrolysis of milk proteins, resulting in more  $\gamma$ -casein and small molecular mass peptides monitored by SDS pages. The highest protein hydrolysis occurred after 24 h of fermentation, as observed from the degree of hydrolysis (24.05%) and peptide content (1.53 mg/mL). The highest AGI activity was obtained after 18 h of milk fermentation (36.12%). Milk fermentation using *L. plantarum* Dad-13 produced potential AGI peptide. Peptide fractions with a molecular mass of >2 kDa had high  $\alpha$ -glucosidase inhibitory activity in fermented milk. Milk fermentation using *L. plantarum* Dad-13 produced AGI peptides that have the potential to be further developed as antidiabetic functional foods.

## 1. Introduction

According to the World Health Organization (2024), diabetes affects around 422 million people globally, with the majority of them living in low- and middle-income countries, and diabetes causes 1.5 million deaths annually. The high number of diabetics in the world encourages research on functional foods for diabetics, such as foods with  $\alpha$ -glucosidase inhibitor activity (AGI).  $\alpha$ -glucosidase inhibitors have been used as a proven therapy for diabetic patients. Glucosidase inhibitors inhibit the enzyme in the brush border of the small intestine, effectively inhibiting glucose absorption and preventing an increase in postprandial blood glucose levels (Yin *et al.*, 2014).

According to Di Stefano *et al.* (2018), phenolic compounds, peptides, non-starch polysaccharides, and triterpenoids have a role in glucosidase inhibitory activity. Several peptides from various sources are also known to have AGI activity, including egg white protein

(Yu *et al.*, 2011), albumin (Yu *et al.*, 2012; Sa'adah and Muhtadi, 2022), whey protein hydrolysate (Konrad *et al.*, 2014; Baba *et al.*, 2021), flax (Ren *et al.*, 2016), silkworm cocoon (Zhang *et al.*, 2016), soybean (Rai *et al.*, 2017; Ha *et al.*, 2019; Li *et al.*, 2023), quinoa (Vilcacundo *et al.*, 2017) and oat globulin (Wang *et al.*, 2018). According to Ibrahim (2017), alpha-glucosidase inhibitor action is held by peptides containing 3-6 amino acid residues consisting of serine, threonine, tyrosine, lysine, or arginine at the N terminal, proline residues near the C terminal, and methionine or alanine at the C-terminal position. Hydrophobic amino acids in peptides, particularly Pro and Leu, significantly contribute to  $\alpha$ -glucosidase inhibitor action (Ren *et al.*, 2016). Milk includes proline, leucine, and hydrophobic amino acids (Payne-Botha and Bigwood, 1959), all of which have the potential to form AGI peptides.

The proteolytic activity of lactic acid bacteria (LAB) during fermentation is considered capable of producing

\*Corresponding author.

Email: [endangsrhayu@ugm.ac.id](mailto:endangsrhayu@ugm.ac.id)

peptides with AGI capability. According to de Valdez *et al.* (1985), the proteolytic ability of LAB during fermentation is influenced by fermentation conditions, including substrate, temperature, and fermentation time. In this study, *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 was used to ferment milk. *L. plantarum* Dad-13 is an indigenous lactic acid bacteria isolated from dadih, a traditional fermented buffalo milk from West Sumatra, Indonesia (Rahayu *et al.*, 2022). This bacterium is known to have the ability as probiotics and other functional properties. This study studied the effect of fermentation time on  $\alpha$ -glucosidase inhibitor of fermented milk.

## 2. Materials and methods

### 2.1 Materials

The LAB strain *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 used in this study was from Gadjah Mada University's Center for Food and Nutrition Studies and was cultured in a broth culture medium called De Man-Rogosa-Sharpe (MRS) (Merck). Cow skim milk (Lactona) was purchased at the local supermarket. This investigation used analytical grade chemicals, including  $\alpha$ -glucosidase (AG) from *Saccharomyces cerevisiae* (type I, freeze-dried powder  $\geq 10$  units/mg protein) from Sigma Aldrich (Merck, Germany) and o-phthaldialdehyde (OPA) from Merck (USA).

### 2.2 Culture preparation

The preparation stage was conducted before the trial to guarantee that the lactic acid bacteria isolate was available and uniform throughout the study. The primary material for stock culture was a pure isolate of the lactic acid bacterium strain *L. plantarum* subsp. *plantarum* Dad-13. The preparation step was carried out according to the method of Chen *et al.* (2014), which involved producing a pure culture of lactic acid bacteria on MRS (de Man, Rogosa, and Sharpe-Merck) broth medium at 37°C for 18 h.

### 2.3 Fermented milk and protein extract preparation

Lactic acid bacteria starter was prepared by inoculating bacterial culture into 10% (m/v) skim milk and incubating it at 37°C for 24 h before reculturing into 10% (m/v) skim milk to produce culture starter. The culture starter was then utilized to make fermented milk by culturing it in 12% (m/v) skim milk and incubating for 0, 6, 12, 14, 16, 18, and 24 h at 37°C. Each fermented milk sample was centrifuged twice at 4000 $\times$ g for 15 min for protein extract (Liu *et al.*, 2016).

### 2.4 Determination of proximate content

The total protein, fat, moisture, and ash contents of

skim milk were determined using standard AOAC INTERNATIONAL methods (AOAC INTERNATIONAL, 2005). Moisture content was measured gravimetrically by drying the sample at 105°C to a constant weight, following AOAC Official Method 925.10. Ash content was determined by weighing the sample after incineration in a muffle furnace at 550°C for 6 h, in accordance with AOAC Official Method 945.46. The total nitrogen content was measured using the Kjeldahl method (AOAC Official Method 991.20), and the protein content was calculated by multiplying the nitrogen value by a conversion factor of 6.38. Carbohydrate content was calculated by difference, subtracting the sum of protein, fat, moisture, and ash from 100%, based on AOAC Official Method 986.25 (Menezes *et al.*, 2004).

### 2.5 Determination of amino acid composition

The amino acid composition was determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on the Water Xevo TQD apparatus (Waters, USA), following the AOAC Official Method 2011.04 with minor modifications. Samples were hydrolyzed with 6 mol/L HCl, cooked in an autoclave at 110°C for 12 h, neutralized with 6 mol/L NaOH, and filtered through a syringe filter (0.22  $\mu$ m pore size). The filtrate was then diluted with distilled water (1:50 v/v). The sample injection volume was 2  $\mu$ L. Elution lasted for 6 min using a gradient of mobile phase A (0.1% pentadecafluorooctanoic acid: 0.1% formic acid in water/acetonitrile at a ratio of 99.5:0.5) and mobile phase B (0.1% pentadecafluorooctanoic acid: 0.1% formic acid in water/acetonitrile at a ratio of 1:9). Elution was performed at 50°C with a flow rate of 0.6 mL/min, 3.5 kV capillary and 15 V collision energy. Positive electron ionization mode was used.

### 2.6 Calculation of total lactic acid bacteria (enumeration) and pH value determination

The population of lactic acid bacteria during milk fermentation was determined by diluting and then plating a pouring plate with MRS media (Rahayu and Margino, 1997). Samples were diluted in various dilution series before being poured into MRS agar substrate containing 0.2% CaCO<sub>3</sub>. After an 18-hour incubation at 37°C, colonies with clear zones on Petri dishes for each dilution series were enumerated and quantified in CFU/mL. The pH was measured using a pH meter.

### 2.7 Proteolytic activity

The culture's proteolytic activity was assessed using Walter's (1984) method, which involved creating a supernatant solution of centrifuged fermented milk inoculated with proteolytic LAB and incubating for 0, 6,

12, 14, 16, 18, and 24 h. A total of 0.5 mL of supernatant (sample), 0.5 mL of distilled water (blank), or 0.5 mL of 0.5 mmol/L tyrosine solution (standard) was added to 1 mL of 2% casein and 1 mL of 0.2 M Tris-HCl buffer pH 8. All treatments were incubated for 10 min at 50°C. After that, 2 mL of 10% TCA and 0.5 mL of distilled water were added to the sample, along with 0.5 mL of supernatant solution for the blank and standard. The solution was incubated at 37°C for 10 min and centrifuged at 10,000 rpm for 10 min at room temperature. Next, 1.5 mL of supernatant was added to 5 mL of 0.4 M sodium carbonate and 1 mL of Folin's solution (1:2). The solution was incubated for 20 min at 37°C, and the absorbance was measured at 578 nm. Proteolytic activity is expressed in units (U), equivalent to the quantity of enzyme required to produce one micromole of tyrosine per minute.

### 2.8 Determination of $\alpha$ -glucosidase inhibition

The inhibition of enzyme activity was conducted in line with Zeng *et al.* (2016) with some modifications. This method relies on the reaction of a substrate with an enzyme to produce a colored product. Acarbose, enzyme, and p-Nitrophenyl- $\alpha$ -D-glucopyranoside reagents were prepared using 0.01M phosphate buffer saline (pH 6.8). The reaction mixture included 25  $\mu$ L 10mM p-Nitrophenyl- $\alpha$ -D-glucopyranoside, 25  $\mu$ L 0.01M phosphate buffer saline pH 6.8, and 25  $\mu$ L 1% acarbose or sample. After that, it was incubated for 10 min at 37 degrees Celsius. After adding 50  $\mu$ L  $\alpha$ -glucosidase 1 U/mL, incubate for 30 min at 37°C. The reaction was stopped by adding 100  $\mu$ L of 0.1 M sodium carbonate solution. The absorbance was measured using a spectrophotometer at 405 nm. The experiment included a blank solution with no sample or enzyme, a positive control with  $\alpha$ -glucosidase enzyme, and a negative control with 225  $\mu$ L of phosphate-buffered saline. The inhibition of  $\alpha$ -glucosidase enzyme activity was calculated using the formula:

$$\text{Inhibition (\%)} = \left( 1 - \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of positive control} - \text{absorbance of negative control}} \right) \times 100\%$$

### 2.9 Degree of hydrolysis

Ramachandran and Shah (2008) assessed the degree of hydrolysis using free amino acids from fermented milk. Filtrate (150  $\mu$ L) was vortexed with 3 mL of OPA (o-phthaldialdehyde) for 5 min, and absorbance was measured at 340 nm using a spectrophotometer. The degree of hydrolysis was calculated by comparing the data from control milk (fermentation time 0) to milk fermented after 6, 12, 14, 16, 18, and 24 h.

### 2.10 Protein pattern

According to Laemmli's (1970) method, protein

patterns were examined using SDS-PAGE, with 5% and 13% separating gels. Peptide extracts were diluted in SDS sample buffer [0.5 M Tris-HCl pH 6.8, 87% glycerol (b/v), 10% SDS (b/v), 0.5% bromophenol blue (b/v), and distilled water] in a 1:2 ratio. The sample was heated at 100°C for 4 min. Each well received either 20  $\mu$ L of material or 5  $\mu$ L of standard protein markers. The gel was operated at 220 volts for around 60 min. The gel was steeped in distilled water for 30 min before being stained with 0.2% Coomassie Brilliant Blue R-250 (a solution of 50% methanol, 10% acetic acid, and 40% distilled water). The gel was immersed in the staining solution for 30 min and then changed three times.

### 2.11 Peptides fractionation using dialysis tubing

Dialysis membranes with 2 and 14 kDa cut-offs were used to dialyze fermentation products, as described by Pohl (1990). The dialysis process begins with activating the dialysis membrane with a 10 mM NaHCO<sub>3</sub> solution. The NaHCO<sub>3</sub> solution was heated to a boil before immersing the dialysis bag for 10 min. The dialysis bag was then cleansed with sterile distilled water and prepared for use. Approximately 10 mL of fermented milk was placed in the active dialysis bag. Both ends of the dialysis bag were knotted with twine. The bag was immersed in a pH 7 phosphate buffer solution (20 mM). The dialysis process was carried out for one night at 4°C. AGI activity was tested on freeze-dried samples from both inside (>bag size) and outside (<bag size).

### 2.12 Statistical analysis

All samples were produced in three batches. All analytical data were analyzed by one-way analysis of variance (ANOVA) using SPSS 27.0 software (IBM SPSS Statistics 27). All assays were determined in 3 replicates. Significant treatment means were separated by Duncan's multiple comparison test at the 0.05 significance level.

## 3. Results and discussion

### 3.1 Proximate and amino acid composition of milk

According to the data in Table 1, the proximate composition of the milk used meets the requirements of the Codex Alimentarius standard (Codex Alimentarius Commission, 2011) and the Indonesian National Standard (Badan Standardisasi Nasional, 2006) for skim milk moisture, protein, and fat content. Skim milk was used in this study because it contains more protein required to produce more peptides with AGI capabilities. Peptides derived from cow, goat, sheep, buffalo, and camel milk have multifunctional properties, including anti-microbial, immune modulators, anti-oxidants, enzyme inhibitory effects, anti-thrombotic, and

Table 1. Proximate composition of skimmed milk.

	Milk sample	Codex Alimentarius Standard	Indonesian National Standard
Moisture (%db)	4.04±0.37	<5	<5
Ash (%db)	6.63±0.04		
Protein (%db)	34.48±0.59	>34	>30
Fat (%db)	0.21±0.04	<1.5	<1.5
Carbohydrate by difference (%db)	54.64±0.93		

antagonistic activities against various toxic agents. Some regulate immunological, gastrointestinal, hormonal, and neurological responses, thus playing an essential role in preventing cancer, osteoporosis, hypertension, hyperglycemia, and other disorders (Mohanty *et al.*, 2016).

According to Ren *et al.* (2016) and Ibrahim (2017), AGI includes peptides with 3-6 amino acid residues consisting of serine, threonine, tyrosine, lysine or arginine at the N terminal, proline residues close to the C terminal, and methionine or alanine at the C terminal position. Ren *et al.* (2016) found that hydrophobic amino acids in peptides, particularly proline and leucine, significantly enhance the efficacy of  $\alpha$ -glucosidase inhibitors. Tyrosine, proline, and leucine are essential components for AGI inhibition. Meanwhile, according to Sartorius *et al.* (2019), isoleucine, leucine, lysine, threonine, and valine, the primary amino acids of milk protein, play a role in AGI activity. Based on Table 2, skim milk has potential as AGI because it contains high amounts of amino acids that play a role in AGI activity, such as isoleucine, leucine, tyrosine, proline, threonine, and valine. Moreover, milk must have proper molecular weight, sequence, and amino acid residues to function as a bioactive peptide, especially as an  $\alpha$ -glucosidase inhibitor. These peptides can be generated during protein hydrolysis or fermentation (Zhao *et al.*, 2022) in the process of making fermented milk.

### 3.2 Total lactic acid bacteria, pH and proteolytic activity value of fermented milk in various fermentation times.

The results in Table 3 showed that the total lactic acid bacteria increased as the fermentation duration of fermented milk increased. In contrast, as fermentation time rose, the pH decreased due to lactic acid production. Lactic acid bacteria are gram-positive bacteria that create lactic acid as the primary by-product of carbohydrate fermentation (George *et al.*, 2018). The pH of fermented milk decreased as the quantity of lactic acid bacteria increased and fermentation time increased because more lactic acid was produced.

Based on Table 3, proteolytic activity increased with increasing fermentation time but decreased after 18 h of fermentation. According to De Valdez *et al.* (1985), lactic acid bacteria fermentation is influenced by

Table 2. Amino acid composition of skimmed milk.

Amino acid	Value (ppm)
glutamic acid	26.55
aspartic acid	11.35
isoleucine	11.29
serine	8.21
leucine	7.71
arginin	6.32
phenylalanine	6.20
threonine	5.55
histidine	4.70
valine	3.66
glycine	2.54
proline	1.63
tyrosine	1.45
alanine	1.15
cysteine	0.96
methioninen	0.50
thriptophan	0.12
lysine	0.11
Total	100.00
HOAA	35.76
HIAA	64.24
AAA	7.77
PCAA	11.13
NCAA	37.90

HOAA: total hydrophobic amino acids (alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, cysteine, thriptophan), HIAA: total hydrophilic amino acids (aspartic acid, glutamic acid, arginine, lycine, histidine, serine, threonine, tyrosine), AAA: total aromatic amino acids (phenylalanine, tyrosine, thriptophan), PCAA: total positively charged amino acids (arginine, histidine, lysine), NCAA: total negatively charged amino acids (glutamic acid and aspartic acid).

fermentation temperature and time, which is related to their proteolytic ability. Lactic acid bacteria have proteolytic ability to produce essential amino acids for their growth (Savijoki *et al.*, 2006). Lactic acid bacteria require high essential growth factors, such as peptides and amino acids. However, milk does not contain enough free amino acids and peptides to enable LAB growth. Therefore, LAB has complex proteinase and peptidase systems that allow it to use milk casein as a source of amino acids and nitrogen. The casein degradation stage is initiated by proteases in the cell wall, breaking casein

into oligopeptides. Further degradation into smaller peptides and amino acids that can cross the cell membrane is performed by peptidases (Kirilov *et al.*, 2009). The proteolytic activity of lactic acid bacteria increased when milk fermentation began, then decreased at the end of incubation due to reduced proteinase/peptidase enzyme activity, due to damage from the enzyme activity itself and the acidity of the fermented milk.

Table 3. Total lactic acid bacteria, pH and proteolytic activity value in various fermentation time.

Fermentation time (h)	<i>L. plantarum</i> Dad-13		
	Total LAB (CFU/mL)	pH	Proteolytic activity (unit/g protein)
0	6.01	6.80	0.22±0.01 <sup>b</sup>
6	6.72	5.97	0.19±0.01 <sup>a</sup>
12	8.84	5.78	0.26±0.02 <sup>c</sup>
14	8.17	5.80	0.33±0.01 <sup>c</sup>
16	8.12	5.68	0.31±0.01 <sup>d</sup>
18	8.78	5.68	0.39±0.01 <sup>f</sup>
24	8.57	4.91	0.21±0.01 <sup>b</sup>

Proteolytic activity values are presented as mean±SD (n = 3). Values with different superscripts in the same column are statistically significantly different (P<0.05).

### 3.3 Total peptide, degree of hydrolysis and alpha-glucose inhibitory activity of fermented milk in various fermentation times.

From Figure 1, the results indicated that the total peptides and degree of hydrolysis increased as the fermentation time of fermented milk increased. This phenomenon occurs due to the increased production of peptides due to extended fermentation duration. Total peptides and degree of hydrolysis of 18-hour fermented milk (1.3132 mg/mL and 20.24%) were lower than those of 24-hour fermented milk (1.5319 mg/mL and 24.05%). LAB uses milk protein as the primary source of essential amino acids and growth stimulants. As many milk starter cultures are highly proteolytic, bioactive peptides can be produced by both starter and non-starter bacteria used to manufacture fermented milk products. As the proteolytic system of LAB is very complex, there is some controversy regarding its ability to produce biopeptides. These peptides should be made after peptidase hydrolysis of long oligopeptides that are initially liberated through proteinase activity during milk fermentation. Since peptidase activities are intracellular in LAB, they may contribute only after cell lysis, a rare event in fermented milk due to the short fermentation time (Atanasova *et al.*, 2014).

In fermented milk, the proteolytic system of LAB

plays a vital role because it allows bacteria to grow in milk and makes milk fermentable. The proteolytic ability affects the flavor and texture characteristics of products containing LAB and produces several bioactive peptides that benefit health (Ramachandran and Shah, 2008). AGI activity increased with increasing fermentation time up to 12 h but decreased at 14 and 16 h, then reached the highest value at 18 h and lowered again after 24 h. AGI activity values decreased at 14 h of fermentation and slowly increased again at 16 h of fermentation. It is possible that 20-11 peptides with relatively high inhibitory activity (Ibrahim *et al.*, 2017; Wang *et al.*, 2018) were formed during this 12-hour fermentation. Then, peptides with smaller sizes were formed and fermented for 14 and 16 h. The size of these peptides may not be suitable to inhibit  $\alpha$ -glucosidase activity.

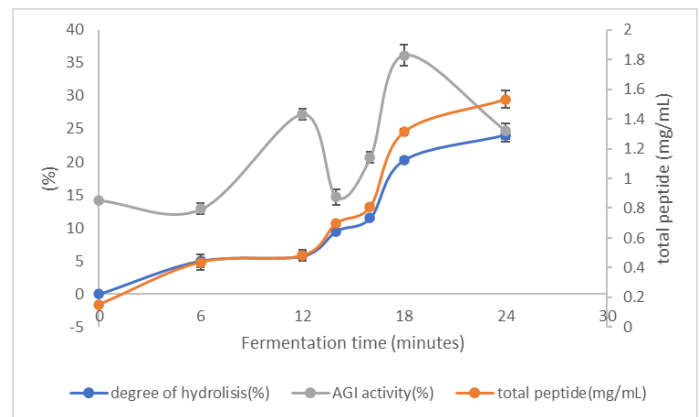


Figure 1. Total peptide (mg/mL), degree of hydrolysis (%) and AGI activity (%) of fermented milk in various fermentation time.

Hydrolysis caused tripeptides and other peptides compatible with AGI activity to form at the fermentation time of 18 h. The size of these peptides followed the AGI activity (Konrad *et al.*, 2014; Zhang *et al.*, 2016; Ibrahim *et al.*, 2017). At 24 h of fermentation, excess hydrolysis led to the formation of smaller sizes that did not match the AGI activity. Peptides with 3-6 amino acids (Yu *et al.*, 2012; Ren *et al.*, 2016; Zhang *et al.*, 2016; Ibrahim, 2017) or peptides with a molecular mass of 3-10 kDa (Konrad *et al.*, 2014) are peptides that have an essential role as AGI. When the fermentation time reaches 18 h, the peptides in fermented milk reach a suitable size to act as AGI. The AGI activity values of all fermented milk were still below the activity value of 5 mg/mL acarbose (50.56%). Acarbose is a drug commonly used to treat type 2 diabetes that acts as a competitive, reversible inhibitor of membrane-bound intestinal  $\alpha$ -glucosidase hydrolase (Ziaee *et al.*, 2017). By delaying the digestion of carbohydrates, acarbose slows glucose absorption, reducing postprandial blood glucose concentrations.

### 3.4 Protein pattern

The protein pattern during milk fermentation was

presented in Figure 2. The protein bands with a molecular weight (MW) of 10, 14, 18, 26-34 kDa were present at all stages of fermentation. At the beginning of the fermentation, bands were seen at 150 kDa and 70 kDa, but after 18 h bands were seen at 34 kDa and below. This occurs because there are peptides with various molecular weights. Peptides with high molecular weights have been degraded into peptides with lower molecular weights during milk fermentation. Based on Bonczar *et al.* (2016), protein with a molecular weight of 150 kDa is immunoglobulin, around 66 kDa is serum albumin, 26-30 kDa is  $\alpha$ -casein, 18 kDa is  $\beta$ -lactoglobulin, 14 kDa is  $\alpha$ -lactalbumin, and <12 kDa is a peptide with a small molecular mass and  $\gamma$ -casein. These results are in accordance with Bonczar *et al.* (2016) statement that the levels of small molecular peptides and  $\gamma$ -casein are higher in fermented milk than in raw milk. It is known that peptides with AGI capability are peptides with molecular weight 3-10 kDa (Konrad *et al.*, 2014), so the statement was in line with the result in this study. Fermented milk that has more small-molecule peptides has the ability to act as an  $\alpha$ -glucosidase inhibitor.

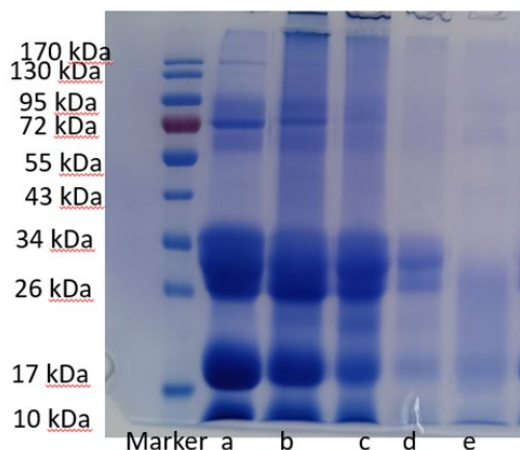


Figure 2. SDS PAGE protein pattern of fermented milk using *L. plantarum* Dad-13 for (a) 0 h, (b) 6 h, (c) 12, (d) 18 h and (e) 24 h fermentation time.

### 3.5 AGI activity at various molecular weight peptides

Based on Figure 3, the peptide fraction with a molecular weight of more than 14 kDa has the highest AGI activity compared to other peptide fractions. The results showed that AGI activity in peptide fractions of 2-14 kDa and >14 kDa did not show significant differences in the Duncan comparison test with a 95% confidence level. This result was not in line with Ibrahim *et al.* (2017) and Hu *et al.* (2023), which stated that peptide fractions with molecular masses <2 kDa have the highest AGI activity, as well as Wang *et al.* (2018) which stated that small peptides with molecular masses between 180 and 500 Da, which is about 2-5 amino acids have high AGI activity. The results of this study were in line with Konrad *et al.* (2014), who explained that AGI

activity is possessed by peptide fractions with molecular masses of 3-10 kDa. Thus, it can be concluded that peptide fractions with a molecular mass of >2 kDa have high  $\alpha$ -glucosidase inhibitory activity in fermented milk.

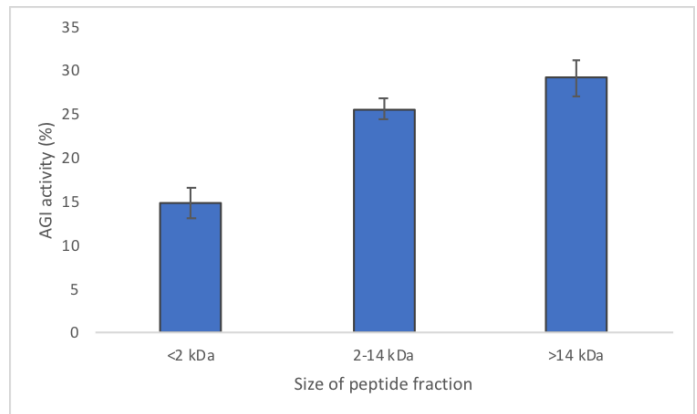


Figure 3. AGI activity at different sizes of peptide fraction.

## 4. Conclusion

The ability of AGI comes from its proteolytic activity. AGI activity increases as fermentation time increases until it reaches a certain point. Milk fermentation using indigenous lactic acid bacteria *L. plantarum* Dad-13 produced potential AGI peptides. The highest AGI activity occurred after 18 h of incubation. Peptide fractions with molecular mass of >2 kDa have high  $\alpha$  glucosidase inhibitory.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgments

The author would like to thank the SDM IPTEK scholarship, Ministry of Research and Technology, Republic of Indonesia, for providing research funding.

## References

- AOAC INTERNATIONAL. (2005). Moisture in solids and flour (AOAC Official Method 925.10). In Official Methods of Analysis of AOAC INTERNATIONAL, 20th ed. Gaithersburg, USA: AOAC INTERNATIONAL.
- AOAC INTERNATIONAL. (2005). Ash of milk (AOAC Official Method 945.46). In Official Methods of Analysis of AOAC INTERNATIONAL, 20th ed. Gaithersburg, USA: AOAC INTERNATIONAL.
- AOAC INTERNATIONAL. (2005). Nitrogen (Total) in milk: Kjeldahl method (AOAC Official Method 991.20). In Official Methods of Analysis of AOAC INTERNATIONAL, 20th ed. Gaithersburg, USA:

- AOAC INTERNATIONAL.
- AOAC INTERNATIONAL. (2005). Proximate Analysis of Milk-Based Infant Formula (AOAC Official Method 986.25). In Official Methods of Analysis of AOAC INTERNATIONAL, 20th ed. Gaithersburg, USA: AOAC INTERNATIONAL.
- AOAC INTERNATIONAL. (2005). Protein in Raw and Processed Meats (Automated Dye-Binding Method) (AOAC Official Method 2011.04). In Official Methods of Analysis of AOAC INTERNATIONAL, 20th ed. Gaithersburg, USA: AOAC INTERNATIONAL.
- Atanasova, J., Moncheva, P. and Ivanova, I. (2014). Proteolytic and antimicrobial activity of lactic acid bacteria grown in goat milk. *Biotechnology and Biotechnological Equipment*, 28(6), 1073-1078. <https://doi.org/10.1080/13102818.2014.971487>
- Baba, W.N., Mudgil, P., Kamal, H., Kilari, B.P., Gan, C.Y. and Maqsood, S. (2021). Identification and characterization of novel  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory peptides from camel whey proteins. *Journal of Dairy Science*, 104 (2), 1364-1377. <https://doi.org/10.3168/jds.2020-19271>
- Badan Standardisasi Nasional. (2006). Susu Bubuk (SNI 01-2970-2006). Jakarta, Indonesia: Badan Standardisasi Nasional. [In Bahasa Indonesia].
- Bonczar, G., Walczykca, M. and Duda, I. (2016). The changes of proteins fractions shares in milk and fermented milk drinks. *Acta Scientiarum Polonorum Technologia Alimentaria*, 15(4), 379-389. <https://doi.org/10.17306/J.AFS.2016.4.36>
- Payne-Botha, S. and Bigwood, E.J. (1959). Amino-acid content of raw and heat-sterilized cow's milk. *British Journal of Nutrition*, 13(4), 385-389. <https://doi.org/10.1079/BJN19590052>
- Chen, P., Zhang, Q., Dang, H., Liu, X., Tian, F., Zhao, J., Chen, Y., Zhang, H. and Chen, W. (2014). Screening for potential new probiotic based on probiotic properties and  $\alpha$ -glucosidase inhibitory activity. *Food Control*, 35(1), 65-72. <https://doi.org/10.1016/j.foodcont.2013.06.027>
- Codex Alimentarius Commission. (2011). Milk and Milk Products (CODEX STAN 207-1999). 2<sup>nd</sup> ed. Rome: Food and Agriculture Organization of the United Nations.
- De Valdez, G.F., De Giori, G.S., Holgado, A.P.D.R. and Oliver, G. (1985). Effect of the Rehydration Medium on the Recovery of Freeze-Dried Lactic Acid Bacteria. *Applied and Environmental Microbiology*, 50(5), 1339-1341. <https://doi.org/10.1128/aem.50.5.1339-1341.1985>
- Di Stefano, E., Oliviero, T. and Udenigwe, C.C. (2018). Functional significance and structure-activity relationship of food-derived  $\alpha$ -glucosidase inhibitors. *Current Opinion in Food Science*, 20, 7-12. <https://doi.org/10.1016/j.cofs.2018.02.008>
- George, F., Daniel, C., Thomas, M., Singer, E., Guilbaud, A., Tessier, F.J., Juneless, A.M.R., Borges, F. and Foligne, B. (2018). Occurrence and dynamism of lactic acid bacteria in distinct ecological niches: a multifaceted functional health perspective. *Frontiers in Microbiology*, 9, 2899. <https://doi.org/10.3389/fmicb.2018.02899>
- Ha, T.J., Park, J.E., Kang, B.K., Kim, H.S., Shin, S.O., Seo, J.H., Oh, E., Kim, S. and Kwak, D. (2019).  $\alpha$ -Glucosidase Inhibitory Activity of Isoflavones and Saponins from Soybean (*Glycine max* L.) and Comparisons of Their Constituents during Heat Treatments. *Journal of the Korean Society of Food Science and Nutrition*, 48(9), 953-960. <https://doi.org/10.3746/jkfn.2019.48.9.953>
- Hu, J., Lai, X., Wu, X., Wang, H., Weng, N., Lu, J., Lyu, M. and Wang, S. (2023). Isolation of a novel anti-diabetic  $\alpha$ -glucosidase oligo-peptide inhibitor from fermented rice bran. *Foods*, 12(1), 183. <https://doi.org/10.3390/foods12010183>
- Ibrahim, M.A., Bester, M.J., Neitz, A.W.H. and Gaspar, A.R.M. (2017). Structural properties of bioactive peptides with  $\alpha$ -glucosidase inhibitory activity. *Chemical Biology and Drug Design*, 91(2), 370-379. <https://doi.org/10.1111/cbdd.13105>
- Kirilov, N., Petkova, T., Atanasova, J., Danova, S.V., Iliev, I., Popov, Y., Haertle, Y. and Ivanova, I.V. (2009). Proteolytic activity in lactic acid bacteria from Iraq, Armenia and Bulgaria. *Biotechnology and Biotechnological Equipment*, 23(Sup1: XI Aniversary Scientific Conference), 643-646. <https://doi.org/10.1080/13102818.2009.10818506>
- Konrad, B., Anna, D.B., Marek, S., Marta, P., Aleksandra, Z. and Jo'zefa, C. (2014). The evaluation of dipeptidyl peptidase (DPP)-IV,  $\alpha$ -glucosidase and angiotensin converting enzyme (ACE) inhibitory activities of whey proteins hydrolyzed with serine protease isolated from Asian pumpkin (*Cucurbita ficifolia*). *International Journal of Peptide Research and Therapeutic*, 20, 483-491. <https://doi.org/10.1007/s10989-014-9413-0>
- Li, W., Fu, X., Zhang, T., Li, H., Chen, T. and Liu, X. (2023). Isolation and identification of an  $\alpha$ -glucosidase inhibitory peptide from extruded soybean protein and its hypoglycemic activity in T2DM mice. *Food and Function*, 14(9), 4288-4301. <https://doi.org/10.1039/d3fo00580a>
- Liu, B., Kongstad, K.T., Wiese, S., Jäger, A.K. and Staerk, D. (2016). Edible seaweed as future

- functional food: Identification of  $\alpha$ -glucosidase inhibitors by combined use of high-resolution  $\alpha$ -glucosidase inhibition profiling and HPLC-HRMS-SPE-NMR. *Food Chemistry*, 203, 16-22. <https://doi.org/10.1016/j.foodchem.2016.02.001>
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685. <https://doi.org/10.1038/227680a0>
- Mohanty, D.P., Mohapatra, S., Misra, S. and Sahu, P.S. (2016). Milk derived bioactive peptides and their impact on human health - a review. *Saudi Journal of Biological Sciences*, 23(5), 577-583. <https://doi.org/10.1016/j.sjbs.2015.06.005>
- Paludetti, L.F., Jordan, K., Kelly, A.L. and Gleeson, D. (2018). Evaluating the effect of storage conditions on milk microbiological quality and composition. *Irish Journal of Agricultural and Food Research*, 57 (1), 52-62. <https://doi.org/10.1515/ijafr-2018-0006>
- Pohl, T. (1990). Concentration of Proteins and Removal of Solutes in Methods in Enzymology. Vol. 182. Cambridge, UK: Academic Press. [https://doi.org/10.1016/0076-6879\(90\)82009-q](https://doi.org/10.1016/0076-6879(90)82009-q)
- Rahayu, E.S., Aini, N.N., Mariyatun and Utami, T. (2021). Current Taxonomic Name of Indigenous Probiotic Strains, presented at the 6<sup>th</sup> International Conference of Indonesian Society for Lactic Acid Bacteria and Gut Microbiota. Retrieved from website: <https://probiotics.wg.ugm.ac.id/2021/10/29/current-taxonomic-name-of-indigenous-probiotic-strains/>
- Rahayu, E.S and Margino, S. (1997). Materi workshop: Bakteri Asam Laktat, Isolasi dan Identifikasi. Retrieved from website: [http://cfns.ugm.ac.id/wp-content/uploads/sites/861/2020/05/1997\\_Isolasi-dan-Identifikasi-Bakteri-Asam-Laktat\\_ESR2\\_compressed.pdf](http://cfns.ugm.ac.id/wp-content/uploads/sites/861/2020/05/1997_Isolasi-dan-Identifikasi-Bakteri-Asam-Laktat_ESR2_compressed.pdf) [In Bahasa Indonesia].
- Rai, A.K., Sanjukta, S., Chrasia, R., Bhat, I., Bhardwaj, P.K. and Sahoo, D. (2017). Production of bioactive hydrolysate using protease,  $\beta$ -glucosidase and  $\alpha$ -amylase of *Bacillus* spp. isolated from kinema. *Bioresource Technology*, 235, 358-365. <https://doi.org/10.1016/j.biortech.2017.03.139>
- Ramachandran, L. and Shah, N.P. (2008). Proteolytic Profiles and Angiotensin-I Converting Enzyme and  $\alpha$ -Glucosidase Inhibitory Activities of Selected Lactic Acid Bacteria. *Journal of Food Science*, 73(2), 75-81. <https://doi.org/10.1111/j.1750-3841.2007.00643.x>
- Ren, Y., Liang, K., Jin, Y., Zhang, M., Chen, Y., Wu, H. and Lai, F. (2016). Identification and characterization of two novel  $\alpha$ -glucosidase inhibitory oligopeptides from hemp (*Cannabis sativa* L.) seed protein. *Journal of Functional Foods*, 26, 439-450. <https://doi.org/10.1016/j.jff.2016.07.024>
- Sa'adah, A. and Muhtadi. (2022). Inhibitory Activity of the  $\alpha$ -Glucosidase Enzyme by Albumin Isolated from Giant Gourami (*Osphronemus Goramy*), Rice Eel (*Monopterus albus*), and Mackerel Tuna (*Euthynnus affinis*). Proceedings of the 4<sup>th</sup> International Conference Current Breakthrough in Pharmacy (ICB-Pharma 2022). USA: Atlantis Press. [https://doi.org/10.2991/978-94-6463-050-3\\_15](https://doi.org/10.2991/978-94-6463-050-3_15)
- Sartorius, T., Weidner, A., Dharsono, T., Boulier, A., Wilhelm, M. and Schön, C. (2019). Postprandial effects of a proprietary milk protein hydrolysate containing bioactive peptides in prediabetic subjects. *Nutrients*, 11, 1700. <https://doi.org/10.3390/nu11071700>
- Savijoki, K., Ingmer, H. and Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology*, 71, 394-406. <https://doi.org/10.1007/s00253-006-0427-1>
- Vilcacundo, R., Villaluenga, C.M. and Ledesma, B.H. (2017). Release of dipeptidyl peptidase IV,  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory peptides from quinoa (*Chenopodium quinoa* Willd.) during in vitro simulated gastrointestinal digestion. *Journal of Functional Foods*, 35, 531-539. <https://doi.org/10.1016/j.jff.2017.06.024>
- Walter, H.E. (1984). Proteinases. In Bergmeyer, H.U. and Grassl, M. (Eds.). *Methods of Enzymatic Analysis*, p. 271-276. Michigan, USA: Verlag Chemie. <https://doi.org/10.1016/B978-0-12-091304-6.X5001-0>
- Wang, F., Zhang, Y., Yua, T., Hea, J., Cuia, J., Wang, J., Cheng, X. and Fana, J. (2018). Oat globulin peptides regulate antidiabetic drug targets and glucose transporters in Caco-2 cells. *Journal of Functional Foods*, 42, 12-20. <https://doi.org/10.1016/j.jff.2017.12.061>
- World Health Organization (WHO). (2024). Diabetes. Retrieved on April 20, 2024 from WHO Website: [https://www.who.int/health-topics/diabetes#tab=tab\\_1](https://www.who.int/health-topics/diabetes#tab=tab_1)
- Yin, Z. Zhang, W. Feng, F. Zhang, Y. and Kang, W. (2014).  $\alpha$ -Glucosidase inhibitors isolated from medicinal plants. *Food Science and Human Wellness*, 3(3-4), 136-174. <https://doi.org/10.1016/j.fshw.2014.11.003>
- Yu, Z., Yin, Y., Zhao, W., Yu, Y., Liu, B., Liu, J. and Chen, F. (2011). Novel peptides derived from egg white protein inhibiting alpha-glucosidase. *Food*

- Chemistry*, 129(4), 1376-1382. <https://doi.org/10.1016/j.foodchem.2011.05.067>
- Yu, Z., Yin, Y., Zhao, W., Liu, J. and Chen, F. (2012). Anti-diabetic activity peptides from albumin against  $\alpha$ -glucosidase and  $\alpha$ -amylase. *Food Chemistry*, 135 (3), 2078-2085. <https://doi.org/10.1016/j.foodchem.2012.06.088>
- Zeng, Z., Luo, J., Zuo, F., Zhang, Y., Ma, H. and Chen, S. (2016). Screening for potential novel probiotic *Lactobacillus* strains based on high dipeptidyl peptidase IV and  $\alpha$ -glucosidase inhibitory activity. *Journal of Functional Foods*, 20, 486-495. <https://doi.org/10.1016/j.jff.2015.11.030>
- Zhang, Y., Wang, N., Wang, W., Wang, J., Zhua, Z. and Li, X. (2016). Molecular mechanisms of novel peptides from silkworm pupae that inhibit  $\alpha$ -glucosidase. *Peptides*, 76, 45-50. <https://doi.org/10.1016/j.peptides.2015.12.004>
- Zhao, Q., Wei, G., Li, K., Duan, S., Ye, R. and Huang, A. (2022). Identification and molecular docking of novel  $\alpha$ -glucosidase inhibitory peptides from hydrolysates of Binglangjiang buffalo casein. *Food Science and Technology*, 156, 113062. <https://doi.org/10.1016/j.lwt.2021.113062>
- Ziaee, A., Esmailzadehha, N. and Honardoost, M. (2017). Comparison of adjunctive therapy with metformin and acarbose in patients with Type-1 diabetes mellitus. *Pakistan Journal of Medical Sciences*, 33(3), 686-690. <https://doi.org/10.12669/2Fpjms.333.12669>