Antimicrobial activity assay of Plantaricin F produced by L. lactis pNZ8148-plnAF against Candida albicans

A Z Mustopa1, N Rasmentari1,2, Kusdianawati2, L Triratna1 and M Nurfatwa1*

1Research Center for Biotechnology, Indonesian Institute of Science, Jalan Raya Bogor km 46, Cibinong 16911, Bogor, Indonesia
2Faculty of Technobiology, Sumbawa University of Technology, Nusa Tenggara Barat, Indonesia

*E-mail: maritsa.nurfatwa@gmail.com

Abstract. Plantaricin is a bacteriocin produced by Lactobacillus plantarum. It has potential as probiotic, antimicrobial, easily degraded by proteolytic enzyme and Generally Recognized as Safe (GRAS). The objective of this study was to produce plantaricin F expressed from Lactococcus lactis pNZ8148-plnAF, purified using gel-filtration chromatography and investigated its antimicrobial activity using disc diffusion method against Candida albicans using nystatin as a positive control. Crude plantaricin F yield was 1.23 grams. Gel-filtration chromatography using Sephadex G-50 of plantaricin F yielding in 25 fractions. 5 fractions (fraction no 7, 9, 17, 18, 19) from 25 fractions have antimicrobial activities based on the clear zone on disc diffusion method.

1. Introduction

Bacteriocin is an antimicrobial compound produced by lactic acid bacteria (LAB). Bacteriocin can be used as a food preservative and pathogen growth controller. Plantaricin is one type of bacteriocin produced by LAB (Lactobacillus plantarum) [1]. Plantaricin has several benefits such as inhibiting bacterial growth in meat [2], inhibiting Listeria growth [3], inhibiting Staphylococcus aureus and Escherichia coli [4], and generally recognized as safe (GRAS) for consumption [5,6].

Plantaricin F (as class IIb bacteriocin) was isolated from L. plantarum and heterogenous recombinant Lactococcus lactis [4]. Mustopa [7] has succeeded in isolating L. plantarum S34 which produced plantaricin from bekasam (fermented meat). Plantaricin has inhibitory activity against Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, and Escherichia coli. Plantaricin F biosynthesis was controlled by plantaricin A. Plantaricin A was fused with plantaricin F and cloned into pNZ8148 using L. lactis as an expression host. Plantaricin F was produced using strong inducible promoter nisA [7-11]. Kusdianawati et al. [6] and Mustopa et al. [12] also have cloned plantaricin E in E. coli as a host.

Candida albicans is flora normal of the oral cavity, gastrointestinal tract and vagina. Candida as fungal species can cause mucosal and tissue infections especially in hospitalized and immunocompromised patients. The study of antibiotic alternatives for C. albicans infection has attracted great attention since it possesses drug-resistant ability using its biofilms forming [13]. Pertami et al. stated that antimicrobial activity produced by lactic acid contained in Lactococcus
acidophilus can disrupt the balance of acid in C. albicans, and will damage the cells [14]. Thus, recombinant plantaricin F antimicrobial activity was investigated against C. albicans. Recombinant plantaricin F were expressed from L. lactis pNZ8148-plnAF bacteria.

2. Materials and Methods

2.1 Materials

L. lactis pNZ8148-plnAF isolates (laboratory collection), recombinant plantaricin F, Candida albicans (ATCC 10231), M17 broth (Himedia), glucose (Merck), bacteriological agar (Oxoid), yeast extract (Oxoid), peptone (Oxoid), dextrose (Sigma-Aldrich), NaCl (Merck), chloramphenicol (Bio Basic), nisin (Sigma-Aldrich), HCl tris buffer, ammonium sulfate (Sigma-Aldrich), distilled water, ethanol and G-50 sephadex gel (Sigma-Aldrich).

2.2 Plantaricin F Recombinant Production

Plantaricin F recombinant production began with 10% L. lactis pNZ8148-plnAF bacteria inoculated into 15 mL M17G media (0.5% glucose and chloramphenicol). Furthermore, pre-culture into 150 mL M17G media. Bacterial culture was incubated at 30°C without agitation for 2-3 hours or up to Optical Density (OD) reach 0.5 at a wavelength (λ) 600 nm. Induced by adding 10 mg/mL nisin and incubated during 4-5 hours without agitation until OD600 reached 1. The recombinant plantaricin F from L. lactis pNZ8148-plnAF was centrifuged at 10,000 rpm at 4°C for 30 minutes. The supernatant was conditioned to pH 6.5 by using 1 N NaOH [10].

2.3 Plantaricin F Recombinant Purification

2.3.1 Ammonium Sulfate Partial Purification

Precipitation was carried out on cell-free supernatant produced from 150 mL of L. lactis pNZ8148-plnAF cultures. Precipitation was done by adding ammonium sulfate gradually until saturation of the solution reached 45%. The addition of ammonium sulfate was carried out at 4°C using a low-speed magnetic stirrer. After all the ammonium sulfate dissolved, it was incubated at 4°C overnight. The precipitation solution was then centrifuged at 10,000 rpm for 30 minutes at 4°C and produced pellet. Pellet was dissolved with 10 mM Tris HCl buffer pH 7.4 [15,16].

2.3.2 Gel Filtration Chromatography Purification

Gel filtration chromatography was carried out using a chromatographic column containing Sephadex G-50 gel at 4°C. The gel was washed first using sterile distilled water and then quantified with 10 mM Tris HCl buffer pH 7.4. Plantaricin F samples from centrifugation were put into columns for further elution. Elution was carried out with the same buffer with a flow rate of 1 mL / min. The results were collected in Eppendorf tube (fractions). Gel filtration chromatography separated the target protein based on the size of the protein contained in the solution. This method makes larger proteins pass through the column first compared to smaller proteins. The chromatographic column contains a solid phase which has pores of a certain size. Larger proteins cannot enter the pores so that it eluted first through the column, while smaller proteins will be trapped in these pores [17].

2.4 Plantaricin F Recombinant Antimicrobial Activity Assay against C. albicans

Antimicrobial activity assay of recombinant plantaricin F against C. albicans was carried out using the agar diffusion method. C. albicans was grown in 5 mL Yeast Extract Peptone Dextrose (YPD) culture media for 48 hours at 24-26°C. The culture was diluted using 0.85% NaCl with 1:8 ratio. The dilution was taken 3 mL and added into 17 mL YPD agar media. Media containing C. albicans was homogenized and poured into petri dish. After it became solid, 20 µL recombinant plantaricin F, negative control (YPD media) and positive control (nystatin 1 ng / mL) were dropped onto paper discs then placed on the surface of the media. Petri dish was incubated at 24-26°C for 48 hours. The antimicrobial activity of recombinant plantaricin F was observed by the clear zone formed in the petri dish [18].
3. Results and Discussion

3.1 Production of Crude Protein from L. lactis pNZ8148-plnAF

Recombinant plantaricin F is bacteriocin designed to be produced on large scale using heterologous (L. Lactis) host cells. Plantaricin F naturally is two-peptide bacteriocin (plantaricin EF) produced by L. plantarum. Through genetic engineering, plantaricin EF was separated from its two peptide pairs into plantaricin E (plnE) and plantaricin F (plnF). Then, the plnF gene was fused with the plnA gene, a signal peptide and produced naturally by L. plantarum. The use of plantaricin A signal peptide is intended for plantaricin F to be secreted by L. lactis strain NZ3900 into M17G media to facilitate further downstream processes [4]. The process elaborated before resulting in recombinant L. lactis pNZ8148-plnAF recombinant bacteria.

The production of crude protein from L. lactis pNZ8148-plnAF using the expression vector pNZ8148 has been successfully carried out by evaluating the results in Tables 1. The crude protein produced should have recombinant F protein produced by recombinant L. lactis. This was consistent with the study of Sogandi et al. [16] that the success of recombinant plantaricin F production was shown by an increase in L. plantarum cell density from 0.1 to 3.6 (OD 600) at incubation for 20 hours and gradually decreased to 2.7 (OD 600) when incubating 32 hours. Plantaricin from cell-free supernatant was collected by centrifugation and the protein was concentrated by ammonium sulfate precipitation to produce crude protein. Production of crude plantaricin was done in the stationary phase. Some researchers have previously stated that the stationary phase was a good phase for producing plantaricin because it has a high level of plantaricin protein activity, as found in plantaricin ST194BZ [19], plantaricin MG [20], plantaricin LB-B1 [3], and plantaricin ST71KS [21].

| Sample | Pellet (g) |
|--------|------------|
| F1     | 0.30       |
| F2     | 0.37       |
| F3     | 0.31       |
| F4     | 0.25       |
| Total Pellet | 1.23 |

Table 1. Pellet Weight of L. lactis pNZ8148-plnAF Metabolites.

Protein expression of L. lactis pNZ8148-plnAF culture (OD 0.5) during the incubation process for plantaricin F recombinant production was carried out by nisin induction (10 μg/mL). Borrerio et al. stated that the induction of nisin using 10 μg/mL produced the largest inhibitory zone in bacteria [21]. Nisin concentration used was closely related to plantaricin production. A low concentration of nisin unable to activate the Nisk gene, resulting in the absence of phosphorylation reaction and nisin operon in the NisA promoter will not be induced. NisA promoters regulate the expression of genes involved in the process of biosynthesis of plnF genes in recombinant L. lactis (pNZ8148-plnAF). High concentration of nisin will inhibit the growth of host cells, the production of plnAF genes will not be optimal [4,12]. L. lactis NZ3900 is a special bacterial strain used for food selection. In this strain, the lacF gene in lactose operon has been deleted and unable to grow on media containing lactose. The medium used as a carbon source for L. lactis NZ3900 is M17 media with 0.5% glucose addition. The plasmid pNZ8148 is a high copy plasmid that has the chloramphenicol resistant gene as a marker of selection. Therefore, the addition of the antibiotic chloramphenicol to the media will prevent other bacteria from growing in culture [22,23].

3.2 Plantaricin F Recombinant Purification

Purification of recombinant plantaricin F was carried out by ammonium sulfate precipitation and Sephadex G-50 gel filtration chromatography. Plantaricin F was precipitated using ammonium sulfate
with 45% concentration [10]. The ionic strength is stronger at high concentrations, causing salt to bind more water molecules (salting-out). Under these conditions, the protein will precipitate [24]. During the salting-out process to precipitate recombinant plantaricin F protein, it is important to keep the salt concentration from decreasing in solution, to prevent the deposition of other unwanted proteins (polluting proteins). During the precipitation process the stirring was carried out for keeping salt concentration maintained. Precipitation of proteins using ammonium sulfate has several advantages such as to stabilize the structure of the protein, relatively inexpensive, and easily available [25].

![Figure 1. 25 Fractions of Plantaricin F Sephadex G-50 Filtration Chromatography.](image_url)

Recombinant plantaricin F purification using G-50 Sephadex gel filtration chromatography can be seen in Figure 1. Sephadex G-50 gel was capable of filtering protein particles size of 1.5-30 kDa, it can filter out recombinant plantaricin F protein particles (3.85 kDa) [4]. Plantaricin F purification using gel filtration chromatography obtained 25 fractions, but only a few fractions were used for further antimicrobial activity assay. Fractions 4-22 have high absorbance value and were tested against C. albicans. From the previous study of Hermiastuti [26], the absorbance value is proportional to protein content in solution. The fraction that has high absorbance value also has high protein content. This shows that there was a dominant protein produced by bacteria in the fraction (figure 1).

### 3.3 Plantaricin F Recombinant Antimicrobial Activity Test Against C. albicans

Several Sephadex G-50 gel chromatography fractions used in further tests for antimicrobial activity were 4 - 22 fractions. The negative control used was YPD media and the positive control used was nystatin antibiotics. The results of antimicrobial activity from several fractions can be seen in table 2 and figure 2.

Based on table 2, several fractions used in antimicrobial activity assay against C. albicans have only few inhibitory activity, namely fraction 7, fraction 9, fraction 17, fraction 18, and fraction 19. The assay indicator of antimicrobial activity was characterized by the appearance of inhibition zones/clear zone around the disc paper [27]. According to Vandepitte [28], clear zone is form from microbial sensitivity to antimicrobial agent expressed by the width clear zone diameter. Inhibition zones from 5 fractions were less than optimal. This occurred because of the temperature in the production process and purification of recombinant plantaricin F. The optimum temperature for the
reaction activity of plantaricin was at 4°C [12,15,29]. In the bioassay process, incubation was not carried out at 4°C, which can cause changes in protein environmental conditions. The temperature changes will result in a decrease in protein activity. Incubation of 4°C for 1-2 hours needs to be done with the aim to give the sample time to absorb well into the YPD media [30]. Some studies also stated that bacteriocin activity decreases with a longer period of incubation time. The existence of this recovery activity is likely due to microbes becoming resistant to antimicrobial activity [2].

**Table 2.** Antimicrobial Activity of Recombinant Plantaricin F Fractions against *C. albicans.*

| No | Sample          | Results  |
|----|----------------|----------|
| 1. | Control + (Nystatin) | +        |
| 2. | Control – (YPD) | -        |
| 3. | S34¹           | +        |
| 4. | S34²           | +        |
| 5. | Fraction 4     | -        |
| 6. | Fraction 5     | -        |
| 7. | Fraction 6     | -        |
| 8. | Fraction 7     | +        |
| 9. | Fraction 8     | -        |
| 10.| Fraction 9     | +        |
| 11.| Fraction 10    | -        |
| 12.| Fraction 11    | -        |
| 13.| Fraction 12    | -        |
| 14.| Fraction 13    | -        |
| 15.| Fraction 14    | -        |
| 16.| Fraction 15    | -        |
| 17.| Fraction 16    | -        |
| 18.| Fraction 17    | +        |
| 19.| Fraction 18    | +        |
| 20.| Fraction 19    | +        |
| 21.| Fraction 20    | -        |
| 22.| Fraction 21    | -        |
| 23.| Fraction 22    | -        |

+ : inhibitory activity  
- : no inhibitory activity

Antimicrobial activity assay against *C. albicans* carried using positive control nystatin 1 ng/mL. It can be seen that there was antimicrobial activity from table 2, namely the presence of clear zones around the disc paper. Related to Paramita et al., nystatin can inhibit *Candida albicans* (ATCC 10231) with resistant categories ranging from concentrations 350 µg/mL (6.1 mm), 400 µg/mL (6.7 mm), and 450 µg/mL (8.1 mm) [31]. Another article also stated that pantaricin J, plantaricin E and plantaricin F had been found to have activity against *Candida albicans* (strain SC5314) using cell viability assay [32]. Samples S341 and S342 were used to compare for recombinant plantaricin F. Samples S341 and S342 are S34 plantaricin proteins from *Lactobacillus plantarum*. S34 were isolated from typical food meat traces of Way Kanan Regency (Lampung Province) which had previously been tested and were proven to have antimicrobial activity [8].
4. Conclusions
Plantaricin F (from L. lactis pNZ8148-plnAF production) has antimicrobial activity against C. albicans. Another confirmation method such as BCA assay and SDS-Page should be done to determine the protein concentration on fraction 7, 9, 17, 18 and 19 which gave inhibitory activity.

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