IDENTIFICATION OF A BACTERIOCIN PRODUCING BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* PD14

Hoa Thi Minh Tu[1, *], Nguyen La Anh[2], Le Thanh Binh[1]

[1]*Institute of Biotechnology, VAST 18 Hoang Quoc Viet, Cau Giay, Hanoi*

[2]*Food Industrial Research Institute, Ministry of Industry and Trade*

[*]Email*:* *hoathiminhtu@gmail.com*

Received: 25 November 2012; Accepted for publication: 15 August 2013

ABSTRACT

The strain *Lactococcus* PD14 isolated from cow's fresh milk was identified as *Lactococcus lactis* subsp. l*actis.* The bacteriocin produced by PD14 was isolated and purified by absorption-desorption method described and then this bacteriocin was purified by solid-phase extraction-SPE and HPLC method with column C18. The result of tricine- SDS-PAGE indicated the molecular weight of bacteriocin was about 3.5 kDa, the same as nisin.

The prenisin encoding gene was cloned and sequenced successfully for PD14. The n*is* gene of the strain PD14 was 99 % homology with *nisA* and *nisZ* while 98 % with *nisF*, 97 % with *nisQ*. The deduced amino acid sequence of prenisin was determined, which had minor difference from published sequence of *nisA* in one amino acid. At the position aminoacid -18, prenisin of PD14 had valine while the known prenisin A had phenylalanine. The matured nisin of PD14 was completely similar to the known amino acid sequence of nisA. Thus, the strain *Lactococcus lactis* subsp. *lactis.* PD14 produced a bacteriocin which was identified as nisin A.

Keywords: bacteriocin, nisin, identification, amino acid sequence, *Lactococcus lactis* subsp. *Lactis*, homology.

1. INTRODUCTION

Nisin is the first lantibiotic was discovered in 1928 but the structure was published by Gross and Morell in 1971, it contains unusual amino acid residues, namely dehydroalanine. Nisin is a polypeptide of size 3.5 kDa, consisted by 34 amino acids produced by *L. lactis* subsp. *lactis* [1, 2], which exhibits antimicrobial activity against a wide range of Gram-positive bacteria, especially that associated with food spoilage, such as *S. aureus, B. cereus, Clostridium* and *L. monocytogenes* [3, 4]. The unusual biosynthesis of nisin is by post-translational modification of serine and threonine to form dehydro amino acids that react with cysteine to form thioether lanthinine rings mades typical characteristics of this bacteriocin class [4]. Many studies published indicate that nisin is safe and granted GRAS (generally recognized as safe) by the FDA and allowed by the FAO and WHO to use as a food preservative [1, 5, 6]. Nisin is

worldwide used over than 50 countries for different food preservation. The major use of nisin is in dairy industry and canned vegetables and meats. The application of nisin in food preservation has been recently extending to active package field as an antimicrobial packaging material [7, 8], dehydrobutyrine, lanthionine, and 3-methyllanthionine [1, 4, 9, 10].

The *nis* gene cluster for nisin biosynthesis has been studied in details. It is demonstrated that the cluster is located in a conjugative transposon locates on the chromosome [1, 11, 7]. The transposon also encodes for sucrose fermentation and reduced bacteriophage sensibility. The cluster consists by 11 genes, i.e. *nisA, nisB, nisT, nisC, nisI*, *nisP*, *nisR, nisK*, *nisF*, *nisE*, *nisG* arranged in three multi-cistronic operon. *NisB* and *nisC* play the role for maturation of the lantibiotic, while *nisT* involved in transport across the cell membrane. *NisI* encodes an immunity protein, *nisP* is responsible for a putative serine protease involving in processing. *NisR, nisK* encode a putative regulatory protein and putative histidine kinase, respectively. *NisF, nisE, nisG* encode ATP-binding cassette transporter, which are together with *nisI* responsible for immunity [12].

Several lantibiotics have been discovered, among them five other natural variants of nisin are A, Z, Q, F and U [3, 13, 14]. The difference between these nisins are from one to few amino acids (aa) in their structure. Nisin A, Z, Q and F have similar activities, but differ in a few aa. Nisin A and nisin Z differ in a single one, while nisin A differs from nisin Q six aa [15, 16, 17] and differs nisin F in two aa. Nisin F differs nisin Q in four amino acids [2].

A bacteriocin was produced by strain *Lactococcus lactis* subsp. *lactis* strain PD14 isolated from cow's fresh milk in the farm at Hanoi outskirt, that was described in the previous paper [18]. The properties of bacteriocin from PD14 showed wide antibacterial range and heat resistance [19]. Thus, it has led to an interest to determine encoding gene sequence for PD14's bacteriocin and its deduced amino acid sequence in comparison with known published nisins.

2. MATERIAL AND METHODS

2.1. Microbial strains and media

The strain *Lactococcus* PD14 and *Lactobacillus plantarum* JCM1149- indicator strain were received from the collection of biomaterial technology laboratory, Institute of Biotechnology. These strains were cultured on MRS medium those composition is (g/l) peptone 10; meat extract 10; yeast extract 5; glucose 20; Tween 80 1; K_2HPO_4 2; CH_3COONa 5; ammonium citrate 2; $MgSO_4$ .7 H_2O 0.2; $MnSO_4.H_2O$ 0.05; pH = 6.5 ÷ 6.8.

Escherichia coli DH5α was cultured in Luria-Bertani broth (LB) on a shaker with speed of 220 rpm/min, overnight at 37 °C.

2.2. Isolation and purification of bacteriocin

The bacteriocin produced by PD14 was isolated and purified by absorption-desorption method described by Rongguang et al [20] and then this bacteriocin was purified by solid-phase extraction-SPE and HPLC method with column C18.

2.3. Determination the size and activity of bacteriocin directly on SDS-polyacrylamide gel

Tricine-SDS-polyacrylamid gel electrophoresis (PAGE) was conducted according to the method of Schagger with some modification for determination of size and activity of bacteriocin [21]. Bacteriocin sample producing by PD14 strain was loaded repeatedly into gel hole. The electrophoresis was stopped running when the dyes was appeared at the position less than 0.5cm from the end of the gel. The gel then cut into two parts as seen in Fig. 1, one half was dyed in coomassie, the other half was washed with 35 % ethanol, 2 % glycerol for 30 - 60 min and rinsed with sterile distilled water 15 - 30 min. The gel finally placed on a sterile pettri dish available a layer of solid agar and then poured over one layer of semi-liquid agar contained *L. plantarum* JCM1149. The dish was kept in the refrigerator for 4 h, and then transferred to 37 °C incubation for further 24 h.

2.4. DNA extraction and Cloning *nis* gene

Total DNA was prepared by Sambrook et al [22]. Total DNA was isolated by using kit of Promega Wizard® plus SV minipres DNA, A1330.

Nis gene was amplified by PCR using a couple of degenerate primers with the following sequences: NisF1:5→3´ATGAGTACAAAAGATTTTNAACTT and NisR1: 3→5 TTATTTNCTTACGTGAA

For the PCR, 2 µl of the total purified DNA and the two primers NisF1, NisR1 were taken. Thermal cycle was performed by Kwaadsteniet [10], denaturation at 94 ^{o}C for 4 min, 35 thermal cycles of 94 °C for 1 min, 48 °C for 30 sec, 72 °C for 7 sec and one cycle for 7 min at 72 °C.

The PCR product was purified by QiaQuick PCR Purification (Qiagen, Hilden, Germany). Ligation was conducted by ligation vector pGEM®-T, ligase (Takara) for 1 h at 16 °C. The transformation of ligated product in competent *E. coli* DH5α was performed as described Sambrook et al [22]. The isolated recombinant plasmid from transformed colonies (white colonies) was then purified and cut by restrict enzyme *Eco*RI. The restricted sample was analysed by electrphoresis for *nis* gene discovery and it was used for sequence reading. The reading was carried out on Beckam coulter CIQ TM8000 by using GenomeLab Dye Terminator Cycle Sequencing (Quick Start Kit). Nucleotides was treated by SeqEd1.03 and compared by Blast of NCBI.

Sequencing gene nis of PD14 strain were compered with the sequences in GenBank by sofwere Clustalw.

3. RESULT AND DISCUSION

3.1. Electrophoresis for determination of bacterioin size

The result of Tricine - SDS-PAGE was indicated in Fig. 1, where gel A (haft A) showed only one band with the size about 3.5 kDa. On the gel B (haft B), at the same position was appeared an inhibitory zone. It meant that *L. plantarium* JCM1149 was inhibited by the bacteriocin which was located on the gel at the position as 3.5 kDa indicated by the marker. Therefore, it can be concluded the bacteriocin produced by strains PD14 has a molecular weight of about 3.5 kDa as nisin.

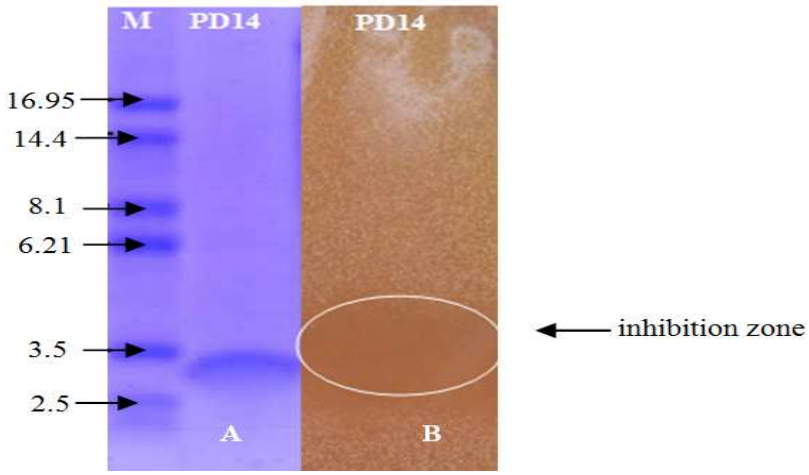

Figure 1. Electrophoresis of bacteriocin producing by *L. lactis* PD14; M: Marker MW-17S (Sigma), A: first part of the gel dyed with comasie, B: second part of the gel with inhibition zone formation (due to *L. plantarum* JCM1149 was inhibited).

3.2. Detection of *nis* gene in *Lactococcus lactis* subsp. *lactis* PD14

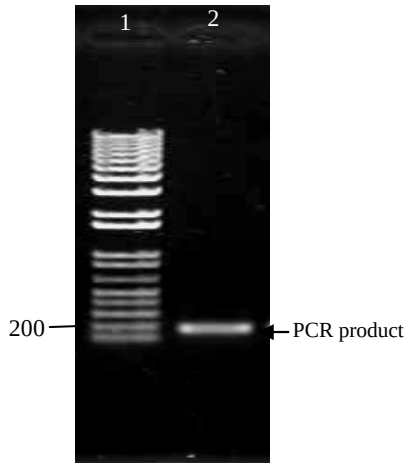

Figure 2. Elecrophoresis of *nis* gene amplified on gel agarose 0.8%
1. Marker Fermentas GeneRuler 1 kb Ldr. Plus RTU #SM1333
2. PCR product.

By PCR method, the use of a couple of degenerate primers as above- mentioned designed on sequences encoded for nisin A, nisin Z, nisin Q and nisin F indicated the existing *nis* gene in the strain PD14 (Fig. 2). The PCR product was a DNA fragment of 171bp (including 69 bp of leader and 102 bp of nisin mature).

The pGEM®-T Easy Vector Systems are convenient systems for the cloning of PCR(c) products and size 3.018 kb. This Vector contains multiple restriction sites within the MCS. These restriction sites allow for the release of the insert by digestion with a single restriction enzyme. In this study, the restriction enzymes *Eco*RI was used for the release of the insert by digestion.

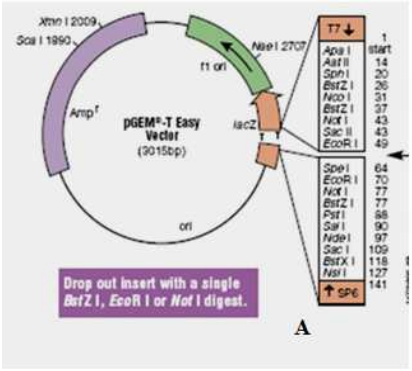

Figure 3. A - pGEM®-T Easy.

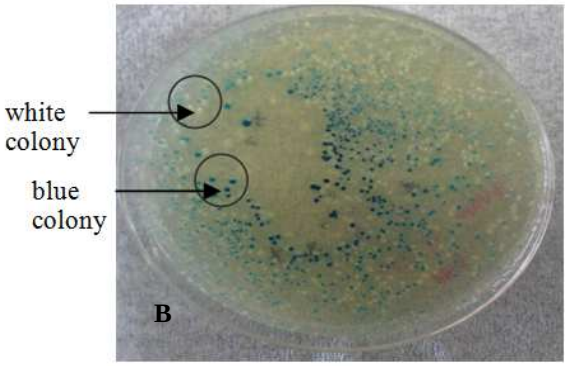

Figure 3. B – Blue and white.

The colonies of E. *coli* containing the recombinant plasmid were selected on solid LB medium containing ampicillin, X-gal and IPTG. The selected eight colonies in which six were white and two were blue colonies. Isolation of recombinant plasmid DNA was described in Promega's Protocols and Applications Guide. The result analyzed by 0.8 % agarose gel electrophoresis illustrated as in Fig. 4.

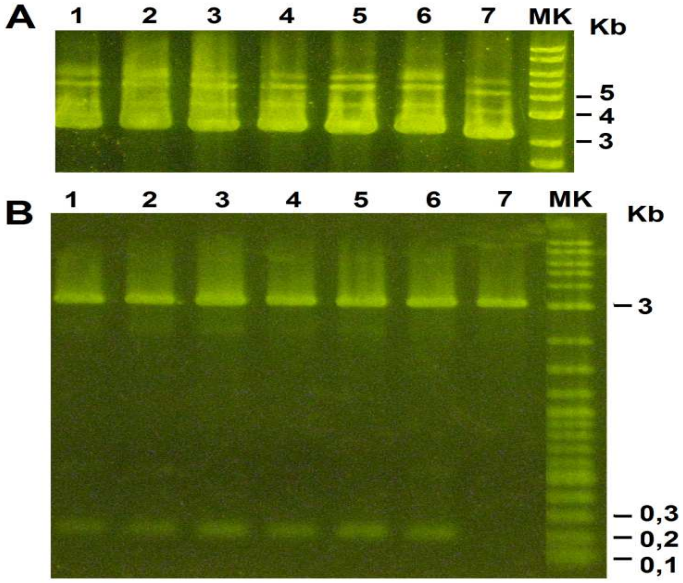

Figure 4. **A**. Lane: 1, 2, 3, 4, 5, 6: gene *nis* selected from white colonies; lane: 7 from blue colonies): Marker Biolabs (0.1 – 10 kb).

B. Lane: 1, 2, 3, 4, 5, 6, 7 plasmid from colonies containing *nis* gene in which (1, 2, 3, 4, 5, 6 from white colonies while 7 from blue colonies) after digestion with *Eco*R I: Marker Biolabs (0.1 – 10 kb).

To confirm the *nis* gene was inserted into vector, plasmid products were tested with restriction enzymes digestion with *Eco*RI. The results cut with restriction enzymes was checked on 0.8 % agarose gel electrophoresis (Fig. 4A).

Observation of the electrophoresis image as seen in Fig 4B showed that plasmide ADN products after digestion with *Eco*RI which appeared two bands. A band was corresponding to the

size of plasmids isolated from blue colonies. The other band has size appropriate the band of PCR products. The obtained results indicated that lane 1, 2, 3, 4, 5, 6 containing *nis* gene from the strain PD14.

The plasmid DNA products were purified by QiaQuick PCR Purification (Qiagen, Hilden, Germany). For *nis* gene sequencing, it was use of Genome Lab Dey Terminator Cycle Sequencing Kit (Quick Start Kit), reading on Beckam coulter CIQ TM8000. Nucleotides were treated by SeqEd1.03 Programme and compared to the Blast of NCBI.

The result of homology comparision *nis* gene of strain PD14 was carried out with gene *nisA* (AY526091.1), *nisZ* (AB375441.1), *nisf* (EU057979.1) and *nisQ* (AB100029.1) by CLUSSTAL (1.81). The obtained results indicated that, *nis* gene of strain PD14 was 99 % homology with *nisA* and *nisZ* while with *nisF* and *nisQ* was 98 % and 97 %, respectively as below:

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PD14    ATGAGTACAAAAGATTTGAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCA
NISA    ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCA
NISZ    ATGAGTACAAAAGATTTTAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCA
NISF    ATGAGTACAAAAGATTTCAACTTGGATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCA
NISQ    ATGAGTACAAAAGATTTGAACTTAGATTTGGTATCTGTTTCAAAAACAGATTCTGGCGCT
        ***************** ***** ***************** ** * ****** ** **

PD14     TCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATG
NISA     TCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATG
NISZ     TCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATG
NISF     TCACCACGCATTACAAGTATTTCGCTATGTACACCCGGTTGTAAAACAGGAGCTCTGATG
NISQ     TCAACACGTATTACCAGCATTTCGCTTGTACACCAGGTTGTAAAACAGGTGTTCTGATG
         *** **** ***** ** ******** ******** ************** * *******

PD14     GGTTGTAACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAA
NISA     GGTTGTAACATGAAAACAGCAACTTGTCATTGTAGTATTCACGTAAGCAAATAA
NISZ     GGTTGTAACATGAAAACAGCAACTTGTAATTGTAGTATTCACGTAAGCAAATAA
NISF     GGTTGTAACATGAAAACAGCAACTTGTAATTGTAGCGTTCACGTAAGCAAA---
NISQ     GGATGTAACCTGAAAACAGCAACTTGTAATTGTAGCGTTCACGTAAGCAAATAA
         ** ****** ***************** ******* **************
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The deduced amino acid sequences nis gene of strain PD14 by CLUSTALW (1.81) and its comparison with *nisA, nisZ, nisF* and *nisQ* were as follows:

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                -18             -8       -2 +1                  15       21      27   30
PD14   MSTKDLNLDL VSVSKKDSGA SPRITSISLC TPGCKTGALM GCNMKTATCH CSIHVSK
Nis_A  MSTKDFNLDL VSVSKKDSGA SPRITSISLC TPGCKTGALM GCNMKTATCH CSIHVSK
Nis_Z  MSTKDFNLDL VSVSKKDSGA SPRITSISLC TPGCKTGALM GCNMKTATCN CSIHVSK
Nis_F  MSTKDFNLDL VSVSKKDSGA SPRITSISLC TPGCKTGALM GCNMKTATCN CSVHVSK
Nis_Q  MSTKDLNLDL VSVSKTDSGA STRITSISLC TPGCKTGVLM GCNLKTATCN CSVHVSK
       *****.**** *****_**** *_******** *******_** ***.*****. **.****
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Nis gene of the strain *Lactococcus lactis* subsp. *lacti*. PD14 was 99 % homology with *nisA* and *nisZ* while 98 % with *nisF*, 97 % with *nisQ*. The deduced amino acid sequence of prenisin from PD14 differed *nisA* by one amino acid at -18 position (its L: valine while F: phenylalanine in prenisin A), and there were 98 % homology with *nisA*, 96 % with *nisZ*, 94 % with *nisF* and 7 % with *nisQ*. The final sequence consideration demonstrated that *nis* gene of the strain PD14 was completely homology with *nisA*, and the strain had already identified as *Lactococcus lactis*

subsp. *lactis*. Therefore, it can be confirm that the bacteriocin produced by the strain PD14 is nisin A.

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TÓM TẮT

NGHIÊN CỨU ĐỊNH TÊN BACTERIOCIN DO CHỦNG *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* PD14 TỔNG HỢP

Hoa Thi Minh Tu[1], Nguyen La Anh[2], Le Thanh Binh[1]

[1]Viện Công nghệ sinh học, Viện HLKHCNVN, 18 Hoàng Quốc Việt, Cầu Giấy, Hà Nội

[2]Viện Công nghiệp thực phẩm, Thanh Xuân, Hà Nội

*Email: hoathiminhtu@gmail.com

Chủng vi khuẩn lactic *Lactococcus* PD14 phân lập từ sữa bò tươi đã được định tên là *Lactococcus lactis* subsp. *lactis* [19]. Chủng này sinh tổng hợp bacteriocin qua phân tích điện di Tricine - SDS- PAGE cho thấy có kích thước khoảng 3,5 kDa, giống như nisin. Bacteriocin này có phổ tác dụng chủ yếu với các vi khuẩn Gram dương, trong đó đặc biệt ức chế mạnh nhóm vi khuẩn gây bệnh, gây ngộ độc thực phẩm như *B. cereus, S. areus, L. monocytogenes*. Ngoài ra, các đặc tính chịu nhiệt, bền axit, nhạy cảm với protease rất tương đồng với nisin.

Việc nghiên cứu xác định tên một hoạt chất sinh học luôn là một ưu tiên hàng đầu, làm cơ sở cho các bước tiếp theo, đặc biệt cho những nghiên cứu ứng dụng.Với việc tách và tinh sạch thành công gene *nis* mã hóa tổng hợp nisin đã mở ra cơ hội để xác định trình tự gene *nis*, trình tự axit amin trong việc xác định tên bacteriocin này. Kết quả giải trình tự gene *nis* của chủng PD14 cho thấy 99 % tương đồng với *nisA* và *nisZ*, 98 % với *nisF* và 97 % với *nisQ*. Ngoài ra, phân tích trình tự axit min của prenisin ở chủng PD14 chứng tỏ nó chỉ khác với nisin A bởi chỉ một axit amin ở vị trí -18 (phần đầu) và có độ tương đồng 98 % với nisA, 96 % với nisZ, 94 % với nisF và 87 % với nisQ.

Ngoài ra, chủng PD14 đã được xác định là *Lactococcus lactis* subsp. *lactis*. Vì vậy, với tất cả các kết quả này là những bằng chứng có thể khẳng định bacteriocin do chủng PD14 tổng hợp là nisin A.

Từ khóa: amino acid sequence bacteriocin, nisin, identification, *Lactococcus lactis* subsp. *Lactis*, homology.