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Original Article

Rapid screening and evaluation of site saturated structural variants of lantibiotic, nukacin ISK-1

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ABSTRACT: Here we reports the feasibility of introducing saturation mutagenesis (NNK mutagenesis) in nukacin ISK-1 structural gene and to create a large number of structural analogues of this peptide to study the structure function relationship. Three individual residues as Lys1, Pro8 and His12 from the parent peptide were scanned where mutation frequencies were estimated to be around 70% in all cases. A rapid screening method for identification of a large number of nukacin ISK-1 variants form whole cell using a modified MALDI-TOF/MS method have also been developed. As many variants appeared multiple times, therefore after DNA sequencing it could be obtained a total of 37 nukacin ISK-1 variants were obtained for these three positions. We evaluated the potency of each variant by a modified spot on lawn method regardless of their productivity and hence concluded that Lys1 and Pro8 positions are highly variable whereas His12 position is relatively conserved to retain the antimicrobial activity.

KEYWORDS: lantibiotic, NNK scanning, site-saturation mutagenesis

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INTRODUCTION

Lantibiotics are peptide-derived antimicrobial agents that are ribosomally synthesized and posttranslationally modified to their biologically active form[1]. They contain unusual amino acids such as lanthionine (Lan) and/or 3-methyllanthionine (MeLan) and dehydrated amino acids i.e. dehydroalanine (Dha) and/or dehydrobutyrine (Dhb) which contribute to various biological activities and structural stability of lantibiotics. Nukacin ISK-1, a representative example of type-A(II) lantibiotic and produced by *Staphylococcus warneri* ISK-1, possesses 27 amino acids[2-4]. DNA sequence analysis of the gene cluster of nukacin ISK-1 suggested that it consisted of nukacin ISK-1 structural gene (*nukA*), modification gene (*nukM*), leader peptide processing and secretion ABC transporter gene (*nukT*), and the self-protection genes (*nukFEGH*)[5-6].

As lantibiotics are gene encoded and can be easily manipulated by genetic engineering, therefore investigation of the structure-function relationships within lantibiotics is principally motivated by the desire to create new and more effective lantibiotic analogues. The continuation of rational and random mutagenesis strategies will be central to the efforts in unlocking the structure-function relationships within lantibiotics and thus provide blueprints for the design and optimization of more potent antimicrobial peptides. To this end, the development of *in vivo* expression systems could play a key role in the production and screening of such peptides. Previously Nagao *et al.* (2007) have reported the heterologous expression system for

nukacin ISK-1 biosynthetic gene cluster in *Lactococcus lactis* under nisin-controlled expression (NICE) system[7]. Using this expression system it is possible to generate a large number of nukacin ISK-1 variants efficiently to analyze its structure-function relationship. An efficient combined methodology that will describe both the generation of large number of structural analogues and screening and evaluation of the variants effectively will be highly applicable for further lantibiotic engineering as well.

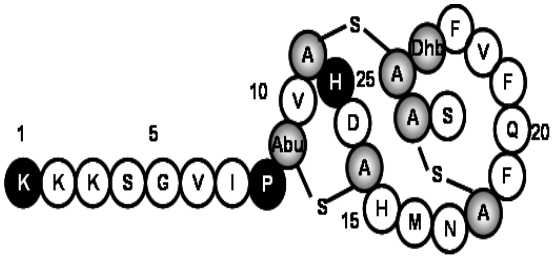

Fig. 1: Proposed structure of nukacin ISK-1. Scanned residues are highlighted by a dark black shade. Unusual amino acids are indicated by a dark shade. Dhb, Dehydrobutyrine; Abu-S-A, 3-methyllanthionine; A-S-A, lanthionine.

We carried out saturation mutagenesis of the nukacin ISK-1 structural gene (*nukA*) at positions Lys1, Pro8 and His12 using NNK method (Fig. 1). NNK codon degeneracy (N: adenine/cytosine/guanine/thymine; K: guanine/thymine) is a conventional way to perform saturation mutagenesis that involves 32 codons and relates to all 20 proteinogenic amino acids as building blocks. The diversity of an NNK library in terms of the number of structurally different (distinct) transformants is much higher with respect to other mutagenesis methods[8-9].

A major drawback in lantibiotic bioengineering is the rapid identification and evaluation of variants. Therefore in this study we developed a rapid screening method for identification of nukacin ISK-1 variants using MALDI-TOF/MS and evaluation of variants using a modified spot on lawn method. MALDI-time of flight MS (MALDI-TOF/MS) is effective for peptides and proteins with molecular masses ranging from 0.5 to 30 kDa and has been used to determine the masses of purified bacteriocins as well as lantibiotics since a long time[10-13]. Very frequently this method has been modified as to use whole bacterial cell in order to obtain mass spectral fingerprints that allowed taxonomic identification of bacteria, monitoring of protein expression, and detection of secondary metabolites[14-16]. The preparation of samples requires only very limited handling, and the greatest advantage of this technique lies in its speed that enables high-throughput screening. In this connection we developed a modified procedure for detection of nukacin ISK-1 variants from whole cell without time-consuming sample preparation. Evaluation of nukacin ISK-1 variants was carried out only by verifying their antimicrobial activity against a highly sensitive gram-positive bacterium namely, *Lactibacillus sakei* subsp. *sakei* JCM 1157^T. Therefore this study sheds light on the methodology for rapid generation of large number of nukacin ISK-1 variants using NNK method as well as screening of variants by MALDI-TOF/MS and evaluating by spot on lawn assay.

MATERIAL AND METHODS

Bacterial strains, culture media and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. A recombinant strain of *Lactococcus lactis* (pInukdA) was grown in M17 media (Merck, Darmstadt, Germany) supplemented with 0.5% glucose (GM17) at 30^0 C. Indicator strain *Lactibacillus sakei* subsp. *sakei* JCM1157^T was grown in MRS media at 30^{0}C. pNZA plasmid that contains the *nukA* gene downstream to *nisA* promoter was used for genetic manipulation in order to create nukacin ISK-1 variants using NNK methods. Antibiotic solution chloramphenicol (Cm, 5 µg/ml) and erythromycin (Em, 5 µg/ml) was used for selection of transformed *L. lactis* colonies.

Plasmid construction and optimization of NNK method

In Order to create mutation in nukacin ISK-1 structural gene (*nukA*) by NNK, we used pNZA plasmid that contains *nukA* structural gene, as a template. Selected three positions of NukA (Lys1, Pro8 and His12) were considered for genetic manipulation. Forward primers used to create mutation in these three positions are Lys1F (TTAGGAGCTNNKAAAAAGTCAGGAGTA), Pro8F (GGAGTAATCNNKACTGTGTCA CAC) and His12F (GGAGTAATCCCAACTGTGTCANNKGATTGC CATATG) and the reverse primer for Lys1 is NNKR1 (GACTTCATTCAATTCATCTTCTTG) and for Pro8 and His12 is NNKR2 (TGACTTTTTCTTAGCTCCTAAGACTTC).

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Description[a]	Source and Reference
Strains		
Lactococcus lactis NZ9000	MG1363 derivative; *nisRK::pep*	17
Lactococcus lactis pInukdA	NZ9000 strain harboring pInukdA plasmid	18
Lactobacillus sakei subsp. *sakei* JCM 1157^T	Indicator strain	JCM
Plasmids		
pNZ8048	Cm^r; P_{nisA}; *L. lactis* expression vector	19
pNZA	pNZ8048 derivative containing *nukA* downstream of P_{nis}	18
pNZ9520	Em^r; pIL253 derivative containing *nisRK*	20
pInuk	Em^r; pNZ9520 derivative containing *nukAMTFEGH* downstream of P_{nisA}	18
pInukdA	pInuk derivative containing *nukMTFEGH* downstream of P_{nisA}	18

[a]Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance.
JCM, Japan Collection of Microorganisms, Wako, Japan.

Inverse PCR using KOD$^+$ polymerase was performed according to the following procedures: final volume of 100 µl containing 20 pmol of primers, 0.5 ng of template DNA, 2 mM of Mg^{2+}, 0.2 mM of dNTP and 1 unit of DNA polymerase. PCR amplification conditions were pre-heat at 94°C for 3 min, followed by 30 cycles of incubation with denaturation (94°C) for 30 s, annealing for 30 s, and extension (68°C) for 4 min. Annealing temperature was adjusted according to the Tm values of different primers. After PCR, the product was purified by QIAquick PCR purification kit (QIAGEN) and then treated with *DpnI* (TOYOBO) for 3 h at 37°C. Purified products were then incubated with T4 kinase for 1 h at 37°C and ligated with Ligation High (DNA ligation kit, TOYOBO) at 16°C overnight (O/N). The ligated plasmids were then purified and transformed into the *Lactococcus lactis* pInukdA strain by electroporation.

Sample preparation and MALDI-TOF/MS

Prior to MALDI-TOF/MS analysis of bacterial colonies, 100 colonies for each three positions of nukacin ISK-1 were picked using sterile tooth pick from the plates and inoculated in 1 ml GM17 culture media containing Cm and Em. After overnight (O/N) incubation at 30^0C, 40 µl of each culture broth was transferred to another fresh 1 ml GM17 media containing 10 ng/ml nisin A for induction and another round O/N. The culture broth was then centrifuged at 12000 rpm for 5 min to collect the cell pellet. After washing with 600 µl deionized H_2O one loop of cell pellet was spotted on the MALDI-TOF/MS detection plate and air dried. A 1.5 µl matrix solution (10 mg/ml of α-cyano-4-hydroxy cinamic acid solubilized in 50% actonitrile/0.1% trifluroacetic acid) was added to each well and air dried. MALDI-TOF/MS spectra were recorded with a AXIMA-CFR plus mass spectrometer (Shimadzu corporation, Kyoto, Japan and Kratos Analytical, Manchester, U.K.) under the following settings: nitrogen laser (337 nm); reflectron mode; detection of positive ions. The acceleration potential was set to 45 kV using a gridless-type electrode. The mass spectra were averaged over 200 individual laser shots. MALDI-TOF/MS spectra were acquired in automatic mode, from m/z 2700 to 3200 and internally calibrated with purified nukacin ISK-1 peak (m/z 2960). Observing the molecular mass of individual colonies that produced nukacin ISK-1 variants were again DNA sequenced for further confirmation.

Antimicrobial activity assay

The 'spot-on-lawn' method according to Mayr-Harting *et al.* (1972) was used to determine the peptide variants activity against indicator strain *Lactobacillus sakei* subsp. *sakei* JCM1157^T [21]. Fractions collected by solid phase (Sep-pak) purification process were filter-sterilized using 0.22 µm cellulose acetate filter (Advantec, Toyo Rashi Kaisha Ltd., Japan) and 10 µl of peptide samples were spotted onto MRS agar (1.2% w/v) plates overlaid with 5 ml of lactobacilli agar

AOAC (Difco, Md., USA) containing a 1% overnight culture of the indicator strain. The plates were incubated overnight at the 30°C. Antimicrobial activity of each peptide variant was classified by comparing the diameter of inhibition zone to nukacin ISK-1 producing strain.

RESULTS

Generation of nukacin ISK-1 variants by NNK

Three very important positions of nukacin ISK-1 (Lys1, Pro8 and His12) were selected for saturation mutagenesis by NNK method (Fig. 1). Lys residues of nukacin ISK-1 were predicted to be crucial for interaction with the membrane of the target organism. Pro8 residue is the flanking residue that connects the N-terminal linear and C-terminal globular region of nukacin ISK-1 and His12 residue is relatively conserved among different type-A(II) lantibiotics. Using specific primers and inverse PCR we created mutation in each three position of nukacin ISK-1 structural gene (*nukA*) where pNZA plasmid was used as a template and later we transformed this mutagenized plasmid into *Lactococcus lactis* pInukdA strain that harbors all the biosynthetic machinery except nukacin ISK-1 structural gene for production of nukacin ISK-1 variants and obtained a large number of transformed colonies in each case. To validate the feasibility of this method for site-saturation mutagenesis we determined the mutation frequencies for each position by DNA sequencing of 10 randomly selected colonies and the mutation frequency were estimated to be around 70% in all cases (Table S1). Surprisingly, after DNA sequencing, we identified some codon changes in the nucleotide sequence of *nukA* gene that caused loss of production of peptide variants.

Detection and screening of peptide variants by MALDI-TOF/MS

To demonstrate the applicability of identifying nukacin ISK-1 variants by MALDI-TOF/MS we initially investigated the sensitivity of this method using cell pellet from 1 ml and 200 µl GM17 liquid culture of wild type nukacin ISK-1 producing strain and found nukacin ISK-1 peak in both culture volume (Fig. S1). But peak intensity was much higher for the cell pellet collected from 1 ml GM17 culture. Nukacin ISK-1 could also be detected in the supernatant of 1 ml culture pellet suspended with 50% acetonitrile/0.1% TFA (Fig. S1). We then stored the cell pellet form 1 ml culture in -30^{0}C for different time intervals such as 1 and 5 days and then analyzed by MALDI-TOF/MS to detect nukacin ISK-1, we could successfully detect nukacin ISK-1 in both storage time interval (data not shown).

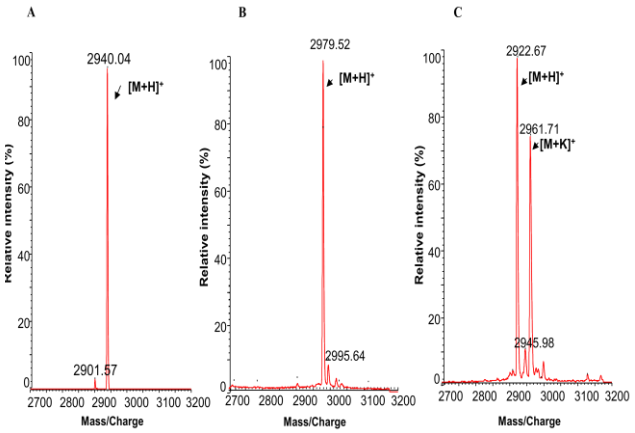

Fig.2: Mass chromatogram of some nukacin ISK-1 variants detected by MALDI-TOF/MS. **A**, Lys1Ile variant; **B,** Pro8Ile variant; **C,** His12Val variant

We screened 100 colonies for each three positions of nukacin ISK-1 (Lys1, Pro8 and His12) using cell pellet from 1 ml GM17 culture by MALDI-TOF/MS. Some of the representative mass chromatograms are shown in Fig 2.

In case of Lys1, we identified 37 mutant colonies by comparing their observed molecular mass (form MALD-TOF/MS) and calculated molecular mass (from DNA sequence). And among these 37 mutant colonies, after DNA sequencing of the mutated *nukA* gene, we identified 10 mutant colonies with different amino acid substitution replacing Lys1. We also ascertained that some variants appeared multiple times within the investigated colonies. In favor of Pro8 and His12 scanning, we could identify 14 and 13 variants after screening a specified number of colonies and DNA sequencing respectively.

Evaluation of nukacin ISK-1 variants

Rapid evaluation of nukacin ISK-1 variants was achieved by an immensely used spot on lawn assay which was slightly modified in this case. Sep-pak partially purified peptide variants were spotted on overlaid *Lb. sakei* plates and potency of each variant was measured by observing their zone of inhibition against the indicator strain. Antimicrobial activity of each variant from Lys1, Pro8 and His12 positions are summarized in Figure 3. Here, it is appropriate to consider that smaller or loss of inhibition zone might happen due to low or

Substituted Amino acid	Non Polar											Polar and Uncharged				Polar and Charged				
Position	G	A	V	L	I	P	M	C	Y	W	F	S	T	Q	N	H	K	R	E	D
Lys1	+	+		+	+		–				–	++			++		WT	++	–	
Pro8	±	++	+		+	WT	+	+	+	+	+	++		++		+		++	+	
His12	–	–	–	–			–	–		–	–	–		–		WT	±		–	–

Fig. 3: Inhibitory activity of different nukacin ISK-1 variants obtained by substitution of three different positions of the parent peptide as Lys1, Pro8 and His12. The characteristics of the amino acids are provided in the first row and the substituted amino acids are shown in the second row. The activities were determined using 10 µl of 50X concentrated sep-pak C18 partially purified peptide solution against *Lb. sakei*. Peptide variants showed similar or larger size of zone of inhibition compared to WT are represented as (++), smaller size of zone of inhibition as (+) and no or very little zone size are (- or ±). The blank boxes represent the mutant colonies which were not obtained in this experiment.

loss of production of peptide variants similarly larger zone of inhibition might be involved in either higher productivity, higher solubility or higher specific activity of the peptides.

In this connection, we identified that most of the Lys1 substituted variants showed their antimicrobial action against the indicator strain. Only few, in which Lys was substituted with Met, Phe and Glu did not generate any antimicrobial activity due to low level of productivity (data not shown). On the other hand, among 14 of Pro8 substituted variants, 13 showed inhibitory actions against the strain tested (Fig. 3). Only Pro8Gly variant, which was produced almost 10 times less than the wild type, did not show any inhibitory activity (Fig. 3). Replacement of this residue with Gln and Arg however hamper the production but could retain the same as nukacin ISK-1. This also indicated that these two variants might have higher potency than the wild type. His12 residue is relatively conserved among type-A(II) lantibiotics. Therefore substitution of this residue with any other residue may cause loss of antimicrobial activity. This was also found in this study as replacement of this residue with most of the amino acid resulted loss of antimicrobial activity except for His12Lys (Fig. 3). This variant showed very low level of production as confirmed by LC/MS (data not shown).

The methodology we applied here for quick evaluation of nukacin ISK-1 variants provided us an impression of potency of different variants and positional importance of different residues. This procedure can therefore be adopted for the rapid characterization of different lantibiotic peptide variants.

DISCUSSION

Due to the emergence of antibiotic-resistant bacteria in recent years, there is a pressing need for the development of novel antimicrobial agents. Among many candidates, studies on antimicrobial peptides such as lantibiotics are being encouraged for their possible use as alternative antibiotics[22]. In addition, engineering of new peptides might hold considerable promise to produce potential antibiotics. So far, many expression and gene replacement strategies have been developed to produce engineered lantibiotics (*in vivo* engineering) [23-25]. Heterologous expression system has been used in many cases for the engineering of many lantibiotics such as nisin, pep5, laciticin 3147 and mersacidin. In this study we employed the same system for the generation of nukacin ISK-1 variants. Until now many variants have been generated from type-A(II) lantibiotics by only using site-directed mutagenesis, which produced very limited information regarding the structure function relationship of this peptide sub-group. Therefore a systematic mutagenesis study that will describe the role of each and individual amino acids is required for understanding the structure based function as well as engineering for future applications.

In this study we developed a systematic mutagenesis method, NNK scanning, for evaluating the role of three very important residues of nukacin ISK-1 *e.g.* Lys1, Pro8 and His12. We identified a total of 37 variants in these there positions although we have expected more as the mutation frequency was estimated to be 70% in all three positions. Generation of lantibiotic variants have many restrictions such as the mutant

prepeptide required successful posttranslational modification, processing and exportation of the mutant. This did not happen in all the cases. Therefore we also predicted that the variants that could not be obtained might have resulted from blockage in the maturation process. Despite this we could illustrate that site-saturation mutagenesis using NNK method can be used for nukacin IKS-1 structure function relationship study.

Another significant attempt in this study was rapid screening of a large number of colonies for identification of nukacin ISK-1 variants using MALDI-TOF/MS. So far generation of a large number of lantibiotic variants have been delayed due to the absence of rapid and accurate screening procedure that we could bypass using MALDI-TOF/MS. The main advantage of this procedure lies in its speed: once the bacteria have grown, only few minutes is required to prepare the sample and complete the analysis. In addition, up to 384 samples can be prepared simultaneously and spotted on a single target plate, and the subsequent analysis can be performed automatically, which allows screening of numerous sample in a single day. We screened 100 colonies for each three positions of nukacin ISK-1 and identified 37 structural variants. It should be mentioned here that many variants appeared multiple times during the screening procedure. We have selected only the mutant colonies that showed higher intensity in the mass chromatogram. By observing the peak intensity we also attained an appropriate rough idea about the productivity of different nukacin ISK-1 variants.

We evaluated the antimicrobial activity and productivity of different nukacin ISK-1 variants using agar based spot on lawn method and LC/MS. Potency of the secreted peptide variants was evaluated against a highly sensitive indicator strain, *Lb. sakei* and hence we identified that Lys1 and Pro8 residues of nukacin ISK-1 are significantly variable with respect to their position. His12 is conserved for nukacin ISK-1 antimicrobial activity as no variants in this position showed any activity except His12Lys. Productivity of different Lys1 and Pro8 negative variants (Lys1Phe, Pro8Gly) specified that negative activity were found due to low production which was not similar with His12 substituted variants. Some variants (His12Ala, His12Val, His12Met) at this position produced relatively similar activity as nukacin ISK-1 but failed to retrieve the bioactivity, as this position is highly significant for the antimicrobial activity of nukacin ISK-1. The methods for evaluating nukacin ISK-1 variants described here would be a useful tool for the rapid evaluation of a large number of lantibiotic variants for structure function study.

Our initial proof-of-concept investigations presented here focused on the creation of a large bank of nukacin ISK-1 variants using NNK method and rapid screening of these variants using MALDI-TOF/MS. We also successfully established a methodology for the quick evaluation of nukacin ISK-1 variants with respect to their antimicrobial activity and productivity.

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Supporting information:

Table S1: Mutation frequency in NNK scanning method.

Fig. S1: Detection of nukacin ISK-1 by MALDI-TOF/MS.

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Table S1: Estimation of mutation frequency in NNK method. After performing mutagenesis in the *nukA* gene using specific primer, 10 transformed colonies from each position were picked up, plasmid extracted and DNA sequenced of mutated *nukA* gene.

Lys1

Colony no.	Types of mutant
K1	K1R
K2	Frame shift mutation
K3	K1G
K4	K1L
K5	K1A
K6	K1G
K7	K1M
K8	K1K
K9	Start codon mutation
K10	K1E

Pro8

Colony no.	Types of mutant
P1	P8W
P2	P8A
P3	Frame shift mutation
P4	P8R
P5	P8A
P6	P8S
P7	Frame shift mutation
P8	P8V
P9	P8R
P10	P8G

His12

Colony no.	Types of mutant
H1	H12A
H2	H12V
H3	Frame shift mutation
H4	H12L
H5	Frame shift mutation
H6	H12R
H7	H12S
H8	H12F
H9	H12K
H10	H12A

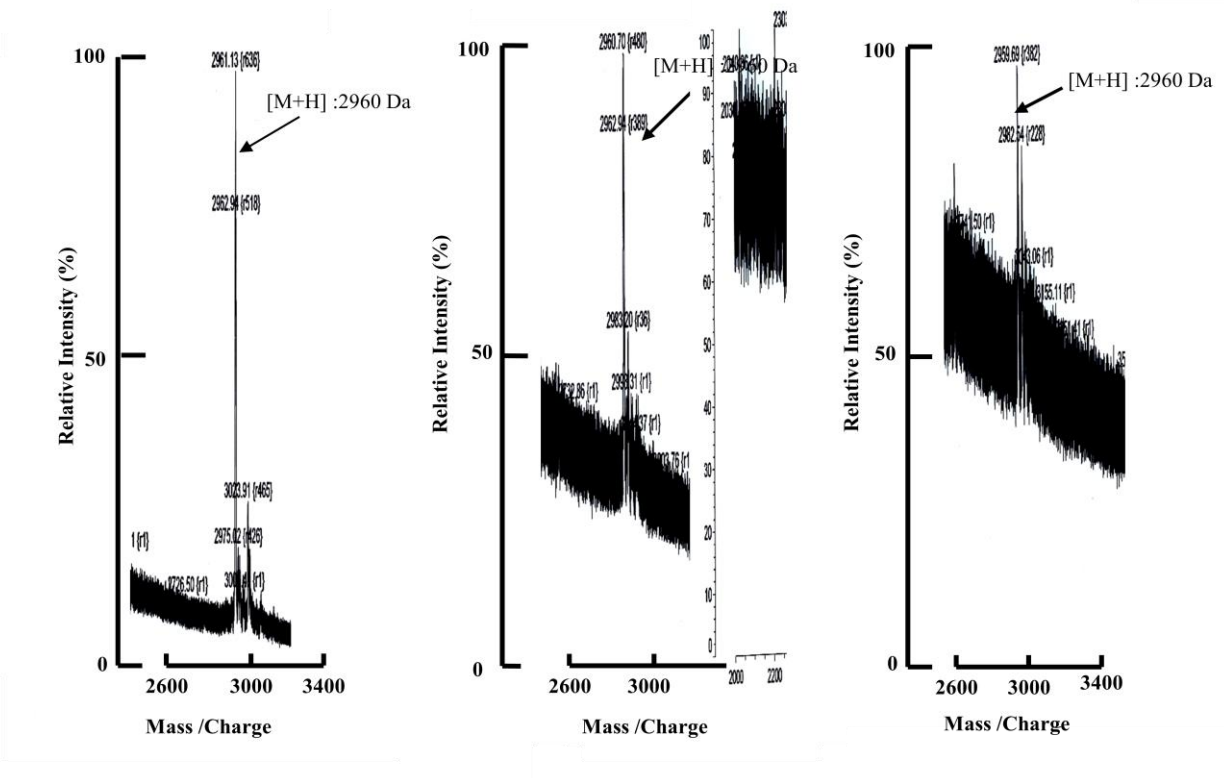

Fig S1: Detection of nukacin ISK-1 by MALDI-TOF/MS from different culture volume and condition using *Lactococcus lactis* (pInukdA+ pNZA) strain. **A**. cell pellet from 1 ml GM17 culture; **B**. Cell pellet from 200 µl GM17 culture; **C**. cell suspension form 1 ml GM17 culture treated with 50%actonitrile/0.1% TFA. M: Nukacin ISK-1