

Heterologous expression and purification of the dehydratase NisB involved in the biosynthesis of lantibiotic nisin

Asuman Karakas Sen · Mike J. Ridout · Arjan Narbad

Received: 10 March 2011 / Accepted: 31 August 2011 / Published online: 20 September 2011
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Abstract Nisin biosynthesis protein NisB, encoded by the *nisB* gene, is a membrane-associated enzyme of 993 amino acid residues which carries out the selective dehydration of the serine and threonine residues of the precursor nisin, leading to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. In this study, the *nisB* gene of *Lactococcus lactis* F15876 was cloned into the expression vector pET-15b, under the control of the isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible T7 promoter, expressed as an N-terminal His₆-tag fusion protein in *Escherichia coli*, and purified by nickel-affinity chromatography. The identity of the recombinant NisB protein was confirmed by western blot analysis using antibodies specific for NisB or the His₆-tag. The circular dichroism spectrum of His₆-tagged NisB was also obtained, which provided secondary structure information. We report here the heterologous expression of the *nisB* gene of a nisin A-producing *Lactococcus lactis* strain in *Escherichia coli* and purification of His₆-tagged NisB under native conditions.

Keywords *Lactococcus lactis* · NisB · His-tagged recombinant protein · Heterologous expression · Circular dichroism spectroscopy

Introduction

Lantibiotics are ribosomally synthesised and post-translationally modified antimicrobial peptides produced by bacteria. This class of bacteriocins contains atypical amino acids, such as dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine (Lan) and β -methylanthionine (MeLan) residues (Chatterjee et al. 2005; Field et al. 2010). The lantibiotic nisin is used as a safe and natural preservative in the food industry (Delves-Broughton et al. 1996). The biosynthesis of nisin requires the products of 11 biosynthesis genes. Prenisin, the inactive translation product of the *nisA* gene, undergoes several post-translational modifications, of which the key reaction is the selective dehydration of serine and threonine residues in the precursor molecule, leading to Dha and Dhb, respectively. NisB, which is a membrane-associated enzyme, catalyses this dehydration reaction (Chatterjee et al. 2005; Field et al. 2010; Siegers et al. 1996). Some of these modified amino acids form Lan and MeLan residues as a result of the addition of neighbouring cysteine thiol groups to the unsaturated side groups, catalysed by the cyclase NisC (Li et al. 2006).

Nisin belongs to the type-A(I) subgroup of lantibiotics that contains other well-characterised linear lantibiotics, including subtilin, epidermin and Pep5 (Chatterjee et al. 2005). In these groups of peptides, the Lan and MeLan residues are formed by the action of LanB and LanC proteins respectively. LanB proteins have no close sequence similarity with known proteins in the databases other than members of the LanB family. LanC proteins have a close sequence similarity with members of the LanC family and with the C terminus of proteins that belong to the LanM family (Chatterjee et al. 2005; Siezen et al. 1996). The

A. Karakas Sen · M. J. Ridout · A. Narbad
Institute of Food Research, Norwich Research Park,
Colney, Norwich NR4 7UA, UK

Present Address:

A. Karakas Sen (✉)
Department of Food Technology, Yenisehir Vocational School,
University of Uludag,
Yenisehir, Bursa 16900, Turkey
e-mail: akarakas@uludag.edu.tr

LanC-like proteins (LANCL) have 20–25% sequence identity with the NisC protein. These proteins are found in a wide range of organisms that do not produce lantibiotics, such as *Drosophila melanogaster*, *Xenopus laevis*, *Arabidopsis thaliana*, and some mammals (Li et al. 2006). NisB shares the closest homology (42% identical residues) with the protein NsuB from *Streptococcus uberis*, a natural nisin variant nisin U producer organism (Wirawan et al. 2006).

NisB dehydrates the serine and threonine residues in the precursor peptide (Chatterjee et al. 2005; Karakas Sen et al. 1999). However, no experimental evidence for the in vitro dehydration function of NisB protein has been reported to date (Field et al. 2010). The structural genes, the *nisB* gene and other genes responsible for the biosynthesis of nisin have been sequenced (Dodd et al. 1990; Kuipers et al. 1993). NisB is a large membrane-associated protein of 993 amino acid residues with a calculated molecular weight of 117.5 kDa (Engelke et al. 1992). Its sequence contains a number of potential amphipathic membrane helices, and it has been proposed that nisin modification enzyme NisB forms a membrane-associated complex with the cyclase NisC and the transporter NisT (Siegers et al. 1996; van den Berg van Saparoea et al. 2008).

The purpose of the study reported here was to employ recombinant methods to produce substantial amounts of NisB which can later be used for the in vitro investigation of the dehydration function of NisB protein. In past studies,

some difficulties were encountered by ourselves as well as by others in attempts to isolate a potentially unstable membrane-bound complex (Karakas Sen et al. 1999; Kupke and Gotz 1996). In this study, we have used a His-tagged approach for the purification of NisB protein. Here we report the heterologous expression of the *nisB* gene of a nisin A-producing *Lactococcus lactis* strain in *Escherichia coli* and its partial purification.

Materials and methods

Microbiological techniques, strains and plasmids

The *L. lactis* and *E. coli* strains used in this study are listed in Table 1. *L. lactis* FI5876 (Dodd et al. 1990) was grown in M17 medium (Oxoid, Basingstoke Hampshire, UK) supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 30°C without agitation. *E. coli* strains were routinely grown in L broth at 37°C on an orbital shaker. Media plates were made by the addition of 1.5% (wt/vol) agar to liquid broth. Antibiotics (Sigma-Aldrich, St. Louis, MO) were added as selective agents when appropriate: ampicillin 50 µg ml⁻¹ (for selection in broth) and 200 µg ml⁻¹ (for selection of transformants on plates); chloramphenicol 34 µg ml⁻¹. The more stable carbenicillin (50 µg ml⁻¹) was used in place of ampicillin in cultures grown for protein purification. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-

Table 1 Strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source/reference
Strains		
<i>Lactococcus lactis</i> FI5876	MG1614::Tn5301	Dodd et al. 1990
<i>Escherichia coli</i> TOP10	Host strain for plasmid constructions	Invitrogen, Carlsbad, CA
<i>E. coli</i> BL21 (DE3)	Expression host	Studier and Moffatt 1986
<i>E. coli</i> BL21-Gold(DE3) pLysS	Expression host, pLysS (Cm ^r)	Stratagene, La Jolla, CA
FI9748	<i>E. coli</i> TOP10/pFI2268, Amp ^r	This study
FI9751	<i>E. coli</i> TOP10/pFI2269, Amp ^r	This study
FI9752	<i>E. coli</i> TOP10/pFI2270, Amp ^r	This study
FI9753	<i>E. coli</i> BL21-Gold(DE3)pLysS/pFI2270, Amp ^r , Cm ^r	This study
FI9754	<i>E. coli</i> BL21-Gold(DE3)pLysS/pET15b, Amp ^r , Cm ^r	This study
FI9755	<i>E. coli</i> BL21 (DE3)/pFI2270, Amp ^r	This study
FI9756	<i>E. coli</i> BL21 (DE3)/pET15b, Amp ^r	This study
Plasmids		
pCR 2.1-TOPO	3.9-kb general PCR cloning vector, Amp ^r	Invitrogen, Carlsbad, CA
pET-15b	5.7-kb His-tagged expression vector, Amp ^r	Novagen, UK
pFI381	Derivative of pMTL23 containing <i>nisB</i> gene	IFR, UK
pFI2268	Derivative of pCR 2.1-TOPO containing <i>NdeI/MunI nisB</i> gene fragment	This study
pFI2269	Derivative of pFI381 containing <i>NdeI/NdeI nisB</i> fragment	This study
pFI2270	Derivative of pET-15b containing <i>NdeI/NdeI nisB</i> fragment	This study

Amp^r, Ampicillin resistance; Cm^r, chloramphenicol resistance

galactopyranoside (X-Gal) was used at a final concentration of 40 $\mu\text{g ml}^{-1}$. The *E. coli* cells were induced with 1 mM isopropyl β -D-1-thiogalactoside (IPTG) for recombinant protein expression.

Molecular techniques

Plasmid DNA isolation, DNA manipulation and transformation of *E. coli* strains with recombinant plasmids were carried out as previously described (Dodd et al. 1990). Restriction enzymes and other DNA-modifying enzymes from various sources were used according to the suppliers' recommendations. Restriction DNA fragments were isolated from an agarose gel by electrophoresis on a DEAE-cellulose membrane using the method described by Dretzen et al. (1981). Caesium chloride (CsCl) gradient DNAs of pFI2268 and pFI381 (Table 1) were prepared according to Sambrook et al. (1989). Primers were synthesised on an Applied Biosystems DNA synthesiser (model 381A; Applied Biosystems, Foster City, CA). The PCR was performed with the following thermal cycling programme: an initial cycle of 2 min at 96°C, followed by 5 cycles of 1 min at 92°C, 1 min at 37°C and 1 min at 72°C, then by 20 cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C and terminated with a final cycle of 1 min at 92°C, 1 min at 55°C and 10 min at 72°C. Fragments generated for the construction of vectors were amplified with DyNAzyme λ DNA polymerase (Flowgen Biosciences, Nottingham, UK) and cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) before nucleotide sequence confirmation. For routine PCR screening of recombinant clones, AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA) was used. The nucleotide sequences of PCR-generated fragments were confirmed on purified plasmid DNA with an Applied Biosystems DNA sequencer (model 373A) and the manufacturer's Taq DyeDeoxy Terminator Cycle Sequencing kit.

Cloning of the *nisB* gene into expression vector pET-15b

The N-terminal region of the *nisB* gene was amplified by PCR from chromosomal DNA of *L. lactis* FI5876 (Fig. 1A, a) using primers NisB-Fwd and NisB-Rev (Table 2). Primer NisB-Fwd included an *NdeI* site which is in frame with the start codon of the *nisB* gene. Primer NisB-Rev was close to the downstream site of *MunI*. An amplified 332-bp fragment was initially ligated into T/A cloning vector pCR 2.1-TOPO using the TOPO TA cloning kit (Invitrogen), and *E. coli* TOP10 competent cells were transformed with this ligation mixture according to the manufacturer's instructions. Transformants were selected on L-agar plates containing ampicillin and X-gal and were screened by PCR using M13 Reverse and Universal M13 Forward primers

Table 2 Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5'→3') ^a
NisB-Fwd	AGCATATGATAAAAGTTCATTTAA
NisB-Rev	ACTGTACTGTGAACCTTTTCGAA
T7 Promoter-Fwd	TAATACGACTCACTATAGGG
T7 Terminator-Rev	GCTAGTTATTGCTCAGCGG
M13 Reverse	AGCGGATAACAATTTACACAGGA
Universal M13 Forward	GTTTTCCCAGTCACGACGTTG

^aThe restriction site (*NdeI*) in the oligonucleotide sequence is underlined. The transcriptional start codon is given in bold

(Table 2) for the presence of recombinant plasmids. Plasmid DNAs were prepared from the positive clones and analysed by restriction digestion using *EcoRI* for confirmation of the expected construct. The identity of the inserted fragment was further confirmed by DNA sequencing. This plasmid was labelled as pFI2268 (Fig. 1A, b), and the *E. coli* strain harbouring pFI2268 was designated as FI9748 (Table 1).

Plasmids pFI2268 and pFI381 were purified by CsCl gradient centrifugation. The *BamHI/MunI* fragment containing the 5' end of the *nisB* gene was extracted from plasmid pFI2268 and ligated into the equivalent *BglII/MunI* sites of plasmid pFI381 to regenerate an intact *nisB* gene (Fig. 1A, c, d). *E. coli* TOP10 cells were transformed with this ligation mixture, and transformants were selected on ampicillin plates. Some transformants were screened by colony PCR using M13 Forward and Reverse Universal primers to confirm the presence of recombinant plasmid. In addition, plasmid DNA was prepared from transformants and analysed by restriction digestion. A plasmid containing the correct fragment was selected and labelled pFI2269 (Table 1; Fig. 1A, d). The *E. coli* strain harbouring this plasmid was designated as FI9751 (Table 1).

The plasmid pFI2269 was digested with *NdeI* and *BglII*, which generated a 3.0-kb *NdeI/NdeI* fragment containing the intact *nisB* gene. *BglII* was included in the digestion as it cuts the vector into small fragments, facilitating the isolation of the *nisB* fragment from the agarose gel. The *NdeI/NdeI* fragment was ligated with *NdeI*-digested pET-15b (Fig. 1A, e, f) and the ligation mixture was used to transform *E. coli* TOP10 competent cells. Transformed colonies were screened by PCR using primers T7 Promoter-Fwd and NisB-Rev (Table 2), and further confirmation was provided by *NdeI* digestion of plasmid DNA. The orientation of the inserted fragment was checked by sequencing from both ends of the inserted segment using primers T7 Promoter-Fwd and T7 Terminator-Rev. The resulting plasmid, pET-15b-*nisB*, was named pFI2270 (Table 1; Fig. 1A, f), and the competent *E. coli* host strain with pFI2270 was designated FI9752.

Expression of the *nisB* gene in *E. coli*

The pET-15b construct (pFI2270) carrying the *nisB* gene encoding His₆-tagged NisB (His6-NisB) was used to transform the expression hosts *E. coli* BL21-Gold(DE3) pLysS and *E. coli* BL21 (DE3), generating FI9753 and FI9755, respectively. Transformation of these *E. coli* hosts with pET-15b yielded the strains FI9754 and FI9756, respectively (Table 1). *E. coli* cells were grown at 37°C to an OD₆₀₀ of 0.6–0.8 with the appropriate antibiotic (Table 1). His6-NisB expression was induced by adding IPTG and cultivation was then continued at 37°C for 4 h. All subsequent steps were performed at 4°C. Cells were harvested by centrifugation, and the pellet was washed once with 200 mM Tris-HCl, pH 8.0, and resuspended in the same buffer containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was sonicated at 20 amplitude microns using an MSE Soniprep (MSE, London, UK). To prevent heating, the vessel containing the cell suspension was surrounded by ice. Sonication was applied in short bursts of 20 s (with 1-min cooling period) with a total exposure time of 1.7 minutes. The sonicate was centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant was designated as the soluble fraction. The pellet was resuspended in the membrane extraction buffer [100 mM Tris-HCl pH 7.0, 8 M urea, 3% sodium dodecyl sulfate (SDS), 0.5% Triton X-100] containing 1 mM DTT and 1 mM PMSF, vortexed vigorously, and centrifuged for 2 min at 3,000 g. The resulting supernatant was designated as the membrane fraction. The fractions were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis as described by Karakas Sen et al. (1999).

Purification of His6-NisB protein

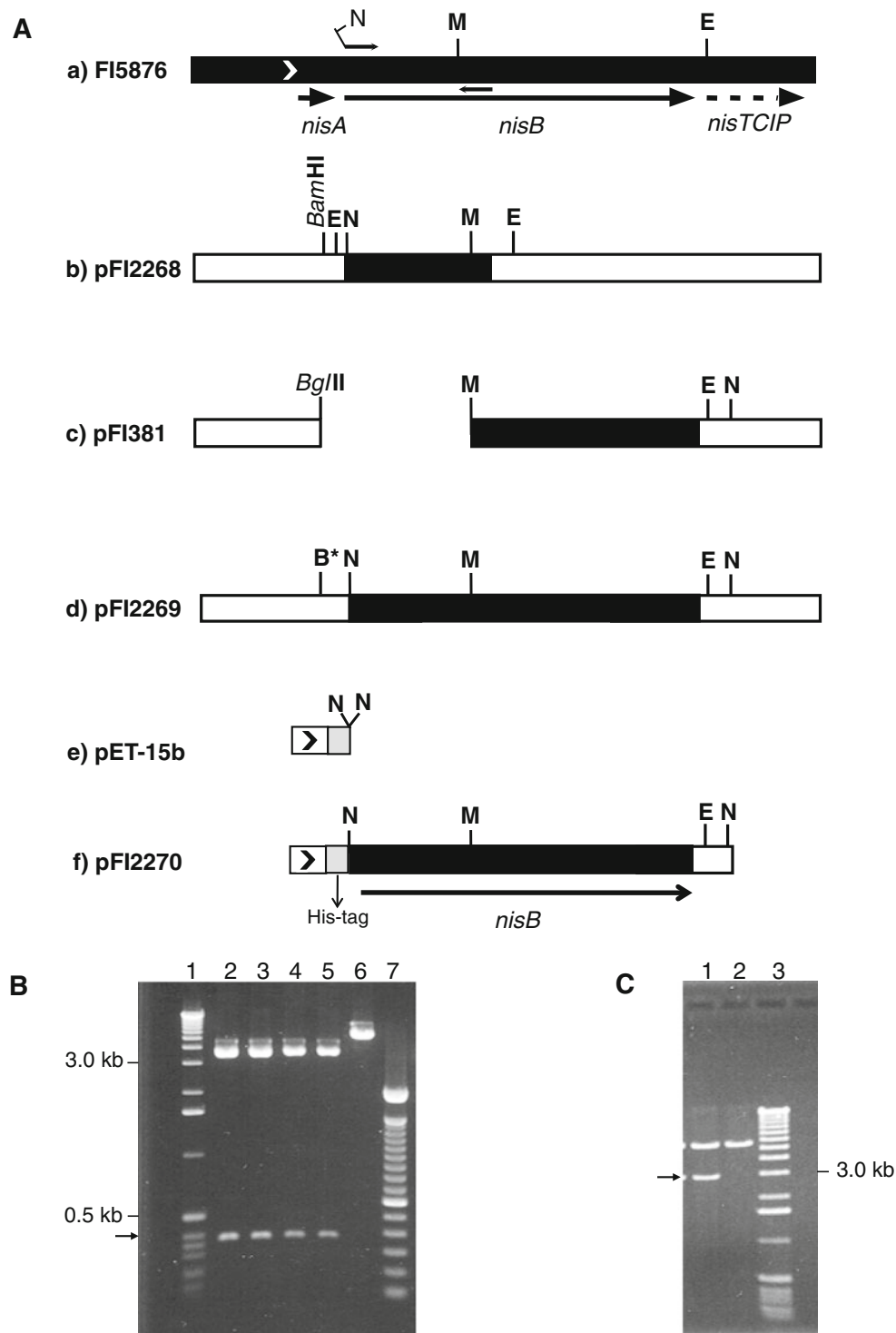
For purification of the His6-NisB protein, the cultures of FI9753 were grown at 37°C to an OD₆₀₀ of 0.6–0.8 in L Broth containing 50 µg ml⁻¹ carbenicillin and 34 µg ml⁻¹ chloramphenicol. Expression of the His6-NisB protein was induced by adding IPTG to a final concentration of 1mM, and cultivation was then continued at 25°C for 5 h. The cells were then harvested by centrifugation. The pellet was washed once with 20 mM phosphate buffer (pH 7.4) and resuspended in the same buffer containing 10 mM imidazole, 500 mM NaCl, 1 mM DTT and 1 mM PMSF. Cells were sonicated as already described. After centrifugation at 20,000 g for 30 min at 4°C, the supernatant was filtered through a 0.45-µm filter and loaded onto a pre-charged nickel affinity column. His6-NisB protein was purified on a HiTrap chelating metal-affinity column (Amersham Pharmacia Biotech, Uppsala,

Sweden) on a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Uppsala, Sweden), including a Single Path Monitor UV-1 Control Unit for monitoring absorbance at 280 nm. Buffers used to run the Ni²⁺ affinity column were the binding buffer (20 mM phosphate buffer pH 7.4 containing 10 mM imidazole and 500 mM NaCl) and the elution buffer (20 mM phosphate buffer pH 7.4 containing 1 M imidazole and 500 mM NaCl). After the protein was loaded onto the Ni²⁺ affinity column, it was washed with the binding buffer. Bound proteins were eluted on an imidazole gradient from 10 mM to 1 M. The collected fractions were analysed by SDS-PAGE for the presence of His6-NisB protein, and His6-NisB was confirmed by western blot analysis using antibodies specific for NisB or the His₆-tag (Roche Diagnostics, Indianapolis, NJ), as previously described (Karakas Sen et al. 1999). To remove salt and imidazole, NisB preparation was eluted through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) and then concentrated by using a Centricon-50 filter (Millipore, Billerica, MA). Protein concentrations were measured by a procedure described by Bradford (1976) using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard.

Circular dichroism spectroscopy

To quantify the secondary structure of the purified His6-NisB, the protein sample of His6-NisB was subjected to circular dichroism (CD) spectroscopy analysis at room temperature using a J-710 spectropolarimeter (JASCO,

Fig. 1 Construction of the expression vector for His6-nisin biosynthesis protein (NisB). **A** Map of *Lactococcus lactis* FI5876 chromosome showing the location of the *nisB* gene and inducible *nisA* promoter (white arrowhead) in relation to other *nis* genes, **b** PCR-amplified *NdeI/MunI* fragment containing the 5' end of the *nisB* gene cloned into the pCR 2.1-TOPO vector, generating plasmid pFI2268, **c** map of pFI381 containing the *nisB* gene, digested with *Bgl*II and *Mun*I restriction enzymes, **d** *Bam*HI/*Mun*I fragment containing the 5' end of the *nisB* gene cloned into pFI381, generating plasmid pFI2269, **e** map of expression vector pET-15b showing the cloning region and T7 promoter (black arrowhead), **f** *NdeI/NdeI* fragment containing intact *nisB* gene cloned into pET-15b, creating expression vector pFI2270. The relevant restriction sites, shown above the maps are *NdeI* (N), *MunI* (M), *EcoRI* (E) and the non-functional *Bgl*II/*Bam*HI hybrid site (*B**). **B** Agarose gel electrophoresis of *EcoRI*-digested plasmid DNAs. Plasmid DNA was isolated from *E. coli* transformants and digested with *EcoRI* to examine the presence of *NdeI/MunI* fragment containing the *nisB* gene. Lanes: 1 DNA molecular weight marker (1-kb DNA ladder), 3 plasmid pFI2268, 2, 4–6 other recombinant plasmids, 7 100-bp DNA ladder. Arrow indicates the position of the *NdeI/MunI* fragment, containing the 5'-292-bp fragment of the *nisB* gene. **C** Agarose gel electrophoresis of the *nisB* expression vector pFI2270 (pET-15b-NisB) and pET-15b digested with *NdeI*. Lanes: 1 pFI2270, 2 pET-15b, 3 1-kb DNA ladder. Arrow indicates the position of the *NdeI/NdeI* fragment containing the intact *nisB* gene



Great Dunmow, Essex, UK) in a nitrogen atmosphere. The secondary structure was monitored in the far-UV (195–250 nm) wavelength region using a cell of path length 0.2 mm. The protein concentration was $70 \mu\text{g ml}^{-1}$. CD spectra were collected as an average of three successive scans using a computer connected to the spectropolarimeter.

The buffer scans were subtracted from the protein scans, and the spectra were then smoothed using software provided by JASCO. The CD data are given as molecular ellipticity $[\theta]$ (mdeg). The His6-NisB protein was analysed in 20 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl.

Preparation of substrate prenisin

Prenisin-His6 is a 62 amino acid-long peptide that includes the unmodified nisin with a 22-amino acid length leader peptide at the N-terminus and 6-histidine residues added at the C-terminus using the same pET-15b system (Novagen, Madison, WI), similar to that used for the heterologous expression of NisB protein. The peptide was again purified using a nickel affinity column.

Results and discussion

In order to express the NisB protein with six histidine residues on the N-terminus, the *nisB* gene of *L. lactis* was cloned into the expression vector pET-15b. The pET expression system (Novagen), originally developed by Rosenberg et al. (1987), is now widely used for the expression of genes from different organisms (Deng et al. 2009; Xie et al. 2002). This system has been designed for the exclusive expression of cloned genes and uses a bacteriophage T7 RNA polymerase-promoter system. The *nisB* gene was initially cloned using a host that does not contain the T7 RNA polymerase gene in order to eliminate plasmid instability (Table 1). Once the plasmid constructions were established in a non-expression host, they were then transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene (Δ DE3 lysogen) under *lacUV5* control, and the expression of the *nisB* gene was induced by the addition of IPTG. NisB is a large protein of 993 amino acid residues, encoded by a 2982-bp open reading frame. As there is always the possibility that PCR amplification of this length of sequence may introduce incorrect nucleotide bases, we developed a different strategy which only involved amplification of the 5'-292-bp fragment of the *nisB* gene. Plasmid pFI381 (Table 1; Fig. 1Ac) carrying the *nisB* gene provided the remaining 3' end of the coding region. In order to identify the appropriate digestion sites within plasmid pFI381, we generated a restriction enzyme digestion map of the *nisB* gene using GCG Software (GCG, Genetics Computer Group, Madison, WI), which revealed that a *MunI* restriction enzyme cuts the *nisB* gene 292 bp downstream of ATG start codon. Hence, two primers, NisB-Fwd and NisB-Rev, were designed from the published sequence (Genbank accession number L16226) to amplify the 5'-292-bp fragment of the *nisB* gene. Using this primer set, we were able to successfully isolate the 5'-292-bp fragment of the *nisB* gene by PCR amplification from the chromosomal DNA of *L. lactis* FI5876, and cloned it into the vector pCR (Table 1; Fig. 1A, a, b). The creation of the expression construct pFI2270 is shown in Fig. 1A. The *EcoRI* enzyme analysis of plasmid pFI2268, which carries

the 5'-292-bp fragment of the *nisB* gene and isolated from *E. coli* FI9748, is shown in Fig. 1B (lane 3), confirming the presence of the 332-bp PCR product. The *NdeI* digestion of expression vector pFI2270 (pET15b-NisB) showed the presence of the 3.0-kb intact *nisB* gene fragment (Fig. 1C, lane 1).

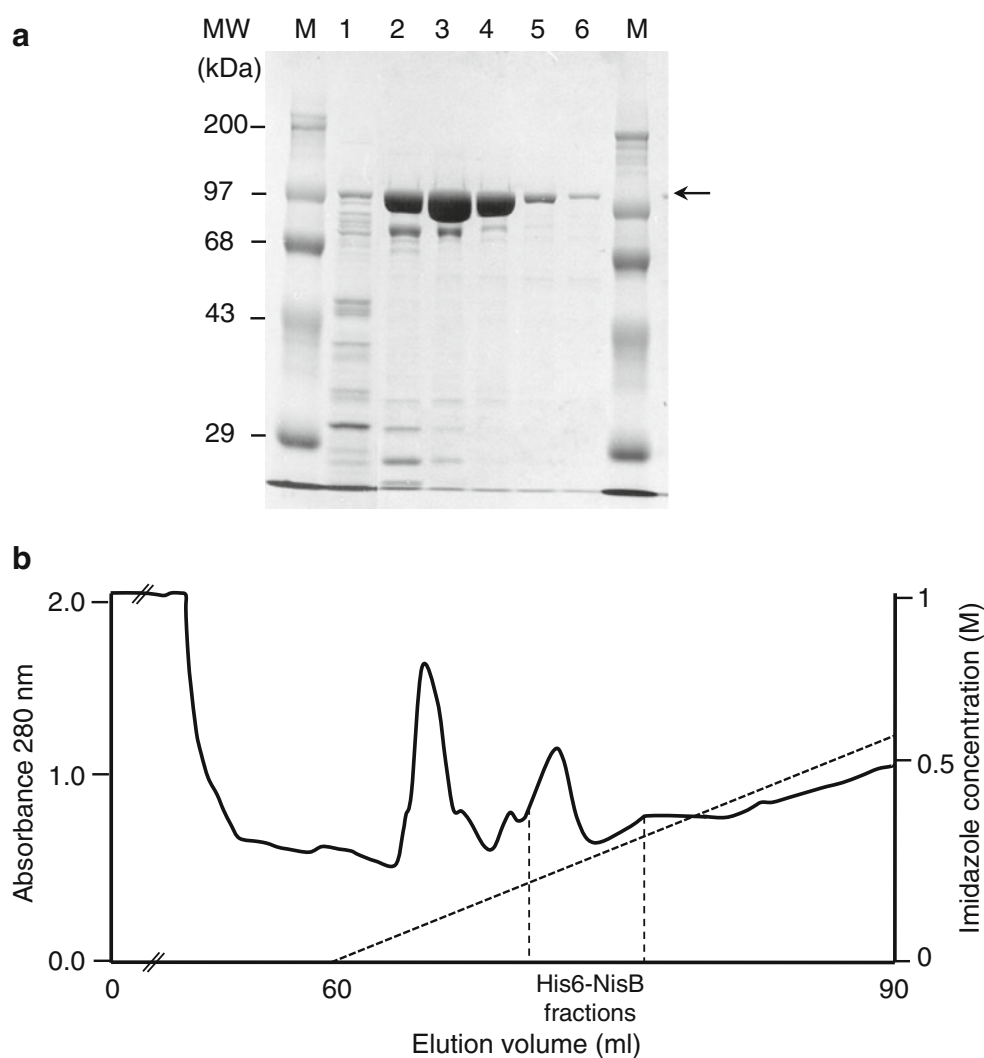
Expression and purification of His6-NisB

NisB protein with N-terminal His₆-tagged (His6-NisB) was expressed in *E. coli* BL21-Gold(DE3)pLysS and *E. coli* BL21 (DE3) strains from the pET-15b-derived construct pFI2270. The *nisB* gene encoding the His6-NisB protein was cloned under the control of a strong T7 promoter for inducible expression. The strain *E. coli* BL21-Gold(DE3)pLysS contains plasmid pLysS, a pET-compatible plasmid that produces T7 lysozyme, hence suppressing the expression of target genes prior to induction with IPTG. The expression of the *nisB* gene in these *E. coli* host strains was confirmed by western analysis with antibodies specific for the NisB protein or the His₆-tag. These results revealed that expression in *E. coli* BL21-Gold(DE3)pLysS yielded more soluble protein than expression in *E. coli* BL21 (DE3) (data not shown). Therefore, the soluble protein fraction of *E. coli* FI9753 strain, *E. coli* BL21-Gold(DE3)pLysS containing expression construct pFI2270, was used for the purification of His6-NisB. Moreover, after induction with IPTG, the lower temperature (25°C) was used to reduce the occurrence of inclusion bodies (Fink 1998). Similar results were obtained when *E. coli* BL21 (DE3) strain was used as the expression host for the purification of SpaB (subtilin dehydratase) (Xie et al. 2002).

To obtain further insight into the biosynthesis of lantibiotics and the involvement of novel post-translational modification reactions, the purification and characterisation of the LanB and LanC proteins and in vitro demonstration of modification reactions are necessary. There have been very few reports in the literature of attempts to purify the LanB proteins. In one study, the *epiB* gene was expressed in *Staphylococcus carnosus* and the EpiB protein purified by a process that involved three chromatographic steps (Peschel et al. 1996). Xie et al. (2002) recently reported that the *spaB* gene was overexpressed as His₆-tag in *E. coli* as a cytoplasmic protein with the aid of the GroEL/ES molecular chaperons and purified using Ni²⁺-affinity chromatography.

In our study, the His6-NisB protein was purified from the soluble protein fraction of *E. coli* FI9753, in a single step on Ni²⁺ affinity HiTrap column with FPLC. The collected fractions were analysed by SDS-PAGE for the presence of His6-NisB protein (Fig. 2a). A protein band of approximately 115 kDa was detected, which is accordance with calculated molecular weight of 119.692 kDa for His6-NisB.

Fig. 2 Purification of His6-NisB protein. The fractions were analysed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (**a**), and the proteins were detected by Coomassie blue staining. *Lanes:* *M* protein molecular weight markers, *1* crude supernatant, *2–6* eluted fractions. **b** Elution profile of NisB on the Ni²⁺ affinity column. *Arrow* indicates the position of His6-NisB protein



His6-NisB protein eluted at 240 mM imidazole concentration, as a single peak (Fig. 2b). The purity of the protein (>90%) was determined by SDS-PAGE, and the identity of His6-NisB was confirmed by western blot analysis using antibodies specific for NisB or the His₆-tag (data not shown).

CD spectra

Circular dichroism spectroscopy is a technique commonly used to study the secondary structure of proteins and peptides in solution (Woody 1996). In this study, the secondary structure of the His6-NisB protein was studied using CD spectroscopy. Figure 3 shows the far-UV CD spectrum (195–250 nm) of His6-NisB, which is characterised by a strong double minima at approximately 209 nm and 222 nm and a stronger maximum at approximately 198 nm; these are characteristic peaks for an α -helical protein (Woody 1996). Fitting data using “SELCON”, an estimate of the secondary structure of His6-NisB was

15.4% α -helix, 35.7% β -sheet, 22.4% turn, and 24.5% random structures.

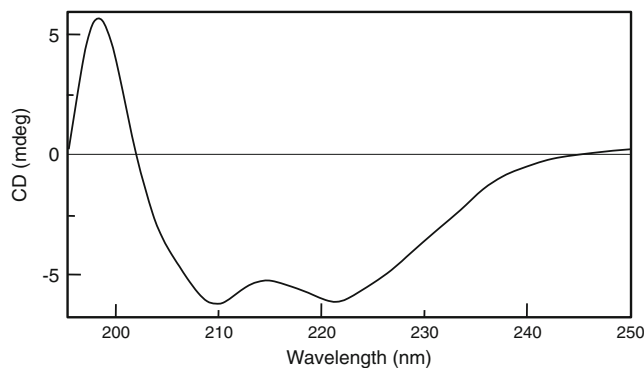


Fig. 3 Circular dichroism (CD) analysis of the His6-NisB protein. The far-UV CD spectrum of His6-NisB was obtained on a JASCO J-710 spectropolarimeter according to the procedure given in the [Materials and methods](#)

In vitro activity of NisB

Studies on the mechanism of action of the modification enzymes can be best performed in vitro. In our study, enzyme assays were carried out using either crude extracts of the NisB from *L. lactis* strain overexpressing the *nisB* gene (Karakas Sen et al. 1999) or the purified NisB protein together with purified His-tagged prenisin as substrate. Some of the cofactors tested during the enzyme assays were pyridoxal 5'-phosphate (P5P), adenosine monophosphate (AMP), adenosine triphosphate (ATP), and MnCl₂. The reactions were performed at 30°C. We used reverse-phase high performance liquid chromatography (RP-HPLC) and electrospray mass spectrometry (ES-MS), or liquid chromatography–mass spectrometry (LC-MS) for the detection and analysis of the prenisin peptide products for the presence of any dehydro-residues. The preliminary enzyme assay results indicated that under the assay conditions used, we were unable to observe the dehydration. Although the in vitro reconstitution of some active LanM enzymes was successful, the in vitro reconstitution of the nisin dehydratase NisB or indeed other LanB enzymes is yet to be achieved despite significant efforts (Field et al. 2010). On the other hand, the overexpression of the *nisB* gene in wild-type nisin- and nisin variants-producing strains resulted in the complete conversion of Ser33 to Dha33, demonstrating the involvement of NisB in the dehydration process (Karakas Sen et al. 1999). Kuipers et al. (2004) demonstrated that in *L. lactis* strain expressing the *nisBT* genes, NisBT was sufficient to dehydrate and export the dehydrated non-lantibiotic peptides and the dehydrated prenisin. In another study, *L. lactis* strain expressing the *nisBTC* genes dehydrated and secreted a wide range of medically relevant non-lantibiotic peptides (Kluskens et al. 2005). More recently Shi et al. (2011) were able to show in vivo modification only in the presence of both NisB and NisC. It is therefore likely that NisB will only function in vitro in the presence of other maturation-specific proteins, implying that lantibiotic biosynthesis enzymes can be used for the creation of a wide range of peptides with novel bioactivities and of biostable analogues. It is also expected that the manipulation of modification enzymes will increase substrate tolerance and thus provide a more flexible approach for the introduction of lantibiotic-associated structures into non-lantibiotic peptides/proteins (Field et al. 2010).

In conclusion, the nisin biosynthesis gene *nisB* from *L. lactis* was successfully cloned into an expression vector and expressed in *E. coli* with an N-terminal extension of six histidine residues (His-tag). The cloning method described here has the advantage of avoiding a number of tedious cloning steps. His6-NisB protein was successfully purified in one step by Ni²⁺ affinity chromatography. The purification was carried out under native conditions in order to

retain the protein activity and yielded a >90% pure product. His6-NisB protein could be obtained in milligram quantities using this procedure for use in in vitro structural and functional studies.

Acknowledgements We thank Nikki Horn (IFR, UK) for the technical assistance with the cloning of *nisB*.

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