Journal of Biomolecular NMR, **18:** 145–151, 2000.
KLUWER/ESCOM

Production of stable isotope enriched antimicrobial peptides in *Escherichia coli*: An application to the production of a ^{15}N-enriched fragment of lactoferrin

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Received 24 May 2000; Accepted 3 August 2000

Key words: antimicrobial peptide, human lactoferrin, isotope labeling, lipopeptide, recombinant peptide

Abstract

A method is described for the production of recombinant isotopically enriched peptides in *E. coli*. Peptides are produced in high yield as fusion proteins with ketosteroid isomerase which form insoluble inclusion bodies. This insoluble form allows easy purification, stabilizes the peptide against degradation and prevents bactericidal activity of the peptide. Cyanogen bromide cleavage released peptide which was conjugated with alkylamines to form lipopeptide. An important advantage of this system is that it allows production of peptides that are toxic to bacteria, which we have demonstrated on a dodecapeptide based on residues 21–31 of human bactericidal protein lactoferrin.

Abbreviations: CNBr, cyanogen bromide; DMF, *N,N*-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; GuHCl, guanidinium hydrochloride; His_6, hexahistidine-tag; HS, homoserine; HSQC, heteronuclear single-quantum coherence; IPTG, isopropyl-1-thio-β-D-galactopyranoside; KSI, ketosteroid isomerase; NOESY, nuclear Overhauser effect spectroscopy; NTA, nitrilotriacetic acid; PCR, polymerase chain reaction; ROESY, rotating frame Overhauser effect spectroscopy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

Introduction

Application of recombinant protein production and particularly their isotopic enrichment has stimulated development of a range of novel multi-dimensional heteronuclear NMR techniques (Bax and Grzesiek, 1993; Wider and Wüthrich, 1999). Isotope enrichment allows determination of structure of larger macromolecules on one hand and on the other provides additional information on molecular dynamics, improvement of the precision of determined structures, discrimination between inter- and intramolecular contacts etc. Peptides are in most cases amenable to assignment and structure determination without the need for isotopic labeling. However, there are many cases where the availability of ^{15}N and/or ^{13}C enriched peptides is beneficial as for example in binding studies of peptides to larger molecules (examples Gizachev et al., 1998; Fraternali et al., 1998) and particularly in the rapid evolving area of solid state NMR for the study of peptides incorporated into the membranes (Marassi and Opella, 1998). Another promising development are techniques for chemical and biochemical ligation of peptides which allow labeling of selected regions of the protein (Muir et al., 1998; Severinov et al., 1998). Bacteria are generally the most cost-effective option for the production of isotopically enriched proteins, but production of peptides is often ineffective since they are generally rapidly degraded. Recently several procedures for preparation of peptides in bacteria have been described which are based on fusion with carrier proteins, which makes them resistant to

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proteolysis and from which the peptides are cleaved off afterwards. In this report we describe the application of peptide production in *E. coli* aimed at isotopic labeling. One of the advantages of the system used in this work is that, besides the high yield of production and ease of preparation, it allows production of peptides that are toxic to bacteria, which we demonstrate on the bactericidal fragment of human lactoferrin. An additional advantage is that the CNBr cleavage product contains a reactive lactone group on its C-terminus which can be derivatized with amino group containing substituents. In this report we describe preparation of ^{15}N labeled antimicrobial dodecapeptide LF12, based on the human lactoferrin (21–31) (Odell et al., 1996; Chapple et al., 1998) and its derivatization with hexylamine.

Materials and methods

Materials

The following materials were used: pET-31b(+) vector (Novagen), T4 DNA ligase (New England Biolabs), *E. coli* strain BL21(DE3)pLysS (ATCC 47092), *E. coli* strain DC2 (CGSC 7139), ^{15}N ammonium chloride (Martek). Oligonucleotides were custom synthesized by The Great American Gene Company (Ramona, CA) in purified (desalted by EtOH) and phosphorylated form. All other reagents were from Sigma and Fluka.

Gene construct preparation

Oligonucleotides for peptide LF12 were LF12-5 (TTTCAGTGGCAACGCAACATTCGTAAAGTGCGCATG) and LF12-3 (GCGCACTTTACGAATGTTGCGTTGCCACTGAAACAT). Primers LF12-5 and LF12-3 were denatured at 95 °C for 10 min and annealed in the ligation buffer and ligated into the pET-31b(+) vector with T4 DNA ligase at 16 °C for 18 h. After transformation into DH5α strain of *E.coli*, colonies harboring the plasmid with insert were identified by PCR using primers KSI (GGCAAGGTGGTGAGCATC) and T7term (TGCTAGTTATTGCTCAGC).

Expression and purification of the fusion protein

Protease deficient strain of *E. coli* (BL21(DE3)pLysS) was used for protein production. Fermentation was performed in shake flasks at 37 °C and 200 rpm.

Table 1. Components of 1 l of the medium for the production of (^{15}N)LF12

1.	985 ml of M9-15N medium, pH 7.4 (autoclaved)	per l
	$Na_2HPO_4 \cdot 2H_2O$	7.5 g
	KH_2PO_4	3.0 g
	NaCl	0.5 g
2.	0.5 ml of trace salts (sterile filtered)	per l
	$CuSO_4 \cdot 7\ H_2O$	6.0 g
	NaI	0.08 g
	$MnSO_4 \cdot H_2O$	3.0 g
	$Na_2MoO_4 \cdot 2H_2O$	0.2 g
	H_3BO_3	0.02 g
	$CoCl_2$	0.5 g
	$ZnCl_2$	20.0 g
	$FeSO_4 \cdot 7H_2O$	65.0 g
	Biotin	0.2 g
	H_2SO_4	5.0 ml
3.	1 g $^{15}NH_4Cl$	
4.	10 ml glucose (300 mg/l) (sterile filtered)	
5.	3 ml 1 M $MgSO_4$ (autoclaved)	
6.	2 ml ampicillin (100 mg/ml)	

Production of the KSI-LF12-His_6 fusion protein was performed in M9 minimal medium, where $^{15}NH_4Cl$ was used for preparation of ^{15}N enriched peptide LF12 (Table 1). When the OD_{600} reached 0.8, transcription of the gene was induced by the addition of 0.4 mM IPTG. Three hours after the induction the cells were harvested, suspended in the lysis buffer (0.1% Na-deoxycholate, 50 mM phenylmethylsulfonylfluoride and 10 mM Tris/HCl pH 8.0) and cells and DNA fragmented by sonication for 6×1 min at 400 W in sonic disrupter (TMX 400, Tekmar). Cell lysate was pelleted for 15 min at 12,000 rpm and 4 °C and pellet washed with lysis buffer, followed by 2 M urea in 10 mM Tris/HCl pH 8.0 and 10 mM Tris/HCl pH 8.0. Inclusion bodies were dispersed in 20 mM Tris/HCl pH 8.0 containing 6 M GuHCl and left to solubilize overnight at room temperature. After centrifugation (15 min at 12,000 rpm and 4 °C), the supernatant was loaded on a 10 ml column of Ni^{2+}-NTA-Agarose (Quiagen), which had been charged with 1 M $NiSO_4$ and equilibrated with buffer containing 5 mM imidazole, 0.5 M NaCl, 6 M GuHCl and 20 mM Tris/HCl pH 8.0. The column was washed first with 5 mM imidazole, 0.5 M NaCl, 6 M GuHCl and 20 mM Tris/HCl pH 8.0 and afterwards with 16 mM imidazole, 0.5 M NaCl, 6 M GuHCl and 20 mM Tris/HCl pH 8.0. Fusion protein was eluted with 300 mM imidazole, 0.5 M NaCl and

6 M GuHCl in 20 mM Tris/HCl pH 8.0. Peak fractions were pooled and dialyzed overnight at 4 °C against H_2O containing 1 mM EDTA pH 8.0 in 10 kDa MW cutoff dialysis bag.

CNBr cleavage of the fusion protein

White pellet resulting from dialysis was solubilized in 70% TFA to a concentration of 10 mg/ml of the KSI-LF12-His_6 fusion protein and transferred to a 100 ml round-bottomed flask. CNBr was added to the sample solution at 100-fold molar excess over all methionyl residues in the fusion protein. Nitrogen was bubbled in for 1 min, and the flask was wrapped in aluminium foil and incubated at room temperature for 24 h. Reaction products were dried under vacuum in a rotary evaporator at 30 °C and washed 3 times with H_2O. A water suspension of the reaction products was stirred at room temperature overnight under nitrogen and protected from light. Afterwards the suspension was centrifuged at 12,000 rpm for 15 min at 4 °C. Soluble peptide LF12 in the supernatant was concentrated by rotary evaporation (Speed Wac DNA 110, Savant) and analyzed by RP-HPLC.

Purification and derivatization of the peptide

Peptide LF12 was purified by RP-HPLC on a Lichrospher 100RP C-18 column. Elution was carried out at room temperature with a linear gradient from 0 to 100% or from 30 to 70% of buffer B over 20 min at a flow rate of 0.5 ml/min with the absorbance monitored at 280 nm (where buffer A was 5% acetonitrile and 0.1% TFA in H_2O and buffer B was 80% acetonitrile and 0.1% TFA in H_2O) using a chromatograph VWM (Knauer). The resultant LF12 peptide fractions were collected and dried under vacuum in a centrifuge evaporator (Savant). Peptide concentrations were determined by measuring absorbance at 280 nm. The CNBr cleavage was also monitored by SDS-PAGE. SDS-PAGE was carried out on a 12% acrylamide resolving gel and stained with Coomassie blue.

Derivatization of the LF12 peptide was performed as follows: 170–460 nmol of purified LF12 was first dried in a centrifuge evaporator (Savant), completely lactonized by addition of 20 μl of 100% TFA and again immediately dried in a centrifuge evaporator. Dried pellet was dissolved in 50 μl of anhydrous DMF, delivered by a gas-tight syringe, and 8 μl of Et_3N was added. Hexylamine at 100-fold molar excess over peptide was added to the lactonized peptide solution and incubated overnight at 45 °C. Conjugate of the peptide was isolated by HLPC, as already described, and analyzed by FAB-MS and antibacterial assay.

Figure 1. Schematic representation of pLF12, expression vector for peptide LF12. Structural genes of pET-31b(+) vector (KSI and His_6) were used for production of ^{15}N isotopically enriched recombinant LF12. The KSI-LF12-His_6 fusion protein is placed under the control of T7 lac promotor, which is recognized as a transcription start site by the T7 RNA polymerase additionally controlled by *lac* repressor.

Analysis of the peptide and its conjugate

Identity of peptides was confirmed by mass spectrometry and NMR. FAB-MS measurements were performed on a mass spectrometer (AutoSpec, MicroMass, Manchester, UK) using glycerol or nitrobenzyl alcohol as matrices and a Ce-ion gun.

Peptide and its conjugate were analyzed by an antimicrobial assay using *E. coli* strain DC2 and BL21 on spotting 10 μl of different concentrations of peptide, peptide conjugate and as a control polymyxin B and ampicillin were on top of the agar. Antibacterial activity was determined as a region of zero cell density on the plate.

NMR experiments

Isolated (^{15}N)LF12 peptide was lyophilized and dissolved at a concentration of 0.7 mM in 20 mM Na-phosphate buffer pH 4.3 in 90% H_2O/10% D_2O. HSQC spectra were recorded on the 600 MHz Inova NMR spectrometer (Varian, CA) equipped with a probe with z-gradients. Spectra were recorded at 30 °C in the sensitivity enhancement mode. Data were apodized and linearly predicted in the indirect dimension. 2D versions of 3D NOESY-HSQC and TOCSY-HSQC experiments with mixing times between 100 and 200 ms were recorded using pulse sequences from the ProteinPack suite from Varian. As the NOE signals were very weak, a 2D ROESY experiment was performed as well.

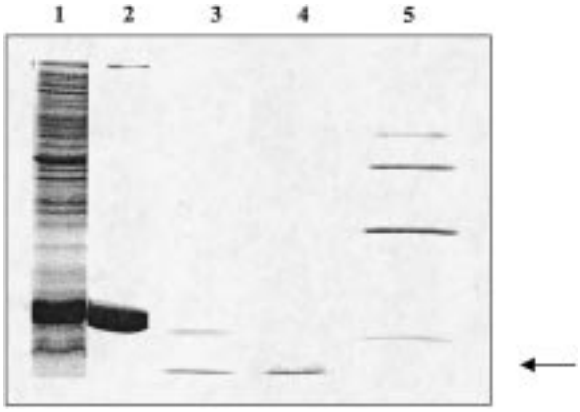

Figure 2. SDS-12% polyacrylamide gel stained with Coomassie Blue R-250. Lane 1, inclusion bodies of the KSI-LF12-His_6 fusion protein; lane 2, purified fusion protein (eluted from His_6-Ni^{2+}-chelating affinity column under denaturing conditions); lane 3, fusion protein after CNBr cleavage (lane at 14.2 kDa represents uncleaved fusion protein); lane 4, purified recombinant LF12 (RP-HLPC); lane 5, MW standards (66.0, 48.5, 29.0, 18.4 and 14.2 kDa). Position of purified recombinant LF12 is indicated by an arrow.

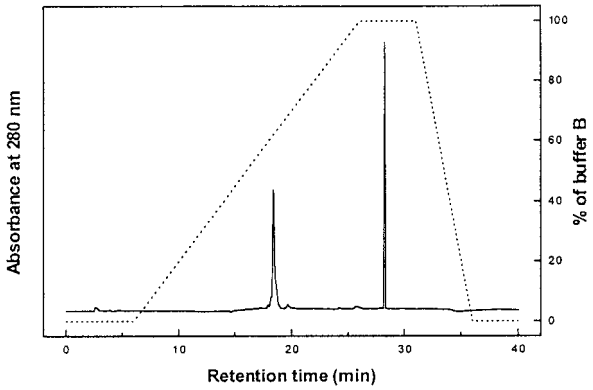

Figure 3. Reversed-phase HPLC purification of recombinant LF12 after CNBr cleavage of the KSI-LF12-His_6 fusion. The peak at 18 min (at approximately 70% of buffer B) represents purified recombinant LF12 and the peak at 28 min (100% of buffer B) uncleaved KSI-LF12-His_6 fusion protein.

Results

Expression and purification of the peptide

We have expressed in *E. coli* an antimicrobial dodecapeptide LF12 where a methionine at position 27 in the original sequence of human lactoferrin was replaced by isoleucine. This modification was not expected to cause major alterations in properties of the peptide as Ile is also found at position 27 in the porcine variant of lactoferrin. The expression plasmid for production of isotopically enriched peptides used in this work contains the gene for ketosteroid isomerase and C-terminal hexahistidine tag (Figure 1). Insertion of coding sequence for the peptide is facilitated by the recognition site for the restrictase *Alw*NI which produces an overhang of 3 arbitrary nucleotides (in this case methionine coding ATG) bounded on each side by trinucleotide recognition site. KSI has been engineered for removal of all methionines so the only CNBr cleavage sites in the fusion protein are on the border of peptide with KSI and hexahistidine respectively (Figure 1). This construct also supports arrangement of several concatenated sequences for the peptide separated by methionine residues (Kuliopulos and Walsh, 1994). KSI-LF12-His_6 fusion protein was produced in *E. coli* in high yield in the form of inclusion bodies. Yield of the KSI-LF12-His_6 fusion protein exceeded 200 mg/l of the bacterial culture in M9 minimal medium. In complex growth medium (LB) the yield of the KSI-LF12-His_6 fusion protein was higher (400 mg of the fusion protein per liter of the bacterial culture). The hexahistidine-tag at the C-terminus of the fusion protein enabled a one-step purification of the fusion protein via Ni^{2+}-chelating affinity chromatography under denaturing conditions (Figure 2). Isolated protein was sufficiently pure for subsequent cleavage with CNBr. The fusion protein contains a relatively large fraction of positively charged residues and was soluble in 70% TFA or 80% formic acid. After CNBr cleavage at methionyl residues, the KSI moiety precipitated while the LF12 peptide was soluble and was separated from other peptides (i.e., terminal His_6 containing peptide) by HPLC. Extensive washing of inclusion bodies without a chromatographic step supplied >90% pure protein which was also sufficient purity for the cleavage step. Final yield of the purified peptide was approximately 6 mg per liter of complex medium. ^{15}N uniformly labeled LF12 was prepared, isolated and the level of ^{15}N enrichment verified by FAB-MS (Figure 4). Experimental molecular weights of natural abundance LF12 and (^{15}N)LF12 determined by FAB-MS were 1613.6 Da and 1640.2 Da; these values are comparable within experimental error with the theoretical values of 1613.8 and 1639.8, respectively.

NMR experiments

The ^{1}H-^{15}N HSQC spectrum of the ^{15}N enriched peptide contains the expected number of crosspeaks, including the side chain amide of tryptophan, therefore confirming that no modification of the tryptophan residue occurred during CNBr cleavage. Assignments

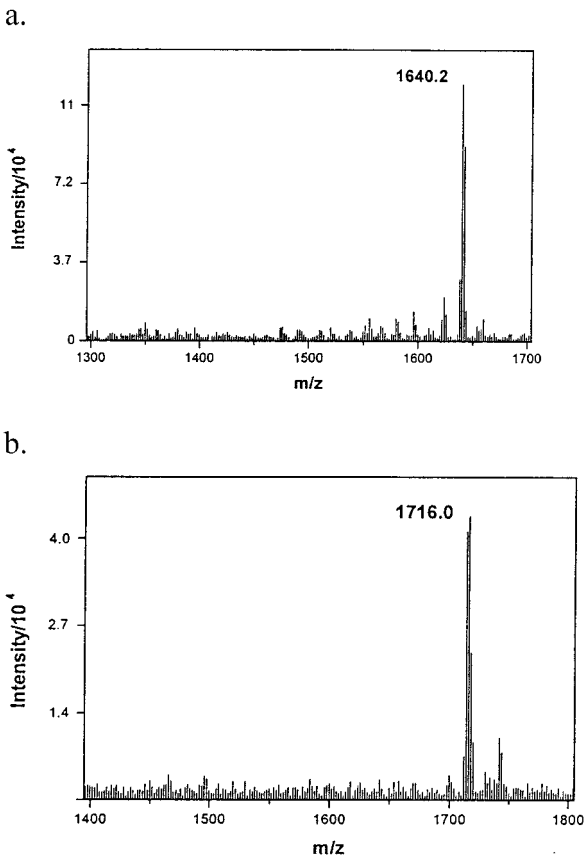

Figure 4. Mass spectra (intensity vs. mass/charge) of (^{15}N)LF12 (a) (theor. 1639.8 Da) and (b) natural abundance LF12 in hexylamidated form (theor. 1714.9 Da).

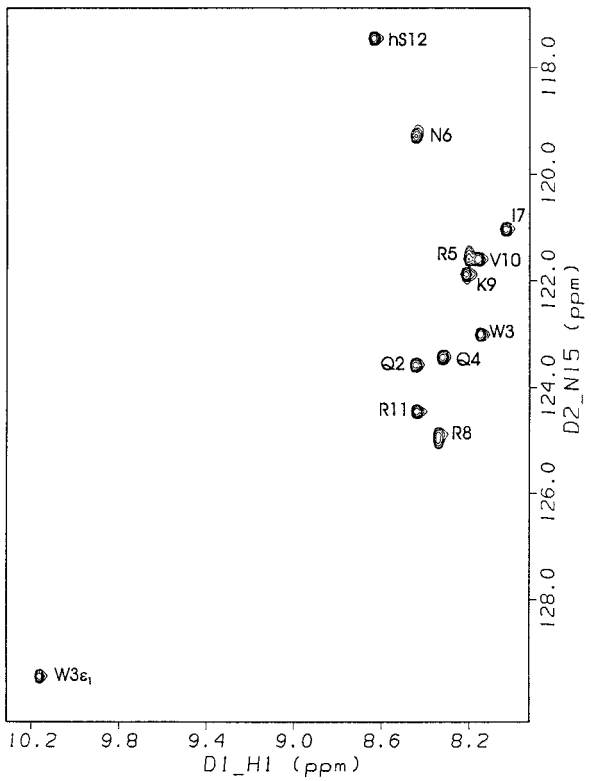

Figure 5. Backbone amide region of ^{1}H-^{15}N HSQC spectrum of (^{15}N)LF12 in 20 mM Na-phosphate buffer pH 4.3 in 90% $H_2$0/10% D_2O at 30 °C with assignments.

of the amide crosspeaks were based on a combination of the homonuclear and ^{15}N edited TOCSY, NOESY and ROESY spectra which were essentially 2D versions of 3D experiments. No NOE crosspeaks were observed that would indicate the presence of a defined tertiary structure of the free peptide in solution.

Addition of alkyl chain to the peptide

Cleavage of KSI-LF12-His_6 fusion protein with CNBr generates LF12 peptide with C-terminal HS-lactone residue (FQWQRNIRKVR-HS-lactone). The reactive HS-lactone group can be used to create peptide conjugates by reaction of lactone group with amines. It is known for hydrophobic and amphipathic antimicrobial peptides that their interaction with membrane plays an important role in their antimicrobial effects (Epand and Vogel, 1999). Polymyxin B, a cationic cyclic peptide antibiotic, has a 6-heptanoyl/octanoyl diaminobutyryl group, which is essential for its antimicrobial activity (Rustici et al., 1993). Alkylamines can therefore be used to modify the hydrophobicity of a peptide. In this article we present derivatization of the recombinant peptide LF12 with hexylamine. This peptide conjugate was purified by HPLC, analyzed by mass spectrometry (MW 1716.0, theor. 1714.9) and assayed for antibacterial activity. Antibacterial activity of recombinant LF12 and its hexylamidated conjugate was determined on *E. coli* strain DC2 (Kondejewski et al., 1996) as well as on strain BL21(DE3)pLysS, which was used for its production. Minimal bactericidal concentration of the recombinant peptide was below 1 μg/ml and decreased approximately fivefold in hexylamidated conjugate and even further with increasing length of the alkyl chain (Majerle et al., manuscript in preparation). Results of this antibacterial assay indicate that recombinant LF12 is functionally equal to the synthetically prepared peptide HLT2, also based on the human lactoferrin (21–31) (Odell et al., 1996; Chapple et al., 1998) confirming that the Met27Ile replacement did not affect its activity and that the C-terminal HS-lactone residue generated by

CNBr cleavage has no adverse effect on antimicrobial activity of LF12 peptide as well.

Discussion

NMR studies of peptides in larger macromolecular assemblies such as membranes, complexes with DNA or receptors can benefit from labeling with NMR-active stable isotopes. A particularly active area of the NMR research is devoted to study of antimicrobial peptides, which are produced as a defense against the microbes by various organisms ranging from animals (mammals, amphibians, insects) to fungi and plants. The possibility to use antimicrobial peptides as a therapeutic alternative due to increase of bacterial resistance to classical antibiotics requires a detailed knowledge of the mechanism of action of antimicrobial cationic peptides which is still much debated (Vorland et al., 1999). Many of those peptides do not have a defined tertiary structure in solution but become ordered upon binding to the membrane. Lipid micelles are often used as a membrane model system, which, due to their correlation times, are within the range of high resolution NMR experiments (Gesell et al., 1997; MacKenzie et al., 1997; Schibli et al., 1999). The closest approximation to biological systems are complexes of peptides with bilayer membranes which can be studied either by solid state NMR of oriented bilayer, magic angle spinning techniques or in magnetically oriented bicelles (Sanders and Landis, 1995; Marassi and Opella, 1998) when the application of isotopically labeled peptides is clearly desired (Marassi et al., 1997). In this paper we report on the preparation of isotopically enriched antimicrobial membrane interacting peptide in bacteria and its chemical modification with applicability in structural studies of membrane interacting peptides. The production cost of such peptides is lower compared to chemical synthesis, particularly once the expression system has been established.

In comparison with proteins only few expression systems for production of recombinant peptides in bacteria have been reported. Intracellular degradation of mostly unstructured peptides is prevented by fusion of peptides to larger proteins or by multimerization (Jansson et al., 1996; Campbell et al., 1997; Jonasson et al., 1998; Kohno et al., 1998). However, systems which produce soluble fusion fail if one desires to produce antimicrobial peptide due to the products' toxicity and one has to resort to producing proteins that are insoluble in *E. coli*. Such a system is fusion with ketosteroid isomerase (Kuliopulos and Walsh, 1994), a protein which is highly insoluble in water as well as in the cytoplasm of bacteria. Antimicrobial peptide moiety is thus sequestered to inclusion bodies and prevented from reaching the cellular membrane. Employing this system in vector pET-31b(+) (Novagen) we have produced ^{15}N-labeled antimicrobial dodecapeptide LF12, based on the sequence of the positively charged portion (residues 21–31) of the loop region of human lactoferrin which is, when solubilized by release from the fusion with KSI, bactericidal to bacteria which produce it.

We can envisage the use of this procedure for many other applications such as for example production of peptides for receptor-ligand binding studies based either on transferred NOE (Clore and Gronenborn, 1982; Sykes, 1993; Ni, 1994), complex formation or chemical ligation of peptides which would extend its use for larger proteins (Muir et al., 1998; Severinov et al., 1998). Recent experiments on binding of isotopically labeled peptides to the receptor anchored in magnetically oriented membrane fragments (Koenig et al., 2000) extend the use of labeled peptides towards determination of angular orientation of the ligands with respect to the membrane axis via the transferred dipolar coupling effect. The single most important limitation of the described method is that the peptide must not contain any internal methionine residues. This restriction is in most cases not exceedingly severe as methionine is one of the residues with the lowest frequency, its mutability is above average (Jones et al., 1991) and can often be substituted, as we have shown, by a conservative replacement with Ile, Leu or Val. CNBr cleavage peptide product contains additional homoserine lactone at the C-terminus, which is very convenient for chemical modification with various functional groups. Our goal is to provide a high-resolution NMR structure of the ^{15}N-labeled recombinant LF12 in lipid environment and in complex with lipopolysaccharides, which would provide structural information on interactions in the initial binding step of peptides such as lactoferricin to Gram-negative bacteria (Vorland et al., 1999).

Conclusions

From our work on LF12 peptide we conclude that the histidine-tagged KSI fusion protein system is very efficient particularly for the production of antimicrobial isotopically enriched peptides. This method is simple

and gives chemically homogeneous peptide product. We succeeded in producing in *E. coli* a ^{15}N enriched antimicrobial peptide, which is toxic to bacteria. Additional advantage of the system is the possibility of producing lipopeptides useful for the study of peptide interaction with membranes.

Acknowledgements

We thank Dr Bogdan Kralj for measuring the mass spectra of peptide and its conjugates. This work was supported by the grant from the Ministry of Science and Technology of Slovenia. NMR experiments were performed on the instrument at the Slovenian National NMR Center at the National Institute of Chemistry in Ljubljana. Mass spectra were determined at the National Mass spectrometry center at the Jozef Stefan Institute in Ljubljana, Slovenia.

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