Purification and structure of mutacin B-Ny266: a new lantibiotic produced by *Streptococcus mutans*

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Abstract Mutacins are bactericidal substances of proteinaceous nature produced by *Streptococcus mutans*. Lantibiotics are antibacterial substances containing post-translationally modified amino acids such as lanthionine. Mutacin B-Ny266 was purified from the cell pellet of *S. mutans* strain Ny266 by ethanol extraction at pH 2.0 followed by reversed-phase chromatography (Sep-Pak® cartridge) and by HPLC on a C_{18} column. The mean purification factor was 3240 ± 81 and the mean yield was 1.0 ± 0.1%. Molecular mass of mutacin B-Ny266 as determined by mass spectroscopy is 2270.29 ± 0.21 Da. The amino acid sequence of the purified active fraction was obtained by Edman degradation after treatment with alkaline ethanethiol. Twenty-one amino acids were detected in this analysis. Mutacin B-Ny266 belongs to the type A lantibiotics. The proposed sequence is: F–K–A–W–U–F–A–Abu–P–G–A–A–K–O–G–A–F–N–U–Y–A. The molecule differs from that of epidermin/staphylococcin 1580 and gallidermin at positions 1, 2, 4, 5 and 6.

Key words: Mutacin; Bacteriocin; Lantibiotic; *Streptococcus mutans*; Amino acid sequence

1. Introduction

Bacteriocins produced by Gram-positive bacteria can be defined as inhibitory substances having a bactericidal mode of action and an essential protein moiety [1]. Bacteriocins produced by *Streptococcus mutans* were termed mutacins by Hamada and Ooshima [2]. Although there are many reports which show that *S. mutans* produces inhibitory substances, only a few such inhibitors have been isolated and characterized as mutacins [3–13]. The well-studied mutacins include: RM-10 [5], C3603 [13], JH 1000 [7], GS-5 [12], MT 3791 and MT 6223 [6,9], mutacin-b [4], mutalipocins [8] and J-T8 [3,10,11].

We previously published a preliminary classification of mutacins [14], but a definitive classification will have to await the complete chemical characterization of these substances. Among the known mutacins, only T8, which belongs to group J [14], was identified as a lantibiotic and its first eight N-terminal amino acid residues were determined by Novák et al. [10]. Lantibiotics are defined as bacterium-derived ribosomally synthesized lanthionine-containing peptides with antibiotic activity [1,15,16]. They generally contain unsaturated amino acids like 2,3-didehydroalanine, and (2)-2,3-didehydrobutyrine. The lantibiotics are divided into two types [1,15–17]. Type A comprises screw-shaped, amphipathic molecules with molecular masses between 2151 and 4635 Da and with 2–7 net positive charges. Type B consists of more globular molecules with molecular masses between 1825 and 2042 Da and with either no net charge or a net negative charge.

In this paper, we present the results of the purification and the determination of the first complete sequence of a lantibiotic produced by *S. mutans*. Mutacin B-Ny266 was previously shown to inhibit 98% of the *S. mutans* strains tested [14] and all tested Gram-positive bacteria including *Listeria monocytogenes*, *Clostridium sporogenes*, *Mycobacterium phleï*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis* [18]. In the present work, the mature peptide was purified to homogeneity and its amino acid sequence was determined as: F–K–A–W–U–F–A–Abu–P–G–A–A–K–O–G–A–F–N–U–Y–A (underlined residues participate in lanthionine or β-methyllanthionine bridges; see Section 3).

2. Material and methods

2.1. Mutacin production

Strain Ny266 of *Streptococcus mutans* was obtained from J.J. van der Hoeven (Dept. of Preventive Dentistry, University of Nijmegen, Nijmegen, The Netherlands). An overnight preculture in M17 broth (BDH Inc., St-Laurent, QC, Canada) was used to inoculate (1%) 1 l of M17 broth in 2-l flasks. The cultures were incubated at 37°C under aerobic conditions in a standing incubator for 24 h.

2.2. Determination of inhibitory activity

Trypticase Soy Agar (Difco Laboratories, Detroit, MI) plates enriched with 0.3% yeast extract were overlaid with 5 ml of melted TSAYE containing a standardized suspension (0.2 ml of an exponentially growing culture at an optical density of 0.1 at 600 nm) of *Micrococcus luteus* ATCC 272 (ATCC, Rockville, MD). In order to assess the activity of mutacin samples, 2-fold dilutions were prepared in 0.11% TFA (Sigma Chemical Co., St. Louis, MO) and 5 μl of each dilution deposited on top of the indicator plates. Mutacin activity was expressed in arbitrary units, which correspond to the reciprocal of the last dilution giving a clear inhibition zone against *M. luteus* after 24 h of incubation at 37°C under aerobic conditions [19].

The specific activity of each preparation was expressed in AU/mg protein. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad, Mississauga, Ontario, Canada).

2.3. Mutacin extraction and purification

One liter of a 24-h culture of *S. mutans* Ny266 was centrifuged at 10000×*g* for 15 min and the mutacin was extracted from the cell pellet with a mixture of 20 ml of HCl (20 mM) and 80 ml of ethanol

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Abbreviations: Abu, 2-aminobutyric acid; ATCC, American Type Culture Collection; AU, arbitrary units; BM-Lan, β-methyllanthionine; BMSEC, β-methyl-S-ethylcysteine; dhA or U, 2,3-didehydroalanine; dhB or O,(2)-2,3-didehydrobutyrine; DSER, PTH-dithiothreitol adduct of dhA; HPLC, high-pressure liquid chromatography; Lan, lanthionine; OPA, *o*-phthaldialdehyde; PTH, phenylthiohydantoin; RP-HPLC, reversed-phase high-pressure liquid chromatography; SEC, S-ethylcysteine; TFA, trifluoroacetic acid; TSAYE, trypticase soy agar yeast extract

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(70%) at pH 2.0 (adjusted with HCl). The suspensions were heated at 70°C for 40 min, cooled on ice, and centrifuged at 10000×*g* for 15 min. This method was adapted from an extraction technique described by Hurst [20]. The supernatants were evaporated with a rotary evaporator at 37°C and the concentrates were applied to C_{18} Sep-Pak® cartridges (Waters Corporation, Milford, MA) and eluted with a discontinuous gradient of acetonitrile (12%, 18%, 24%, 30%, 33% and 60% acetonitrile) were then concentrated with a rotary evaporator and dried in a Speed-Vac® concentrator (Model SC110A, Savant Instruments Inc., Farmingdale, NY). The fractions were re-solubilized in 0.11% TFA. They were further purified by RP-HPLC using an analytical C_{18} column (Vitropack Lichrosorb RP 18, 5 μm; LKB, Pharmacia Biotech, Uppsala, Sweden).

The chromatography was performed on an LKB system (Pharmacia) equipped with two pumps (model 2150), a controller (model 2152), a variable wavelength monitor (model 2151), a Rheodyne M7010 sample injection valve with a 20 μl loop, and a Hewlett-Packard 3396 integrator. Elution was carried out with solvent (A): 0.11% TFA and solvent (B): 60% acetonitrile in 0.1% TFA. The peptides were eluted with a linear gradient of acetonitrile over 44 min at a flow rate of 1 ml/min and detected at 220 nm. The upper half of the peak showing activity (HPLC I) was concentrated in a Speed-Vac® and re-injected under the same conditions to yield fraction HPLC II and finally the pooled sample consisting of the upper third fraction of this peak from eight different batches was injected under the same conditions to yield a unique peak (HPLC III) which was used for chemical analyses.

2.4. Polarity calculation

The polarity was calculated from the percentage of acetonitrile at which the substance eluted according to the formula given by Snyder [21].

2.5. Purity assessment

The purity of mutacin preparations was assessed by capillary electrophoresis. The samples were injected into a 44-cm capillary (2023 Fused silica capillary, 50 μm ID) by hydrodynamic injection on a capillary electrophoresis system (Thermo SP Separation Products, Toronto, Ontario, Canada). The separation was carried out at 25 kV in sodium phosphate buffer (pH 2.5) and 15% (v/v) acetonitrile, at 30°C. The detection was at 200 nm.

2.6. Molecular mass determination

Molecular mass was determined for fraction HPLC III by electrospray mass spectroscopy on a VG/Fisons Quatro II electrospray triple quadrupole mass spectrometer (Fison Instruments, Manchester, UK) at the Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada.

2.7. Amino acid analysis

Purified mutacin B-Ny266 (fraction HPLC III) was submitted to acid hydrolysis (6 N HCl, 110°C, 21 h) and *o*-phthaldialdehyde derivatization according to Sahl et al. [22]. The amino acids were analyzed by RP-HPLC chromatography on a Waters HPLC system (Waters Chromatography Division, Milford, MA) consisting of a Waters Radial-Pak C_{18} 4-μm column (8 mm×10 cm) at 40°C, two pumps (Waters model 510) and an automatic injector (Waters Model 715). The OPA amino acid derivatives were detected at 425 nm (excitation wavelength 338 nm) on a fluorescence detector (Waters, Model 420). The sum of the Lan and/or BM-Lan residues was determined using D,L-lanthionine (Sigma) as a standard. The number of amino acid residues was calculated from the molar ratio relative to Ala and Asx.

Table 1
Mean yield of mutacin B-Ny266 for each purification step[a]

Purification step	Purification factor	Yield (%)
Sep-Pak®	36±27	31 ±22
HPLC I	5± 3	22 ± 2
HPLC II	3± 1	49 ±22
HPLC III	6	31
Total	3240±81	1.0± 0.1

[a]Values presented are the mean ± SD of eight purifications, except for the third HPLC chromatography, which was performed only once.

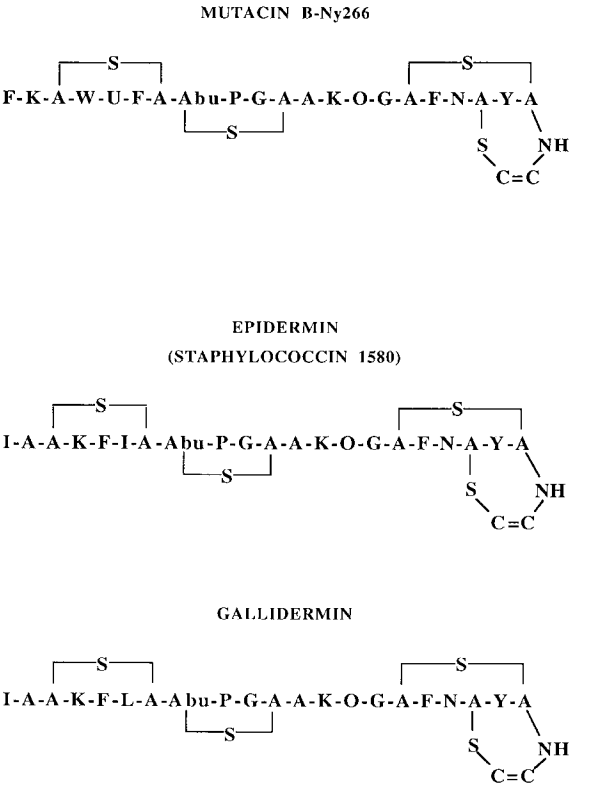

Fig. 1. Comparison of the proposed structure of mutacin B-Ny266 with those of epidermin (staphylococcin 1580) and gallidermin.

2.8. Sequence determination

Automatic Edman degradation was performed on a Pulsed Liquid Protein sequencer (Applied Biosystems model 473, Foster City, CA) after ethanethiol treatment as described by Meyer et al. [23] to permit sequencing of lantibiotics. The results were interpreted according to Meyer et al. [23].

2.9. Protein sequence accession number

The sequence of mutacin B-Ny266 has been deposited in the SWISS-PROT protein sequence database under accession number P80666.

3. Results

The purification procedure was repeated 8 times. The mean cell yield of the cultures in M17 broth was 0.79±0.04 g (mean ± SD) of cell dry weight/l. The specific activity of the cell pellet extracts was 115±43 AU/mg of protein. The specific activity recovered from the supernatants was 22±3 AU/mg of protein. Therefore, only cell pellet extracts were further purified.

The mean values obtained from the eight runs for the purification and yield for each step are presented in Table 1. On the whole, mutacin B-Ny266 was purified 3240±81-fold with a yield of 1.0±0.1%, which corresponds to 3.4±0.3 μg/l of culture. The substance was found to be more than 99.9% pure by capillary electrophoresis. As mutacin B-Ny266 elutes at 33.3% acetonitrile during RP-HPLC, its polarity was calculated to be 8.067.

The electrospray mass spectroscopy analysis yielded a molecular mass of 2270.29±0.21 Da for the purified mature peptide.

Taking into account the limits of amino acid analysis after

Table 2
Amino acid composition of mutacin B-Ny266

Amino acid	Number of residues per molecule		
(one letter code)	Amino acid analysis[a](probable number of residues)	Edman degradation	Calculated molecular weight(Da)
Ala (A)	1.2 (1)	1	71.08
Asx (N, D)	0.8 (1)	1 (N)	114.10
Gly (G)	1.5 (1-2)	2	114.10
Lys (K)	2.5 (2-3)	2	256.35
Phe (F)	2.9 (3)	3	441.53
Tyr (Y)	0.7 (1)	1	163.18
Lan/BM-Lan[b]	2.7 (3)	3 (2 Lan + 1 BM-Lan)	530.65
dhA (U)	0[c]	2	138.13
dhB (O)	0[c]	1	83.09
Pro (P)	NQ[d]	1	97.12
Trp (W)	0[c]	1	186.21
			2195.54
		+ 1H (N-terminal)	1.01
			2196.55

[a]The number of amino acid residues was calculated from the molar ratio relative to Ala and Asx after OPA derivatization.
[b]Lan = lanthionine; BM-Lan = β-methyllanthionine.
[c]dhA, dhB and Trp are acid labile and are not detected by the method used.
[d]NQ, not quantified by OPA derivatization.

acid hydrolysis and OPA derivatization, the amino acid content as determined by acid hydrolysis and Edman degradation are consistent (Table 2). Forty percent are modified amino acid residues.

Edman degradation after treatment with alkaline ethanethiol revealed 21 residues with the following sequence:

$F^1-K^2-(SEC+^3DSER)-W^4-SEC^5-F^6-(SEC+^7DSER)-$

$BMSEC^8-P^9-G^{10}-(SEC+^{11}DSER)-A^{12}-K^{13}-$

$BMSEC^{14}-G^{15}-SEC^{16}-F^{17}-N^{18}-SEC^{19}-Y^{20}-SEC^{21}$

The sample was sequenced with an initial yield of 1060 pmol and a repetitive yield of 87.2%. The level of confidence was higher than 99%.

4. Discussion

Mutacins have always been difficult to obtain from liquid cultures [2,18,24–26]. Novák et al. [10] recently obtained good production of mutacin J-T8 in a chemically defined medium enriched with yeast extract and trypticase soy broth. We obtained a good production of mutacin B-Ny266 in the commercially available medium M17 (BDH). Furthermore, we describe a simple technique permitting the extraction of the mutacin activity from cell pellets and we present, for the first time, the complete amino acid sequence of a mutacin.

Although the specific activity recovered from cell pellets is higher than that recovered from supernatants of bacterial cultures, the total amount of activity in the supernatants was calculated to be, on average, six times that of the cell extracts. Actually, we can recover 40% of this activity, but with a lower purity than from the cell extract (data not shown). We are presently working on a more efficient method to purify mutacin B-Ny266 from these supernatants, which would improve the total culture yields. Although the method of extraction presented permits the recovery of the active substance from cells, we observed a high variability from one extraction to another. Thus, the extraction and purification procedures have to be improved in order to increase the yield and reduce this variability. The procedure nevertheless permitted the purification of the lantibiotic B-Ny266 using only hydrophobicity chromatography. No impurities interfered with the determination of molecular mass and the amino acid sequencing. Furthermore, capillary electrophoresis indicated that the substance was more than 99.9% pure.

At our first sequencing attempt, Edman degradation was blocked at the first dhA (residue 5), the Trp^4 was barely detectable, and a blank was obtained in the third position (preliminary results not shown). The ethanethiol treatment described by Meyer et al. [23] permitted the complete (residue 22 excepted) sequencing of our molecule. These results thus confirm the conclusions of Meyer et al. [23] on the usefulness of this method for the automatic sequencing of lantibiotics.

The results obtained from amino acid analysis and Edman degradation are consistent (Table 2), taking into account the residues that could not be identified by amino acid analysis. Residues dhA, dhB and Trp are acid labile and are not detected by acid hydrolysis and OPA derivatization [22,27], and Pro cannot be quantified by OPA derivatization.

According to Meyer et al. [23], the detection of a SEC signal (residues 5, 16, 19 and 21) during Edman degradation after ethanethiol treatment indicates the presence of a dhA residue.

When this SEC signal is accompanied by a DSER signal (residues 3, 7, 11), it indicates the presence of a lanthionine residue. At cycles 16, 19 and 21, the DSER signal was not observed. These positions would correspond to a dhA residue. However, the DSER signal is always very low [23]. It is possible that, as cycles 16, 19 and 21 are at the end of the sequence, the corresponding DSER signal is undetectable. Furthermore, if we compare the molecule of B-Ny266 with closely similar substances such as epidermin (staphylococcin 1580) and gallidermin (Fig. 1), position 16–21 is occupied by a lanthionine. This would also be in accordance with the results of the amino acid analysis, in which we detected 3 Lan/BM-Lan (Table 2). We can thus hypothesize that these residues could be Lan3–7, BM-Lan8–11 and Lan16–21. The SEC signal detected at position 19 indicates a dhA in the molecule. This dhA could well result from breakage of a link with the S-(2-aminovinyl)-D-cysteine residue, leaving a dhA in position 19

Table 3
Comparison of the first 7 N-terminal amino acids of mutacin B-Ny266 with other known lantibiotics of group A

Lantibiotics	N-terminal sequence[a]	Reference
	1 2 3 4 5 6 7	
	┌──────S──────┐	
Mutacin B-Ny266	F - K - A - W - U - F - A ...	This paper [b]
	┌──────S──────┐	
Epidermin		[c]
Staphylococcin 1580	I - A - A - K - F - I - A ...	[d]
	┌──────S──────┐	
Gallidermin	I - A - A - K - F - L - A ...	[e]
	┌──────S──────┐	
Nisin A	I - O - A - I - U - L - A ...	[f]
Nisin Z		[g]
	┌──────S──────┐	
Subtilin	W - K - A - E - U - L - A ...	[h]
Salivaricin A	K - R - G - S - G - W - I ...	[i]
Streptococcin A-FF22	G - K - N - G - V - F - K ...	[j]
Lacticin 481	K - G - G - S - G - V - I ...	[k]
Lactococcin DR		[l]
Mutacin J-T8	N - R - W - W - Q - G - V ...	[m]
Lactocin S	S - T - P - V - L - A - S ...	[n]
Epilancin K7	X - A - U - V - L - K - O ...	[o]

[a]Amino acids are identified by the one-letter code; U and O represent, respectively, 2,3-didehydroalanine and 2,3-didehydrobutyrine [42].
References: [b]this work; [c][33]; [d][34]; [e][35]; [f][36]; [g][27]; [h][37]; [i][38]; [j][32]; [k][39]; [l][40]; [m][10]; [n][41]; [o][42].

and S-(2-aminovinyl) in the terminal position (which was not detected in our sequence analysis). Although it was not detected, this C-terminal aminovinyl-cysteinyl residue can be assumed from the comparison of the molecular masses obtained by mass spectroscopy analysis (2270.29 ± 0.21 Da) and the total mass obtained from the addition of all the residues calculated from the amino acid sequence (2196.55 Da) (Table 2). The difference between the two masses (73.74 ± 0.21 Da) agrees well with the molecular mass of a S-(2-aminovinyl) residue (73.12 Da). This assumption would again correspond to the known structures of epidermin and gallidermin (Fig. 1).

The signals corresponding to BMSEC indicate the presence of an Abu or a dhB [23]. We observed these signals for residues 8 and 14 during the sequencing. By analogy with the molecules of epidermin and gallidermin, we assigned Abu for residue 8 and dhB for residue 14.

If we hypothesize the same mode of post-translational modification for mutacin B-Ny266 as for other lantibiotics [16,17], the pro-B-Ny266 would thus have the following sequence: F–K–S–W–S–F–C–T–P–G–C–A–K–T–G–S–F–N–S–Y–C–C. We are presently attempting to clone and sequence the corresponding gene in order to confirm the proposed primary structure.

According to the results presented, mutacin B-Ny266 differs from epidermin and gallidermin in positions 1, 2, 4, 5 and 6 and could thus be regarded as type A lantibiotic (Fig. 1 and Table 3). It is surprising that similar lantibiotics are produced by different bacterial genera. Epidermin and gallidermin are produced by staphylococci (*S. epidermis* and *S. gallinarum* respectively) while mutacin B-Ny266 is produced by a streptococcus (*S. mutans*). The Lan3–7, which is a common structure among group A lantibiotics [1], is also present in mutacin B-Ny266. The Lys^2 is also found in subtilin and streptococcin (SA-FF22). The importance of this position for the characteristics of the molecule have so far not been studied. The sequence F1–K2–Lan3 can be found in inverted form as F5–K4–Lan3 in epidermin/staphylococcin 1580 and gallidermin. The significance of this observation eludes us for the moment, but it could point toward a common origin for these three lantibiotics.

Mutacin B-Ny266 possesses a dhA residue in position 5. This dhA5 was found to be important for the activity and stability of nisin [28], and for the sporicidal activity of nisin [29] and subtilin [30]. The possibility of a similar implication of the dhA5 residue in mutacin B-Ny266 activity would be well worth testing. Position 6 of epidermin and gallidermin seems to be implicated in antibacterial activity and resistance to trypsin [31]. While this position is occupied by I and L residues in epidermin and gallidermin, respectively, an F6 residue is found in mutacin B-Ny266, as for streptococcin A-FF22 [32]. Mutacin B-Ny266, epidermin, gallidermin and SA-FF22 must be compared with respect to their resistance to trypsin and their spectrum of action.

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