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ORIGINAL ARTICLE

Lasiocepsin, a novel cyclic antimicrobial peptide from the venom of eusocial bee *Lasioglossum laticeps* (Hymenoptera: Halictidae)

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Abstract In the venom of eusocial bee *Lasioglossum laticeps*, we identified a novel unique antimicrobial peptide named lasiocepsin consisting of 27 amino acid residues and two disulfide bridges. After identifying its primary structure, we synthesized lasiocepsin by solid-phase peptide synthesis using two different approaches for oxidative folding. The oxidative folding of fully deprotected linear peptide resulted in a mixture of three products differing in the pattern of disulfide bridges. Regioselective disulfide bond formation significantly improved the yield of desired product. The synthetic lasiocepsin possessed antimicrobial activity against both Gram-positive and -negative bacteria, antifungal activity against *Candida albicans*, and no hemolytic activity against human erythrocytes. We synthesized two lasiocepsin analogs cyclized through one native disulfide bridge in different positions and having the remaining two cysteines substituted by alanines. The analog cyclized through a Cys8–Cys25 disulfide bridge showed reduced antimicrobial activity compared to the native peptide while the second one (Cys17–Cys27) was almost inactive. Linear lasiocepsin having all four cysteine residues substituted by alanines or alkylated was also inactive. That was in contrast to the linear lasiocepsin with all four cysteine residues non-paired, which exhibited remarkable antimicrobial activity. The shortening of lasiocepsin by several amino acid residues either from the N- or C-terminal resulted in significant loss of antimicrobial activity. Study of *Bacillus subtilis* cells treated by lasiocepsin using transmission electron microscopy showed leakage of bacterial content mainly from the holes localized at the ends of the bacterial cells.

Keywords Antimicrobial peptides · Analogs · Disulfide bridge · Peptide synthesis · Wild-bee venom · CD spectroscopy

Introduction

Rapid development of bacterial resistance to conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents which kill bacteria by fundamentally different modes of action than do traditional antibiotics. Among these, antimicrobial peptides (AMPs) represent one of the most innovative families of anti-infective agents to have been characterized during recent decades (Giuliani et al. 2007; Oyston et al. 2009; Yeung et al. 2011; Zaiou 2007). AMPs, which are part of the innate defense system of practically all living organisms, exhibit activities comparable to those of conventional antibiotics, but the physical nature of their action implies a faster and substantially different mechanism of killing.

Although this mechanism is still not exactly known, it is generally accepted that these positively charged peptides target the anionic bacterial cell envelope, accumulate in the

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cell wall, interact with the membrane surface, infiltrate into the lipid bilayer and disrupt its structure in different ways that are typically described by three mechanistic models known as the carpet, barrel stave and toroidal pore models. Finally this action leads to leakage of cytoplasmic components and cell death (Amiche and Galanth 2011; Epand and Epand 2011; Huang et al. 2010; Oren and Shai 1998; Tossi et al. 2000; Toke 2005; Wimley and Hristova 2011; Yeaman and Yount 2003). Since bacteria have little recourse against this mechanism of action, microbial resistance is less likely to develop. Some AMPs may pass through the membrane without its disruption and interact with putative key intracellular targets through various mechanisms, e.g., similar to those of traditional antibiotics (Giuliani et al. 2007). In addition to their action against Gram-positive and -negative bacteria, some AMPs are active against fungi (Slaninová et al. 2011) or protozoa (Rivas et al. 2009) or they lyse cancer cells (Dennison et al. 2006).

The most studied group of AMPs is comprised of the linear cationic α-helical peptides with length of 10–45 amino acid residues (Tossi et al. 2000). They are rich in hydrophobic and basic amino acid residues and can adopt an amphipathic α-helical secondary structure within the cell membrane environment or in the presence of such membrane-mimicking substances as sodium dodecyl sulfate (SDS), phospholipids or organic solvents like trifluoroethanol. Numerous peptides of this category have been isolated from the venom of stinging hymenopterans such as wasps (Čeřovský et al. 2008a) and ants (Kuhn-Nentwig 2003) and have been extensively studied with regard to their antimicrobial potency. We have found that the venom of wild bees represents another promising source of α-helical amphipathic peptides showing potent antimicrobial properties and low or moderate toxicity to eukaryotic cells. These include, for example, melectin (Čeřovský et al. 2008a, b), and halictines (Monincová et al. 2010) and three pentadecapeptides named lasioglossins (LL-I, LL-II and LL-III) isolated from the venom of the eusocial bee *Lasioglossum laticeps* (Čeřovský et al. 2009).

When we further analyzed the venom extract of *Lasioglossum laticeps,* we found another three AMPs in addition to lasioglossins. Two of them were linear α-helical peptides homologous to lasioglossins containing 12 and 13 amino acid residues. The third one was a unique 27 amino acid residues and two intramolecular bridges containing peptide which we named lasiocepsin. In the present work, we focus on the study of lasiocepsin to describe its isolation, structure determination, synthesis, and structure activity relationships with regard to the importance of disulfide bridges and the chain length requirements.

Materials and methods

Sample preparation and peptide purification

The procedure was the same as described by us (Čeřovský et al. 2008b, 2009; Monincová et al. 2010). In brief, the venom reservoirs were extracted with 25 µl of an acetonitrile/water (1:1) mixture containing 0.5% trifluoroacetic acid (TFA) and then fractionated by RP-HPLC (Fig. 1). The selected fractions were collected, the solvent evaporated, and antimicrobial activity of each fraction against *M. luteus* determined by drop diffusion test. The active fractions were further analyzed by mass spectrometry and Edman degradation (see Figs. 1, 2 of Supplementary Material).

Determination of disulfide bridges

Native isolated lasiocepsin from the venom extract (Fig. 1, peak 22) was dissolved in 50 mM ammonium bicarbonate (13 µl). After addition of 2 µl of trypsin stock solution (0.2 mg/mL), the mixture was incubated at 37°C for 3.5 h. The reaction was quenched by adding 10% TFA (1 µl) and then the entire mixture was subjected to fractionation by HPLC on a Vydac C-18 column (250 × 4.6 mm) using a linear gradient from 2 to 70% acetonitrile/water/0.1% TFA over 60 min. The collected fragments were analyzed by mass spectrometry (MS) to identify their sequences. The patterns of disulfide bridges of synthetic lasiocepsin peptides (both those correctly as well as incorrectly folded) were determined similarly.

SPPS of linear precursors of lasiocepsin peptides

The linear lasiocepsin sequences shown in Table 1 were synthesized manually in 5 mL polypropylene syringes with

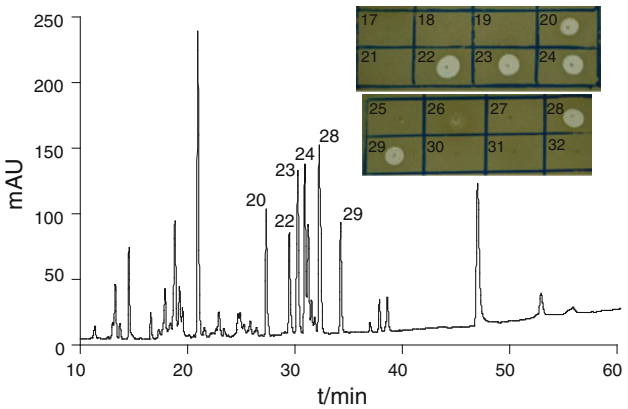

Fig. 1 RP-HPLC profile of *Lasioglossum laticeps* venom extract at 220 nm. An elution gradient of solvents from 5 to 70% acetonitrile/water/0.1% TFA was applied for 60 min at a 1 mL/min flow rate. *Inset* Anti-*M. luteus* activity (clear zones in the drop diffusion test) of individual fractions obtained from RP-HPLC

Table 1 Primary structures and MS data for lasiocepsin and its analogs

Peptide	Sequence	Molecular mass [Da] calcd	found
Lasiocepsin	GLPRKILCAIAKKKGKCKGPLKLVCKC	2891.69	2891.4
Lasiocepsin-NH_2	GLPRKILCAIAKKKGKCKGPLKLVCKC-NH_2	2890.71	2890.5
Las[C8-C27, C17-C25]	GLPRKILCAIAKKKGKCKGPLKLVCKC	2891.69	2891.5
Las[C8-C17, C25-C27]	GLPRKILCAIAKKKGKCKGPLKLVCKC	2891.69	2891.5
Las[C8-C25, Ala17,27]	GLPRKILCAIAKKKGKAKGPLKLVCKA	2829.76	2829.6
Las[C17-C27, Ala8,25]	GLPRKILAAIAKKKGKCKGPLKLVAKC	2829.76	2829.6
Las[Ala8,17,25,27]	GLPRKILAAIAKKKGKAKGPLKLVAKA	2767.84	2767.7
Las[des1-6]-NH_2	LCAIAKKKGKCKGPLKLVCKC-NH_2	2226.27	2226.0
Las[des1-10, Ala25]-NH_2	AKKKGKCKGPLKLVAKC-NH_2	1796.10	1796.0
Las[des15-27, Ala8]-NH_2	GLPRKILAAIAKKK-NH_2	1505.02	1505.9

a Teflon filter in the bottom. Syntheses of C-terminally non-amidated peptides were done using the N^{α}-Fmoc chemistry protocol on a 2-chlorotrityl chloride resin (150 mg, 0.07 mmol) while the C-terminally amidated peptides were assembled on a Rink Amide MBHA resin (100 mg, 0.07 mmol). Fmoc amino acids (4 eq, 0.28 mmol) were coupled using N,N′-diisopropylcarbodiimide (DIPC, 7 eq, 0.49 mmol) and 1-hydroxybenzotriazole (HOBt, 5 eq, 0.35 mmol) as coupling reagents in N,N′-dimethylformamide (DMF, 0.6 mL) as a solvent while utilizing nondestructive monitoring of the conversion of free amino groups with 1% bromophenol blue indicator in DMF (1 µl). For the one-pot cyclization strategy, the four cysteine side chains were protected with the trityl group (Trt). For the regioselective disulfide bond forming strategy, two cysteines in positions 8 and 25 were protected with *N*-acetamidomethyl group (Acm) and the other two in positions 17 and 27 with the Trt group. The peptides were deprotected and cleaved from the resin with a mixture of TFA/thioanisole/H_2O/1,2-ethanedithiol/triisopropylsilane (90:3:2.5:2.5:2) for 3.5 h and then precipitated with *tert*-butyl methyl ether. Crude peptides were further purified by preparative RP-HPLC. The dominant fraction containing the required peptide product was lyophilized and its purity and identity were checked by analytical HPLC and electrospray ionization mass spectrometry (ESI–MS).

One-pot cyclization/folding reaction

The lyophilized linear peptides were dissolved (at concentration 0.2 mg/mL) in 0.1 M ammonium acetate buffer, pH 7.8 and stirred under open air at room temperature. The time course of the disulfide bonds formation was monitored by HPLC until completion. In the case of peptides with four side chain deprotected cysteine residues, the cyclization reaction resulted in three cyclic products with different pattern of disulfide bridges. After 4–12 h (dependent on the peptide sequence) of the folding reaction, the solvent was removed by lyophilization and each component was isolated by preparative HPLC. The final products were then lyophilized and their identities verified by mass spectrometry.

Regioselective disulfide bond formation

The peptides already cyclized through the Cys17–Cys27 disulfide bond (see above) were dissolved (at concentration 0.2 mg/mL) in aqueous 50% methanol containing 0.02 M HCl. Simultaneous Acm removal and formation of the disulfide bridge between Cys8 and Cys25 was achieved by reaction of the peptide with 0.1 M iodine in methanol (5 eq/Acm group), which proceeded with vigorous stirring at room temperature. When the reaction was complete (90 min, HPLC monitoring) the methanol was then

evaporated in vacuum and the iodine extracted with CCl_4. Residual aqueous solvent was concentrated by lyophilization to ca 10% of original volume and then the fully oxidized peptides were purified by preparative HPLC.

Alkylation of linear lasiocepsin peptide

The alkylation of linear peptide was performed on a submilligram scale in order to obtain material for antimicrobial microassay. HPLC purified linear lasiocepsin in µg range (quantity corresponding to HPLC peak eluted from the analytical HPLC column) was dissolved in a solution of iodoacetamide (1 mg) in 50 mM ammonium bicarbonate (50 µl). The mixture was shaken at room temperature in darkness for 1 h and then the reaction was quenched by 10% TFA (3 µl). The cysteine-alkylated peptide (all 4 Cys residues) was isolated by HPLC as a single symmetrical peak which elutes 2 min earlier than the non-alkylated peptide. The solvent was evaporated and the identity of the product was confirmed by ESI–MS: 3,123.6 (found), 3,123.9 (calculated).

Determination of antimicrobial and hemolytic activity

A simple qualitative estimate of antimicrobial properties was undertaken using the drop-diffusion test on Petri dishes by the double-layer technique (Čeřovský et al. 2008b, 2009; Monincová et al. 2010). Quantitative minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multiwell plates as described (Čeřovský et al. 2008b, 2009; Monincová et al. 2010). Routinely, 0.5×10^4 to 1×10^4 CFU of bacteria per well were used for activity determination. Tetracycline in a concentration range of 0.5–50 µM was tested as standard. Antimicrobial testing in a reducing environment was done in the presence of 1.25 mM dithiothreitol (DTT). As test organisms we used *Bacillus subtilis* (*B.s.*) 168, kindly provided by Prof. Yoshikawa (Princeton University, Princeton, NJ, USA); *Escherichia coli* (*E.c.*) B and *Micrococcus luteus* (*M.l.*) No. CCM 144 were from the Czech Collection of Microorganisms, Brno; *Staphylococcus aureus* (*S.a.*) and *Pseudomonas aeruginosa* (*P.a.*) were obtained as multi-resistant clinical isolates; *Candida albicans* (*C.a.*), (F7-39/IDE99) was kindly provided by Olga Hrušková from the Institute of Organic Chemistry and Biochemistry. LC_{50} values of hemolytic activity were determined using human red blood cells of healthy donors (Monincová et al. 2010).

Circular dichroism

Circular dichroism (CD) experiments were carried out using Jasco 815 spectropolarimeter (Tokyo, Japan). All peptide samples were measured in water and in water/trifluoroethanol (TFE) mixtures of different ratios (10, 20 and 50% v/v). The final peptide concentration was kept constant—0.24 mg/mL for all studied peptides. The spectra were collected in a 0.1 cm quartz cell in the range 190–300 nm as averages of four scans at room temperature. We used 0.5 nm point spacing, 10 nm/min scanning speed, 32 s response time, and 1 nm spectral band width. Following baseline correction, the spectra were expressed as molar ellipticity θ (deg cm^2 $dmol^{-1}$) per residue. Secondary structure composition was calculated using the Dichroweb circular dichroism analysis program (Whitmore and Wallace 2008).

Transmission electron microscopy

Bacillus subtilis cells were treated with lasiocepsin for either 10 or 60 min. Untreated control cells were used for negative staining examination. Bacteria were adsorbed on parlodion–carbon-coated copper grids for 5 min. After short washing, the samples were negatively stained by floating on a drop of 0.25% phosphotungstic acid (PTA) with 0.01% bovine serum albumin in dH_2O for 30 s. Excess stain was blotted off with a piece of filter paper and samples were air dried. A JEOL JEM/1200EX transmission electron microscope operating at 60 kV was used for analysis.

Results

Purification and primary structure determination

The extract of bee venoms fractionated by RP-HPLC gave a profile (Fig. 1) with several intense peaks. From the 29 collected fractions (Fig. 1) six fractions exhibited antimicrobial activity against *M. luteus*. The analysis of these fractions revealed that the peptides of peaks 24, 28, and 29 correspond to the previously identified lasioglossins (LL-I, LL-II and LL-III) (Čeřovský et al. 2009). The two peptides of peaks 20 and 23 are short linear peptides of sequences homologous to lasioglossins. The Edman degradation of the peptide of the peak 22 gave the entire sequence in 29 cycles as follows: GLPRKILXAIAKKKGKXKGPL KLVXKX (see Fig. 2 of Supplementary Material). Its molecular mass measured by internally calibrated ESI–QTOF MS was manually calculated from the *m/z* values of multiply (4×, 5× and 6×) charged molecular ions found in the mass spectra, resulting in a monoisotopic molecular mass of 2,891.5 (see Fig. 1 of Supplementary Material). This is in good agreement with the calculated value of 2,891.69, based on the sequence determined by Edman degradation and assuming that all four undetermined amino

acid residues (X) at positions 8, 17, 25, and 27 were cysteine residues that form two disulfide bridges. We named this peptide, lasiocepsin, and it became the object of our study.

The connectivity of disulfide bridges was determined by the ESI–MS identification of peptide fragments isolated by HPLC from tryptic digest of lasiocepsin (Fig. 3 of Supplementary Material). Among several fragments, we clearly identified two peptides containing cystine: ILCA-IAK connected by disulfide bond to LVCK (Mr 1189.5) and CKGPLK connected by second disulfide bond to C (Mr 763.2). Their sequences as well as the sequences of another two identified fragments, GLPR (Mr 441.2) and GPLK (Mr 413.2), clearly fit to the sequence of lasiocepsin with the pattern of disulfide bridges Cys8–Cys25 and Cys17–Cys27.

Peptide synthesis and identification of the disulfide bridges pattern

Linear lasiocepsin and its linear analogs, prepared by standard peptide coupling chemistry, were finally purified by preparative RP-HPLC to provide analytical HPLC purity ranging from 96 to 99%. Their identities were verified by ESI–MS. (Fig. 2a as an example). In the first approach, the one-pot cyclization of lasiocepsin was achieved through an air-mediated oxidation of fully deprotected (by removing the S-Trt protecting group from all four Cys residues) linear peptide. However this oxidative folding of linear lasiocepsin (Las) resulted in a mixture of three cyclic products (Fig. 3). ESI–MS analysis of those peptides revealed a loss of four mass units of each peptide compared to the linear peptide (Fig. 2), thus suggesting formation of two disulfide bridges in all three products. The retention time of the product eluted in 29.5 min (peak 3, Fig. 3) was identical to that of native lasiocepsin. In order to identify the pattern of disulfide bridges in all of these peptides, the peptides were digested by trypsin and the resulting fragments of the digestion were identified by MS (Fig. 3 of Supplementary Material). That experiment showed that the peptide eluted at $t_R = 25.9$ (peak 1, Fig. 3) has the pattern of disulfide bridges Cys8–Cys17 and Cys25–Cys27 and the peptide eluted at $t_R = 27.5$ min (peak 2, Fig. 3) has the pattern Cys8–Cys27 and Cys17–Cys25 (Table 1). The digestion and fragment identification of the peptide of $t_R = 29.5$ min (peak 3, Fig. 3) clearly show the pattern of disulfide bridges identical to that of native lasiocepsin (Table 1).

The method of one-pot cyclization was also used for the cyclization of all lasiocepsin analogs of the sequences shown in Table 1 (see Figs. 4–11 in Supplementary Material for their mass spectra). In the case of the other linear analogs containing four cysteines, their cyclizations resulted in three products of different disulfide pattern analogous to the parent peptide.

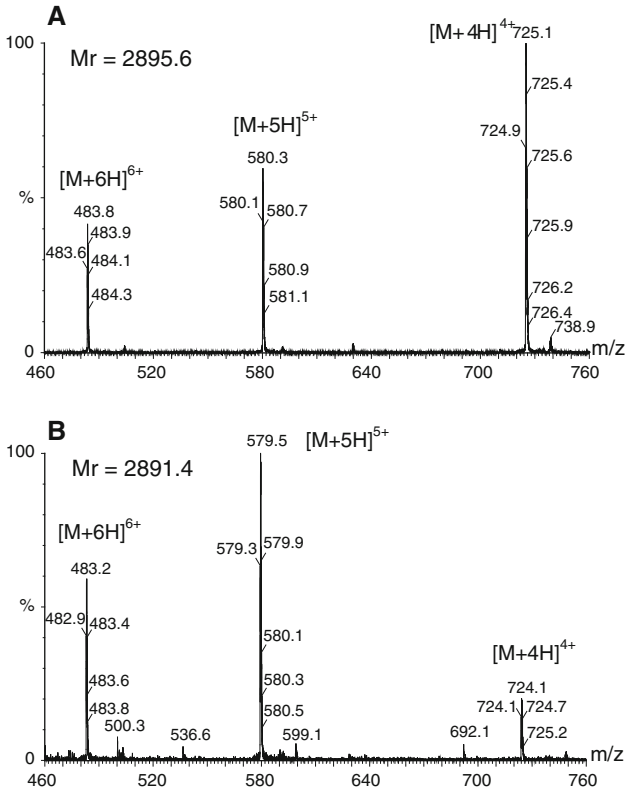

Fig. 2 ESI-QTOF mass spectra of linear lasiocepsin (**a**) and lasiocepsin (**b**)

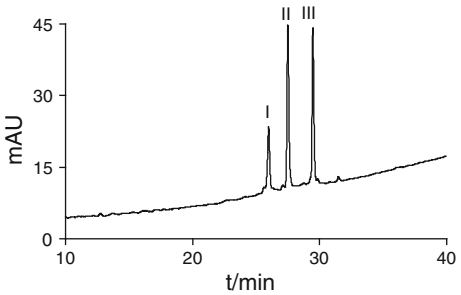

Fig. 3 RP-HPLC profile of the products obtained by an air-mediated oxidation of fully deprotected linear lasiocepsin peptide. Peak I, Las[C8–C17, C25–C27]; peak II, Las[C8–C27, C17–C25]; peak III, lasiocepsin. An elution gradient of solvents from 5 to 70% acetonitrile/water/0.1% TFA was applied for 60 min at a 1 mL/min flow rate

To improve the yield of lasiocepsin, we used a method of regioselective disulfide bond forming strategy utilizing the versatility of S-Acm protection at Cys8 and Cys25 in combination with acid labile S-Trt protection for Cys17 and Cys27 assuring the directed formation of disulfide bonds. Purified linear Las[Cys(Acm)8, Cys(Acm)25] peptide was cyclized by air oxidation to form the first disulfide bond between Cys17 and Cys27, which resulted during 6 h in a single cyclic product (monitored by HPLC). MS

analysis confirmed the identity of one disulfide bridge-containing peptide. After lyophilization of the mono-disulfide product, the second disulfide bond was formed by oxidative removal of Acm by iodine in 50% methanol. In monitoring this reaction by HPLC, the analysis revealed formation of only one peak, at $t_R = 29.5$, which corresponded to the desired product, lasiocepsin. After the workup procedure and final lyophilization of the aqueous mixture to dryness, however, the HPLC analysis of the crude product revealed in addition to the desired lasiocepsin at $t_R = 29.5$ min (Mr 2,891.5) a side product at $t_R = 29.2$. Mass spectrum of this side product (Mr 2,962.5) indicates a shift of the Acm group (difference of 71 Da) from Cys to a side chain of one of the Lys residues. As described, such an $S \rightarrow N$ or $S \rightarrow O$ shift of the Acm group from Cys to a side chain of Asn, Gln, Lys, Ser, Thr or Tyr may occur easily (Vasileiou et al. 2010). To avoid formation of this side product after completion of the reaction and workup procedure, the residual aqueous solution was concentrated by lyophilization to only ca 10% of original volume. Then the lasiocepsin was isolated from this aqueous residual solvent, which does not contain that side product, by preparative HPLC.

CD analyses and structural features

It is evident from the UV CD spectral shape (Fig. 12 of Supplementary Material) that lasiocepsin in water is slightly more α-helical than its misfolded analog Las[C8–C27, C17–C25]. The correct and detailed interpretation of the set of CD spectra is rather complicated, particularly with respect to their intensity. We have therefore employed computerized analysis by a numerical procedure (Dichroweb) (Table 2). According to this analysis, the α-helical content of lasiocepsin ($\alpha_H = 35\%$) is higher than that of his misfolded analog Las[C8–C27, C17–C25] ($\alpha_H = 20\%$), but both peptides have the same total percentage of the structured parts (α_H plus α_B).

CD spectra of analogs containing one disulfide bridge, Las[C8–C25, Ala17,27] and Las[C17–C27, Ala8,25], showed in water mutually quite similar secondary structures with α-helical content of about $\alpha_H = 10\%$ for both peptides. These spectra also revealed rather high content of β-structure ($\alpha_B = 68\%$ for Las[C17–C27, Ala8,25] and $\alpha_B = 73\%$ for Las[C8–C25, Ala17,27]). The peptide Las[Ala8,17,25,28] having no disulfide bonds exhibited only 8% of α-helix in water and the amount of β-structure was comparable to those of Las[C17–C27, Ala8,25] and Las[C8–C25, Ala17,27] peptides. In the presence of TFE (50%) as helix-promoting solvent, the α-helical content within these peptides increased (Fig. 12 of Supplementary Material) as follows: lasiocepsin ($\alpha_H = 52\%$), Las [C8–C27, C17–C25] ($\alpha_H = 50\%$), Las[C17–C27, Ala8,25] ($\alpha_H = 38\%$), Las[C8–C25, Ala17,27] ($\alpha_H = 63\%$), and Las[Ala8,17,25,27] ($\alpha_H = 37\%$). This increase of α-helical content was accompanied by reduction especially of the antiparallel β-structure fraction for all studied peptides (see Table 2). C-terminally amidated lasiocepsin exhibited in water and in 50% TFE a significantly lower α-helical content ($\alpha_H = 21$ and 36%, respectively) than did lasiocepsin itself, but it had a higher content of antiparallel β-structure (see Table 2). The shortening of lasiocepsin at its N-terminal by 6 or 10 amino acid residues resulted in a great decrease in α-helical content in water ($\alpha_H = 13\%$ for Las[des1-6]-NH_2 and $\alpha_H = 11\%$ for Las[des1-10, Ala25]-NH_2). Addition of TFE caused an increase of α-helical secondary structure content for both these peptides. While the α-helical content of Las[des1-6]-NH_2 increased significantly ($\alpha_H = 40\%$), the shorter analog Las[des1-10, Ala25]-NH_2 still had a low content of α-helical secondary structure ($\alpha_H = 18\%$), which was equilibrated by an antiparallel β-structure. The content of α-helical secondary structure of C-terminally shortened peptide Las[des15-27, Ala8]-NH_2, which was very low in water ($\alpha_H = 7\%$), dramatically increased ($\alpha_H = 71\%$) at the expense of antiparallel β-structure content (see Table 2).

Table 2 Calculated incidence (%) of secondary structures of lasiocepsin and its analogs determined by CD spectroscopy measured in water and in the presence of 50% TFE

Peptide	Incidence of secondary structures (%) in water/in 50% TFE[a]				
	α_H	α_{Ba}	α_{Bp}	α_{Bt}	α_R
Lasiocepsin	35/52	16/5	7/6	20/18	23/19
Lasiocepsin-NH_2	21/36	26/11	8/8	18/18	27/27
Las[C8–C27, C17–C25]	20/50	31/7	8/6	18/19	23/19
Las[C8–C25, Ala17,27]	11/63	49/4	7/4	17/18	17/12
Las[C17–C27, Ala8,25]	10/38	45/10	7/7	16/18	21/26
Las[Ala8,17,25,27]	8/37	50/14	7/7	17/20	19/23
Las[des1-6]-NH_2	13/40	39/11	8/7	17/19	23/24
Las[des1-10, Ala25]-NH_2	11/18	45/33	7/8	17/18	20/23
Las[des15–27, Ala8]-NH_2	7/71	51/1	7/3	17/15	18/10

[a] α_H, α-helix; α_{Ba}, antiparallel β-sheet; α_{Bp}, parallel β-sheet; α_{Bt}, β-turn; α_R, random coil

Biological activity

We have determined MIC values of antimicrobial activity for lasiocepsin and its analogs against the Gram-positive bacteria *Bacillus subtilis (B.s.)* and *Staphylococcus aureus (S.a.),* Gram-negative *Escherichia coli (E.c.)* and *Pseudomonas aeruginosa (P.a.)*, as well as its antifungal activity against *Candida albicans (C.a.)* (Table 3). Toxicity to red blood cells was determined as LC_{50} values in a hemolytic test and were >200 μM in all cases tested. Lasiocepsin showed antimicrobial activity against Gram-negative bacteria *E. coli* and also against pathogenic *P. aeruginosa*. With the MIC in submicromolar concentration, *B. subtilis* was the most sensitive bacterium. The C-terminally amidated analog lasiocepsin-NH_2 exhibited only a slight increase of antimicrobial activity. The misfolded lasiocepsin analogs with unnatural pattern of disulfide bridges (Las[C8–C27, C17–25] and Las[C8–C17, C25–C27]) showed significant decrease in antimicrobial activity against all bacteria tested as well as in antifungal activity. The N-terminal Las[des15-27, Ala8]-NH_2 fragment as well as the two C-terminal Las[des1-6]-NH_2 and Las[des1-10, Ala25]-NH_2 fragments were inactive in the antimicrobial and antifungal test, with the exception of weak activity against the most sensitive *B. subtilis*.

To investigate the importance of disulfide bridges in lasiocepsin for its conformation and biological activity, we synthesized and tested two lasiocepsin analogs cyclized through one native disulfide bridge in different positions and having the remaining two cysteines substituted by alanine and one linear analog with all Cys residues substituted by alanine. In the quantitative antimicrobial test, the analog cyclized through a Cys8–Cys25 disulfide bridge, (Las[C8–C25, Ala17,27]), showed reduced antimicrobial activity when compared to the native peptide, while the second one, Las[C17–C27, Ala8,25], as well as the linear analog (Las[Ala8,17,25,27]), were practically inactive (Table 3).

In addition, the importance of the presence and connectivity of disulfide bridges for the antimicrobial activity was investigated by microassay utilizing the drop diffusion test (*M. luteus*) on Petri dishes. In this test we compared the activity of lasiocepsin with its misfolded forms, lasiocepsin analogs containing one disulfide bridge, linear lasiocepsin with all—SH groups in reduced form, linear lasiocepsin having all four Cys residues replaced by Ala, and linear lasiocepsin having—SH groups of all Cys residues blocked by alkylation. The results of this test (Fig. 4) clearly show that the correct connectivity of disulfide bridges is important for the activity (Fig. 4a, lanes 1 and 2 against lane 3). The minimal or reduced activity (Fig. 4a, lanes 5, 6 and 7) indicates the importance of disulfide bridges for the activity, as analogs containing one disulfide bridge still showed some activity while the linear analog with all Cys substituted by Ala showed only negligible activity. The antimicrobial activity of linear lasiocepsin with free—SH groups (Fig. 4b, lane 4) was somewhat lower than that of lasiocepsin (Fig. 4b, lane 1) when tested in the drop—diffusion

Table 3 Antimicrobial activity of lasiocepsin and its analogs

Peptide	Antimicrobial activity MIC (μM)				
	B.s.[a]	*S.a.*	*E.c.*	*P.a.*	*C.a.*
Lasiocepsin	0.4 (1,000)[b]	93 (4.4)	8.6 (46.6)	15 (26.6)	3.6 (111)
Lasiocepsin linear	1.5 (267)	>100	25 (16)	37.5 (10.6)	12.5 (32)
Lasiocepsin linear[c]	1.0 (400)	n.t.[d]	20 (20)	25 (16)	15 (26.7)
Lasiocepsin-NH_2	0.3 (1,333)	63 (6.4)	3.0 (133)	16 (25)	8.0 (50)
Las[C8–C27, C17–C25]	0.6	>100	42	>100	25
Las[C8–C17, C25–C27]	4.0	>100	>100	>100	>100
Las[C8–C25, Ala17,27]	0.5	>100	55	65	70
Las[C17–C27, Ala8,25]	4.2	>100	>100	>100	50
Las[Ala8,17,25,27]	12	>100	>100	>100	>100
Las[des1-6]-NH_2	6.3	>100	>100	>100	>100
Las[des1-10, Ala25]-NH_2	65	>100	>100	>100	>100
Las[des15–27, Ala8]-NH_2	35	>100	>100	>100	>100

[a] *B.s. Bacillus subtilis*, *S.a. Staphylococcus aureus*, *E.c. Escherichia coli*, *P.a. Pseudomonas aeruginosa*, *C.a. Candida albicans*

[b] In parentheses therapeutic index calculated according to Chen et al. (2005), i.e. the ratio between two fold the highest concentration of peptides used in the hemolytic test (400 μM) and the antimicrobial activity expressed as MIC (μM). The value of hemolysis at the highest concentration of peptide tested (200 μM) was lower than 1%

[c] In the presence of DTT

[d] Not tested

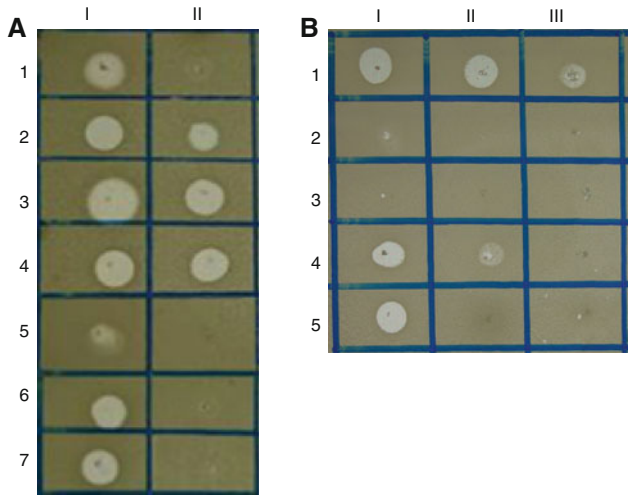

Fig. 4 Antimicrobial activities against *M. luteus* of lasiocepsin and its analogs. *Panel A, lane 1* Las[C8–C17, C25–C27], *lane 2* Las[C8–C27, C17–C25], *lane 3* lasiocepsin, *lane 4* lasiocepsin-NH2, *lane 5* Las[Ala8,17,25,27], *lane 6* Las[C8–C25, Ala17,27], *lane 7* Las[C17–C27, Ala8,25]. *Panel B, lane 1* lasiocepsin, *lane 2* Las[Ala8, 17,25,27], *lane 3* Las with all Cys residues blocked by alkylation, *lane 4* linear lasiocepsin (free –SH groups), *lane 5* linear lasiocepsin (free –SH groups) in the presence of 1.25 mM DTT. Tenfold and 100-fold dilutions were made for each peptide in the second and third column, respectively

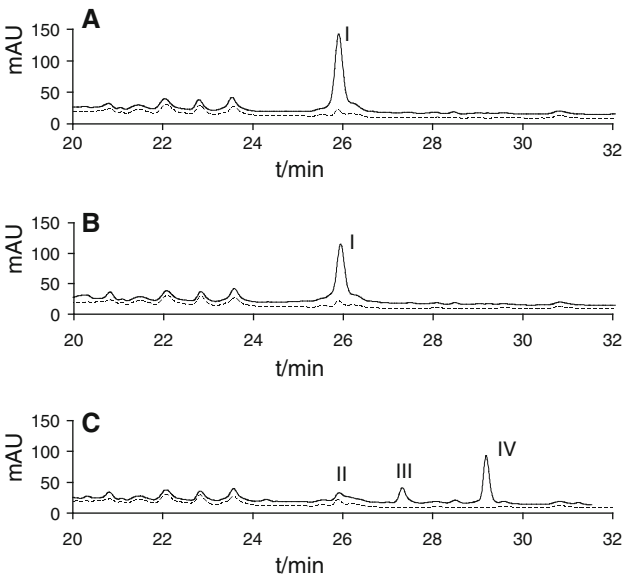

Fig. 5 HPLC analysis of linear lasiocepsin after incubation with *Bacillus subtilis* in the presence of DTT for 10 min (**a**) and 60 min (**b**) and without the presence of DTT for 60 min (**c**). *Peak I*, linear lasiocepsin; *peak II*, presumably Las[C8–C17, C25–C27]; *peak III*, presumably Las[C8–C27, C17–C25]; *peak IV*, lasiocepsin. *Dotted lines* represent the control without peptide. Identities of all peaks were verified by mass spectrometry

assay against sensitive *M. luteus*, while the alkylated species was completely inactive (Fig. 4b, lane 3). In the quantitative test (MIC values), the linear peptide was only about 2–4 times less active than the cyclic one (Table 3). These results indicate that linear lasiocepsin was probably oxidatively folded while carrying out the assay. That assumption was supported by HPLC analysis of the linear lasiocepsin incubated in water and which gave multiple peaks, one of which corresponded to lasiocepsin (Fig. 13A of Supplementary Material). To prevent the effect of oxidative folding of that linear peptide, the parallel experiment (Fig. 4b, lane 5) was carried out in the presence of reducing agent DTT (1.25 mM). Surprisingly, the activity of linear reduced peptide tested in the drop-diffusion assay was similar as of that of supposedly oxidized species (Fig. 4b, lane 4). In this case, the HPLC profile of the mixture of the linear reduced peptide and DTT showed no presence of an oxidized form (Fig. 13B in Supplementary Material).The MIC values of the linear reduced form of lasiocepsin (in the presence of DTT) against all bacteria tested were comparable to those of linear lasiocepsin in the absence of DTT (Table 3). The HPLC analysis (Fig. 5a, b) of the supernatant containing peptide which was obtained after centrifugation of the peptide-bacterium mixture confirmed the existence of the linear lasiocepsin peptide if the experiment was done in the presence of DTT. The HPLC analysis (Fig. 5c) of the parallel experiment without DTT shows the prevalence of lasiocepsin in the cyclic form. In a control test, the DTT itself did not affect the growth of bacteria tested, except of *P. aeruginosa*, whose growth it slightly slowed. The highest antifungal activity was observed with the lasiocepsin itself (MIC equal to 3.6 μM).

Transmission electron microscopy (TEM)

To examine the influence of lasiocepsin on morphology of Gram-positive bacteria (*Bacillus subtilis* as a model system), we treated them with the peptide either for 10 or 60 min. All samples were visualized by negative staining method. Bacteria in the untreated control revealed native morphology represented by electron-dense character and well-preserved bacterial membranes connected with many intact flagella (Fig. 6a). After treatment with lasiocepsin for 10 min (Fig. 6b) many bacteria became electron-transparent. But many of them possessed well-preserved membranes still connected with flagella. Only about 30% of bacterial cells revealed strongly changed morphology with holes in their membranes. Some of the holes were notably large enough to result in extensive leakage of internal bacterial content (Fig. 6b). Incubation with lasiocepsin for 60 min (Fig. 6c, d) led to electron transparency of most bacteria and to huge leakage of bacterial content, particularly from the holes prevalently localized at the ends of the bacterial cells. Surprisingly, the bacterial membranes in the middle part of the cells were often relatively preserved having only small holes in them. This phenomenon

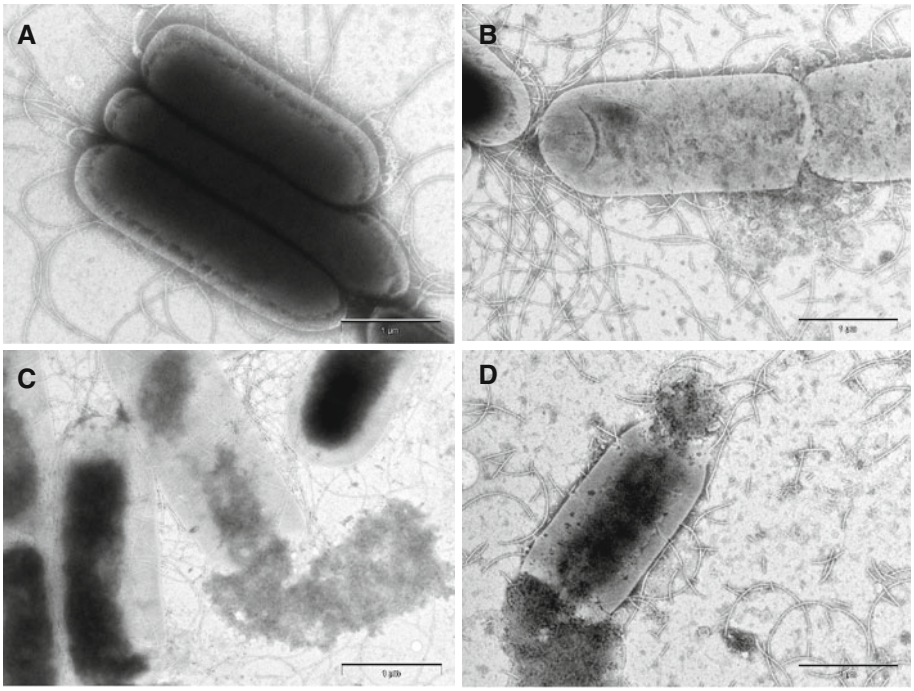

Fig. 6 Electron micrographs of negatively stained *Bacillus subtilis* either untreated (**a**) or treated by lasiocepsin for 10 min (**b**) or 60 min (**c**, **d**). *Scale bars* 1 μm

might be explained by the localization of the domains enriched in negatively charged phospholipids at the polar regions of the *Bacillus subtilis* membrane (Epand and Epand 2009). Such domains are supposed to attract strongly cationic lasiocepsin. Interestingly, we have not observed similar leakage of bacterial content from one big hole in the bacterial membrane after treatment with another antibacterial peptide, lucifensin, which we studied recently (Čeřovský et al. 2011). In that case we observed the leakage of the cytoplasmic content through many pores distributed over the whole bacterial surface.

Discussion

Repeated analyses of the extract of venom from eusocial bee *Lasioglossum laticeps* have shown the presence of several new AMPs. Three of them, which we named lasioglossins (Čeřovský et al. 2009), and other two, belong to the category of linear amphipathic α-helical AMPs and are homologous to one another. The peptide named lasiocepsin, which is the object of our present study, belongs to the category of cyclic AMPs containing two disulfide bridges. Due to the presence of nine basic amino acid residues and the absence of acidic residues, lasiocepsin is a highly cationic peptide with net positive charge of +10. This, in combination with a high content of hydrophobic amino acid residues (9 residues), contributes to its antimicrobial properties.

Unlike lasioglossins, lasiocepsin is not C-terminally amidated. C-terminal amidation is typical for the AMPs belonging to the category of linear amphipathic α-helical peptides. The terminal $-CONH_2$ group provides an extra hydrogen bond that stabilizes the C-terminal end of α-helical structure (Konno et al. 2007) and neutralizes the C-terminal negative charge, resulting together in enhanced antimicrobial activity (Čeřovský et al. 2008a; Kim et al. 2011). In this work, we showed that the C-terminally amidated lasiocepsin analog (lasiocepsin-NH_2) also exhibited slightly improved antimicrobial activity, especially against *E. coli* and *S. aureus* compared to non-amidated lasiocepsin. This might be the result of the negative charge elimination rather than the stabilization of a secondary structure, since its α-helical content as analyzed by CD spectra measurement was lower than that of lasiocepsin.

The sequence comparison of lasiocepsin with other AMPs found in the antimicrobial peptide database http://aps.unmc.edu/AP/main.php (Wang et al. 2009) and in the Basic Local Alignment Search Tool (BLAST) program (NCBI, BLAST server available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) shows no significant homology to other known antimicrobial peptides. It is notable that the lasiocepsin containing two disulfide bridges displays no homology with the neurotoxic peptide apamin (Labbé-Julié et al. 1991) that contains two disulfide bridges and is found in the venom of a honey bee. We may just speculate that the antimicrobial role of lasiocepsin in the venom of *Lasioglossum laticeps* is more or less secondary, while the primary roles, such as possibly neurotoxic activity, pain-causing effect, or other biological activities are unknown.

Intramolecular disulfide bridges in biologically active peptides are usually regarded as a critical factor for their structure and activity. However, the requirement as to the presence of disulfide bridges in cationic antimicrobial peptides for their activity is not fully clarified. Several reports show that some mammalian defensins without disulfide bonds or the misfolded defensins retain antimicrobial activity equal to the natural molecules (Klüver et al. 2005; Mandal et al. 2002; Varkey and Nagaraj 2005; Liu et al. 2008). On the other hand, the presence of all three disulfide bridges in the family of insect defensins seems to be essential for their antimicrobial activity (Kuzuhara et al. 1990). Similarly, the absence of one or both disulfide bridges in the antimicrobial peptide gomesin (Cys to Ser substitution) resulted in a decrease in antimicrobial activity (Fázio et al. 2006). The different linear analogs of antimicrobial peptide protegrin-1, such as those with Cys to Ala or Thr substitution or with Cys residues alkylated with iodoacetamide, exhibited considerably lower activity than did their native form. On the other hand, the linear protegrin-1 (free –SH group on four Cys) was equally active as was the natural peptide containing two disulfide bridges. This was explained by the spontaneous oxidative folding of linear protegrin-1 into native form (Dawson and Liu 2010). In experiments where spontaneous oxidation was prevented by addition of the reducing agent 2-mercaptoethanol, the linear form of protegrin-1 was even more active than the native one (Dawson and Liu 2010). Analogous study with brevinin 1E amide showed that the reduction of its intra disulfide bridge did not affect its antimicrobial activity even in the presence of dithiothreitol (Kwon et al. 1998). Similarly, reduced human β-defensin 1 in the presence of dithiothreitol exhibited potent antimicrobial activity against some Gram-positive bacteria and *Candida albicans*, while its native oxidized form was inactive (Schroeder et al. 2011). In the present study, the linear analogs of lasiocepsin synthesized with Cys to Ala substitution, or when its –SH groups of all four Cys residues were alkylated with iodoacetamide, exhibited substantially reduced activity compared to lasiocepsin, thus suggesting that the disulfide bridges would be essential for its antimicrobial activity. On the other hand, the linear lasiocepsin (free –SH group on four Cys) in the non-reducing environment, as well as in the presence of reducing agent DTT, exhibited antimicrobial activity almost equal to that of the native cyclic peptide. We also showed that spontaneous oxidation into its cyclic form occurs while carrying out the antimicrobial assay (in the absence of DTT) and might be the reason for antimicrobial activity of the linear peptide (HPLC profile, Fig. 13A of Supplementary Material). On the other hand, we proved that the linear lasiocepsin, which did not oxidize in the presence of DTT before addition to the bacterial culture, remained in its linear form during the assay while killing the bacteria. The explanation for that effect might be the ability of lasiocepsin to adopt an active conformation within the bacterial cell envelope without the pairing of cysteine residues.

The analysis of circular dichroism spectra (Fig. 12 of Supplementary Material) showed that the α-helical structure is the crucial secondary structure component of lasiocepsin and its analogs. With regard to their antimicrobial activity, however, α-helicicity is not the only requirement for the mechanism of this peptide's action. All peptides showed the ability to change secondary structure on the addition of TFE, thus resulting in a remarkable increase of α-helical content. As an example, the peptide Las[C8–C25, Ala17,27] had the highest α-helical structure content ($\alpha_H = 63\%$) after addition of TFE (Table 2), but it was only weakly active in the antimicrobial assay (Table 3). The results of CD spectra measurements of the shortened analogs Las[des15–27, Ala8]-NH_2 (N-terminal fragment) and Las[des1-10, Ala25]-NH_2 (C-terminal fragment) show high α-helical content in the N-terminal fragment compared to its C-terminal fragment in the presence of TFE (Table 2). This is in good agreement with the predicted secondary structures of lasiocepsin using the Protein Structure Prediction Server (PSIPRED) (Jones 1999), which indicates a high content of the α-helix in the N-terminal part of lasiocepsin (residues 3–13) while the residues 21–26 represent β-structure (Fig. 14 of Supplementary Material). The determination of three-dimensional structure of lasiocepsin by NMR spectroscopy, which we plan, will contribute to the understanding of lasiopcepsin action mechanism.

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Conflict of interest The authors declare that they have no conflict of interest.

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