HUNGARIAN JOURNAL
OF INDUSTRIAL CHEMISTRY
VESZPRÉM
Vol. 40 (2) pp. 83–86 (2012)

STUDY ON ANALYSIS OF ANTIBIOTIC COMPOUNDS FROM ENTHOMOPATHOGENIC BACTERIA BY FT-IR

D. VOZIK[1], J. MADARÁSZ[2], Z. CSANÁDI[1]✉, A. FODOR[3,4], K. DUBLECZ[4], K. BÉLAFI-BAKÓ[1]

[1]University of Pannonia, Faculty of Engineering, Research Institute on Bioengineering, Membrane Technology and Energetics, 10 Egyetem Str., 8200 Veszprém, HUNGARY
[2]University of Pannonia, Faculty of Engineering, Department of Organic Chemistry, 10 Egyetem Str., 8200 Veszprém, HUNGARY
[3]University of Pannonia, Georgikon Faculty, Institute of Plant Protection, 16 Deák F. Str., 8360 Keszthely, HUNGARY
[4]University of Pannonia, Georgikon Faculty, Department of Animal Sciences, CEPO Group, 16 Deák F. Str., 8360 Keszthely, HUNGARY
✉E-mail: csanadi@almos.uni-pannon.hu

Entomopathogenic bacteria produce antibiotic molecules effective against plant, animal and human plant pathogenic bacteria. They produce broad-spectrum antibiotics, which can be applied in several different fields where suppression of microbes is needed. These antibiotic molecules have different chemical structure such as peptides. Analysis and identification of these molecules provide useful ways in the research and development of drugs and agrochemicals.

Keywords: entomopathogenic bacteria, antimicrobial activity, peptides, analysis

Introduction

Insect pathogenic or entomopathogenic nematodes (EPN) and their symbiotic entomopathogenic bacteria (EPB) can be used as microbial control agents against agricultural insect pests [1]. These nematodes of *Heterorhabditis* and *Steinernema* species are symbiotically associated with the members of the bacteria family *Enterobacteriaceae* as *Photorhabdus* and *Xenorhabdus* species, respectively [2–3]. The EPB have several special functions in this symbiotic relationship. One of them is the production ability of broad-spectrum antibiotics, which keep monoxenic conditions in insect cadavers in soil [4].

Antibacterial resistance is increasing worldwide. The compounds produced by EPN symbiotic bacteria have showed a wide range of bioactivities of medicinal and agricultural interest, such as antibiotic, antimycotic and insecticidal effects [5]. These antimicrobial peptides have been successfully applied in pharmaceutics, plant disease control and many other fields [6]. Antimicrobial peptides have many beneficial characteristics, such as broad-spectrum antibiotic activity, thermal stability, and low molecular weight, and most significantly, compared with most antibiotics, they are not easy to lead to the development of resistance in the target [7]. These compounds were reported as showing in vitro activity against Gram-positive bacteria, including for example the multi-drug resistant strain of *Staphylococcus aureus*. They have diverse chemical structures including peptides as well [5].

Analytical study of these molecules can be achieved in different ways. Edman degradation, developed by Pehr Edman [8], is a method of sequencing amino acids in a peptide. In this method, the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues. A major drawback to this technique is that the peptides being sequenced in this manner cannot have more than 50 to 60 residues (and in practice, under 30). The peptide length is limited due to the cyclical derivatization not always going to completion. The infrared spectrum of a protein provides a wealth of information on structure and environment of the protein backbone and the amino acid side chains [9]. This makes infrared spectroscopy an extremely useful tool for the investigation of protein structure. The absorption of a side chain in a protein may deviate significantly from their absorption in solution or in crystal. The special environment provided by a protein is able to modulate the electron density and the polarity of bonds, thus changing the vibrational frequency and the absorption coefficient. Therefore, the band positions given in reviews should be regarded only as guidelines for interpretation of spectra [10].

In this study, we aimed the isolation and analysis of some peptide-type antibiotic compounds from two EPB strains.

Material and methods

Antibacterial peptide-producing entomopathogenic bacteria were *Xenorhabdus budapestensis* (EMA) and *Xenorhabdus szentirmaii* (EMC). They were cultured in Luria Broth (LB and LBA) liquid and solid media as previously described [11].

Cell-free culture media (CFCM) were prepared as follows: aliquots of the stock culture were added separately into 900 mL sterile medium. The flasks were incubated in a shaker at 200 rpm and 30ºC for 24 h and centrifuged at 13 000 rpm (10 000 g for 30'). After centrifugation the supernatant was filtrated through Express Plus filter (0.22 µm) (Merck Millipore).

Purification of antimicrobial compounds: 1000 mL of CFCM was mixed with activated Amberlite XAD polymeric adsorbent in a 1:20 ratio and incubated for 24 h. The resin-CFCM mixture was filtered through Millipore Express Plus filter of 0.22 µm pore-size and washed subsequently with 200 ml of distilled water, 200-200 ml of 25 V/V%, 50 V/V% and 80 V/V% methanol (MeOH), removing all inactive compounds by this way. After this the resin was washed with 200 ml cc. MeOH:HCl (99:1) and 200 ml i-propanol (*i*PrOH) to eluate biologically active compounds. These samples were evaporated by vacuum distillation and resulted samples with MeOH and *i*PrOH, respectively.

For the analysis of the purified antimicrobial compounds a Thermo Nicolet AVATAR FT-IR-330 FT-IR apparatus was used to determine FT-IR spectra and a Hitachi U-2910 UV-Vis spectrophotometer was used to form the UV spectra. Solid samples were prepared for the IR: some quantity of samples were ground with purified KBr and this mixtures were pressed in a mechanical press to form a transparent pellet through which the beam of the spectrometer can pass. Resolution: 2.000 cm^{-1}, Scans: 16.

Results and discussion

The most precise method for the protein and peptide identification after their separation by high-performance liquid chromatography (HPLC) is mass spectrometry [12]. However, this method is quite expensive. Absorbance at low wavelength (<220 nm) detects peptide bonds and amino acid residues with detection limits between nanomoles and picomoles. Peptides having aromatic residues (phenylalanine (Phe), tyrosine (Tyr), and trypthophan (Trp)) can be detected at 254 or/and 280 nm. For this reason we made a UV spectra of the purified antibiotic activity compounds. Results of these measurements are shown in *Table 1*.

Table 1: UV absorbance (nm) of the antibiotic compounds

EMA		EMC	
*i*PrOH	MeOH	*i*PrOH	MeOH
201	203	200	202
213	218	-	217

IR spectra were made with the above described method. Results of these measurements are shown in *Figures 1–4*.

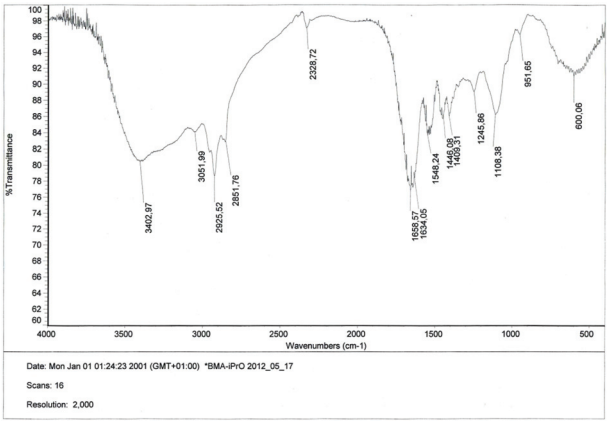

Figure 1: Antibiotic compounds purified with iPrOH from EMA

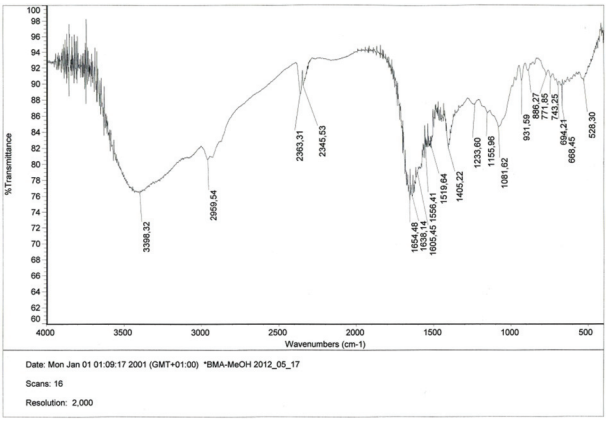

Figure 2: Antibiotic compounds purified with MeOH from EMA

In the case of amino acids only two side chain moieties absorb in spectral regions that are free from overlapping absorption by other groups and thus allow the spectroscopists an unambiguous assignment without further experiments. These are the SH group of cysteine (2550–2600 cm^{-1}) and the carbonyl group of protonated carboxyl groups (1710–1790 cm^{-1}). These groups were obtained in none of the IR spectra.

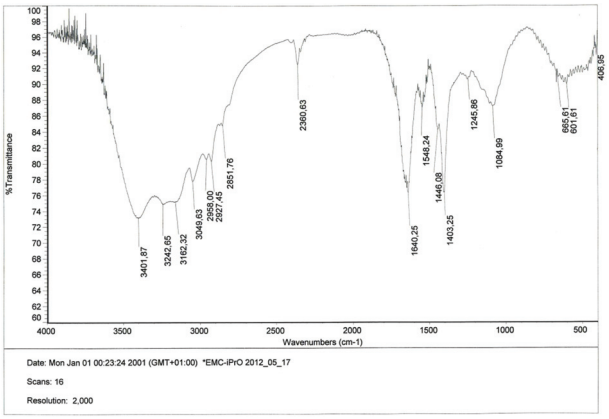

Figure 3: Antibiotic compounds purified with iPrOH form EMC

All other side chain absorption overlap with the absorption of other side chains or of the polypeptide backbone and further experiments are needed to assign an absorption band to a specific side chain moiety.

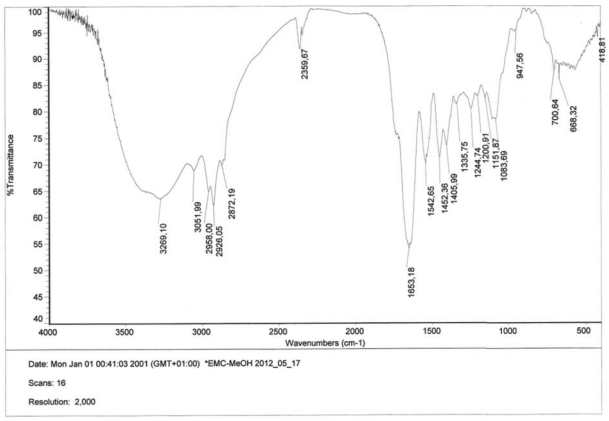

Figure 4: Antibiotic compounds purified with MeOH from EMC

The ν(C=O) vibration of glutamine side chains near 1680 cm^{-1} is a relative strong infrared absorber, the bands are sensitive to H-bonding and the band position is lower the stronger the H-bond is *(Table 2, 3)*.

The ν(C=O) vibration of the deprotonated carboxylate group of the glutamate shows two strong bands near 1400 and 1570 cm^{-1} for symmetric and antisymmetric stretching vibration, respectively *(Tab. 2, 3)*.

The ν(CN) vibration of histidine is a useful band near 1100 cm^{-1} *(Tab. 2, 3)*.

Tyrosine is a relatively strong infrared absorber due to its polar character. The most intense bands originate from ν(CC), the ν(C-O) and the δ(COH) mode near 1517, at 1235–1270 and at 1169–1260 cm^{-1} *(Tab. 2, 3)*.

Table 2: The IR spectra of EMA antibiotic compounds with different eluents

Band position in cm^{-1}		Assignment
*i*PrOH	MeOH	
1659	1654	glutamine (ν(CO) in proteins 1659–1696 cm^{-1})
1634	1638	histidine (ν(C=C) in proteins 1617 cm^{-1})
-	1605	tyrosine (ν(CC) ring in proteins 1615 cm^{-1})
1548	1556	glutamine ($\nu_{as}(COO^-)$ in proteins 1553–1575 cm^{-1})
-	1520	tyrosine (ν(CC) ring, δ(CH) in proteins 1516–1518 cm^{-1})
1446	-	lysine ($\delta(CH_2)$ in proteins 1445 cm^{-1})
1409	1405	glutamine ($\nu_s(COO^-)$ in proteins 1397–1424 cm^{-1})
1246	1234	tyrosine (δ(COH) in proteins 1228–1250 cm^{-1})
1108	1082	histidine (ν(CN), δ(CH) in proteins 1094–1114 cm^{-1})

While the $\delta_{as}(CH_3)$, the $\delta(CH_2)$ and the $\delta_s(CH_3)$ vibration near 1465, 1450 and 1375 cm^{-1} are relatively good group frequencies, the δ(CH) and $\gamma(CH_2)$ vibrations are often coupled to other modes *(Tab. 2, 3)*. The only tryptophan bands with considerable infrared intensity seem to be those at 1334 and 1455 cm^{-1} *(Tab. 2, 3)*.

Table 3: The IR spectra of EMC antibiotic compounds with different eluents

Band position in cm^{-1}		Assignment
*i*PrOH	MeOH	
-	1653	glutamine (ν(CO) in proteins 1659–1696 cm^{-1})
1640	-	histidine (ν(C=C) in proteins 1617 cm^{-1})
1548	1542	glutamine ($\nu_{as}(COO^-)$ in proteins 1553–1575 cm^{-1})
1446	1452	tryptophane (ν(CHb)[a], ν(CCb)[a], ν(CN) in proteins 1455 cm^{-1}) / lysine ($\delta(CH_2)$ in proteins 1445 cm^{-1})
1403	1406	glutamine ($\nu_s(COO^-)$ in proteins 1397–1424 cm^{-1}
-	1336	tryptophane (ν(CCp)[a], ν(CN) in proteins 1334 cm^{-1})/ lysine ($\gamma(CH_2)$, $\gamma(CH_2)$ in proteins 1345 cm^{-1}
1246	1245	tyrosine (δ(COH) in proteins 1228–1250 cm^{-1}
	1201	tryptophane (ν(CC) 1203 cm^{-1}
	1152	proline ($\gamma(CH_2)$, 1168 cm^{-1}
1085	1084	histidine (ν(CN), δ(CH) in proteins 1094–1114 cm^{-1})

"b" and "p" indicate vibrations of the benzene or pyrole moieties, respectively.

Acknowledgement

This work was partly supported by CEPO, the Austrian-Hungarian Cooperation Project for Poultry Excellence Centre, working within the frame of ERFA between 2007–2013 and TÁMOP-4.2.2/B-10/1-2010-0025.

REFERENCES

1. G. C. SMART: Entomopathogenic nematodes for the biological control of insects, Journal of Nematology, 27 (1995), pp. 529–534
2. G. M. THOMAS, G. O. POINAR: *Xenorhabdus* gen. nov., a genus of entornopathogenic, nematophilic bacteria of the family *Enterobacteriaceae*, International Journal of Systematic Bacteriology, 29 (1979), pp. 352–360

3. N. E. BOEMARE, R. J. AKHURST, R. G. MOURANT: DNA relatedness between *Xenorhabdus* spp. (*Enterobacteriaceae*), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov.. International Journal of Systematic Bacteriology, 43 (1993), pp. 249–255
4. R. J. AKHURST: Antibiotic activity of *Xenorhabdus* species, bacteria symbiotically associated with insect pathogenic nematodes of the families *Heterorhabditidae* and *Steinernematidae*, Journal of General Microbiology, 128 (1982), pp. 3061–3065
5. J. M. WEBSTER, G. CHEN, K. HU, J. LI: Bacterial metabolites, In: Entomopathogenic Nematology, CABI Publishing, USA (2002), pp. 99–115
6. Y. J. GORDON, E. G. ROMANOWSKI, A. M. MCDERMOTT: A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs, Current Eye Research, 30 (2005), pp. 505–515
7. Y. XIAO, F. MENG, D. QIU, X. YANG: Two novel antimicrobial peptides purified from the symbiotic bacteria *Xenorhabdus budapestensis* NMC-10, Peptides, 35 (2012), pp. 253–260
8. P. EDMAN, E. HÖGFELDT, L. G. SILLÉN, P. O. KINELL: Method for determination of the amino acid sequence in peptides, Acta Chemica Scandinavica, 4 (1950), pp. 283–293
9. A. BARTH: The infrared absorption of amino acid side chains, Progress in Biophysics & Molecular Biology, 74 (2000), pp. 141–173
10. B. HERNANDEZ-LEDESMA, L. AMIGO, M. RAMOS, I. RECIO: Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion, Journal of Chromatography A., 1049 (2004), pp. 107–114
11. E. M. MOTITU: Evaluating antibacterial potential of entomopathogenic bacterium strains on agriculturally important plant pathogenic bacteria. Master Thesis, University of Pannonia, Georgikon Faculty, Institute of Plant Protection, Keszthely, Hungary (2011)
12. T. HERRAIZ: Sample preparation and reversed phase-high performance liquid chromatography analysis of food-derived peptides, Analytica Chimica Acta, 352 (1997), pp. 119–139