

## **Analysis of individual azurocidin N-glycosylation sites in regard to its secretion by insect cells, susceptibility to proteolysis and antibacterial activity**

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Received: 24 February, 2007; revised: 21 June, 2007; accepted: 06 July, 2007  
available on-line: 26 July, 2007

**Azurocidin is an inactive serine protease homolog with primary sequence similarity to neutrophil elastase, cathepsin G, and proteinase 3. The aim of this study was to investigate possible consequences of differential glycosylation of azurocidin in regard to its secretion, protein stability as measured by susceptibility to proteolysis, and antibacterial activity. Site-directed mutagenesis was employed to generate mutant azurocidin variants lacking individual N-glycosylation sites. Our results show that N-linked glycans may play a role in proper azurocidin folding and subsequent secretion by insect cells. We also demonstrate that N-linked glycosylation contributes to azurocidin stability by protecting it from proteolysis. The lack of N-glycosylation at individual sites does not significantly influence the azurocidin antibacterial activity.**

**Keywords:** azurocidin, glycosylation, protein secretion, antimicrobial activity

### **INTRODUCTION**

Azurocidin, also known as heparin-binding protein (HBP) or 37-kDa cationic antimicrobial protein (CAP37), is an inactive serine protease homolog with the highest primary sequence similarity to neutrophil elastase and proteinase 3 (for references see Wątopek, 2003). Human azurocidin lacks proteolytic activity due to substitutions (H41S and S175G) in the catalytic triad. The protein is constitutively expressed during neutrophil differentiation and targeted to azurophilic granules. Recently, the presence of azurocidin has been shown in the endothelium of Alzheimer's brain microvessels (Pereira *et al.*, 1996a), in the endothelium associated with atherosclerotic plaques (Lee *et al.*, 2002), in pancreatic juice (Gronborg *et al.*, 2004), and several reports have shown that its expression is induced in endothelial (Lee *et al.*, 2002) and corneal epithelial cells (Ruan *et al.*, 2002) in response to inflammatory mediators such as lipopolysaccharide (LPS)

or cytokines. During evolution, azurocidin adopted new functions in host defense against invading organisms by mechanisms not involving proteolytic activity. Azurocidin is a potent chemoattractant for monocytes (Pereira *et al.*, 1990) and microglia (Pereira *et al.*, 2003), stimulates monocyte survival and differentiation and thrombospondin secretion (Ostergaard & Flodgaard, 1992), enhances the LPS-stimulated release of prostaglandin E2 (Heinzelmann *et al.*, 1998), interleukin-6 (Rasmussen *et al.*, 1996), and tumor necrosis factor- $\alpha$  (Rasmussen *et al.*, 1996; Heinzelmann *et al.*, 1998; 1999). Azurocidin also stimulates reversible contraction of fibroblasts and endothelial cells (Ostergaard & Flodgaard, 1992) and activates endothelial cell protein kinase C (Pereira *et al.*, 1996b). Several studies have shown that azurocidin has a potent bactericidal activity against a variety of bacteria (Shafer *et al.*, 1984; 1986; Gabay *et al.*, 1989; Campanelli *et al.*, 1990) and fungicidal activity against *Candida albicans* (Gabay *et al.*, 1989; Campanelli *et al.*, 1990).

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**Abbreviations:** cfu, colony-forming unit; LPS, lipopolysaccharide.

It has been widely shown that N-glycosylation significantly affects intracellular proteolytic processing, secretion efficiency, and posttranslational stability of secreted proteins. In human azurocidin, three N-linked glycosylation sites have been identified (Asn100, Asn114, and Asn145) (Iversen *et al.*, 1997; 1999, Karlsen *et al.*, 1998). The Asn114 glycosylation site is part of a putative endothelial cell protein kinase C activation site, thus it can be important for the cellular activation mechanism. The Asn100 and Asn114 glycosylation sites are located in a long surface loop which serves as a linker connecting two similar  $\beta$ -barrel domains, each composed of six antiparallel  $\beta$ -strands. These glycosylation sites may be involved in the folding of the two domains into the correct position with respect to each other. The Asn145 glycosylation site is situated in a  $\beta$ -strand in the second  $\beta$ -barrel (Iversen *et al.*, 1997; Karlsen *et al.*, 1998). The native protein isolated from neutrophils has a high variety of N-linked glycan structures (Olczak & Watorek, 2002), and the dominant glycans are GlcNAc<sub>2</sub>Man<sub>2</sub>(Fuc), GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAcGalNeu(Fuc), and GlcNAc<sub>2</sub>Man(Fuc). The dominant glycoform found in recombinant protein produced in *Sf9* insect cells contains two GlcNAc<sub>2</sub>Man<sub>3</sub>(Fuc) and one GlcNAc<sub>2</sub>Man<sub>3</sub> unit (Rasmussen *et al.*, 1996). Both native azurocidin purified from neutrophils and recombinant one produced in insect cells show high glycosylation microheterogeneity (Pereira, 1995; Rasmussen *et al.*, 1996; Karlsen *et al.*, 1998). The aim of this study was to investigate possible consequences of differential glycosylation of azurocidin in regard to its secretion, stability as measured by susceptibility to proteolysis, and antibacterial activity.

## MATERIALS AND METHODS

**Cell culture and transfection.** *Sf9* TriEx insect cells (Novagen) were propagated in TriEx insect cell medium at 28°C as recommended by the manufacturer. Transient transfection and protein analyses were performed in 12- or 6-well plates as described previously (Olczak & Olczak, 2006). Transfection with different amounts of wild-type DNA (pAzu1 plasmid; Table 1) was used in preliminary experiments to evaluate the influence of the amount of transfected DNA on azurocidin secretion. No significant dependence was found on the amount of plasmid DNA used (not shown), therefore for all experiments presented in this study 2  $\mu$ g of plasmid DNA was used. Cells and media were collected after 96 h. Before harvesting, the integrity of the cells was checked using Trypan Blue staining. The cells were confluent and viable (viability at least 95%; data not shown). Cells were separated by centrifugation of the suspension at

800 $\times$ g for 10 min at 4°C. Non-concentrated medium was subjected to all analyses.

**Mutagenesis.** Mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) with the pIEx-4 vector containing the entire azurocidin coding sequence (resulting in pAzu1 plasmid) as the template (Olczak & Olczak, 2006). Mutations were generated using the following primers: AzuN100S (5'-GACCGTGAGGCCAGCCT-CACCAGCAGC-3'), AzuN114S (5'-CTGCTCTGCAGAGCGCCACGGTGAAG-3'), and AzuN145S (5'-CCCAGGTTTGTGACGCTGACTGTGACCCC-3'). After mutagenesis, the entire coding sequence of all the mutants was examined by sequencing (Institute of Biochemistry and Biophysics PAS, Warszawa, Poland). Analysis of the predicted azurocidin tertiary structure after introduction of the mutations was performed using the UCSF Chimera program (<http://www.cgl.ucsf.edu/chimera>) with azurocidin models deposited in Protein Data Bank (PDB IDs: 1AE5 and 1A7S; Iversen *et al.*, 1997; Karlsen *et al.*, 1998). We found that neither mutation changed the overall azurocidin structure (not shown).

**Purification of azurocidin.** Recombinant azurocidin was purified from culture medium using cobalt-affinity chromatography as described previously (Olczak & Olczak, 2006). For this purpose, *Sf9* cells were grown in 10 ml of TriEx medium at 28°C with shaking at 145 r.p.m. for 90 h. Native human azurocidin was purified from neutrophils (obtained from "buffy coats" provided by the Wroclaw District Blood Bank) according to Watorek *et al.* (1996) with the following modifications. Briefly, frozen granules were suspended in 50 mM acetate buffer, pH 4.0, containing 1 M NaCl and homogenized using a glass homogenizer. The granule extract was centrifuged at 30000 $\times$ g for 30 min at 4°C and the pellet was re-homogenized five times. Granule extract was adjusted to pH 8.0 using solid Tris and applied to a Sepharose-Trasylol column equilibrated in 50 mM Tris/HCl buffer, pH 8.0, containing 1 M NaCl. After elution of non-adsorbed proteins with the equilibration buffer, human neutrophil elastase and cathepsin G were eluted with 50 mM citrate buffer, pH 4.0, containing 1 M NaCl. Then, elution of azurocidin was accomplished by eluting with 100 mM glycine/HCl buffer, pH 3.0. Fractions containing azurocidin were concentrated using an Amicon ultrafiltration unit (YM1).

**Tryptic digestion.** Culture media containing azurocidin glycosylation variants were adjusted with the medium of cells transfected with an empty vector to the same concentration of recombinant protein. Samples were incubated with trypsin (the molar trypsin:protein ratio was 1:500; Sigma) at 37°C in 100 mM Tris/HCl, pH 8.0, containing 20 mM CaCl<sub>2</sub>. At various time points aliquots were

removed in which trypsin activity was stopped by adding SDS/PAGE sample buffer and immediate boiling. Controls without trypsin were included in the protocol.

**Antibacterial assay.** Radial diffusion assays were performed according to Lehrer *et al.* (1991) and McCabe *et al.* (2002) with the following modifications. *Escherichia coli* K12 Row cells (PCM 1862 strain) were grown overnight in 3% Trypticase soy broth (TSB; Beckton Dickinson). To obtain mid-logarithmic growth phase ( $OD_{600} = 0.55\text{--}0.65$ ), 150  $\mu\text{l}$  of overnight culture was inoculated into 50 ml of fresh TSB and incubated for additional 3–3.5 h at 37°C. Bacteria were harvested by centrifugation at  $2500\times g$  for 7 min at 4°C and washed twice with cold 10 mM sodium phosphate buffer, pH 7.5. Suspension containing  $10^8$  cfu ( $OD_{620} = 0.2$  corresponds to  $5\times 10^7$  cfu/ml) was added to 12 ml of warm 0.8% low electroendosmosis agarose (Sigma) in 10 mM sodium phosphate buffer, pH 7.5, containing 0.02% Triton X-100 (Roth) and 0.02% bovine serum albumin (BSA) (Roth). After dispersing of the bacteria, the suspension was poured into 90-mm diameter Petri dishes. Several dilutions of neutrophil-derived and recombinant azurocidin were added to wells punched in the agarose layer (7.5  $\mu\text{l}$  per well). The plates were incubated for 3 h at 37°C to allow protein diffusion and then overlaid with 10 ml of 1% agar (BIOCORP) containing 6% TSB. Following an 18-h incubation at 37°C the plates were stained for 24 h according to Lehrer *et al.* (1991). The areas of the clearing zones surrounding the wells were measured using a Stemi SV 11 stereomicroscope (Carl Zeiss). All assays were performed at least three times. In all experiments lysozyme (Serva) was used as a positive control. Buffer used for azurocidin dilution served as a negative control.

**Bacterial membrane permeabilization assay.** The assay was performed according to Mak *et al.* (2007) using *E. coli* JM83 strain harboring pCH110 plasmid (Pharmacia-Amersham) encoding  $\beta$ -galactosidase and ampicillin resistance, with the following modifications. Aliquots of 4  $\mu\text{l}$  of bacterial suspension ( $2.5\times 10^8$  cfu/ml) were mixed in wells of 96-well round-bottom polypropylene microtiter plates (Greiner) with 60  $\mu\text{l}$  of azurocidin samples in a buffer at the pH range 4.5–7.5. In addition, several NaCl concentrations (0–0.35 M) were tested. The final amount of azurocidin was up to 2.6  $\mu\text{g}$  per well. The plates were incubated with shaking for 15 min at 37°C. Then, 150  $\mu\text{l}$  of 3.3 mM  $\beta$ -galactosidase colorimetric substrate (*o*-nitrophenyl- $\beta$ -D-galactopyranoside, ONPG; Sigma) in 0.2 M Tris/HCl, pH 8.0, was added to each well and the plates were again incubated for 30 min at 37°C. The reaction was stopped by adding 45  $\mu\text{l}$  of 1.7 M  $\text{Na}_2\text{CO}_3$ . The mixtures (200  $\mu\text{l}$ ) were individually transferred to flat-bottom clear

polyethylene 96-well plates (Nunc) and the absorbance at 420 nm was measured using a Spectra Max 340 microplate spectrophotometer (Molecular Devices). As control samples for 0% perforation served bacteria incubated without azurocidin, and as control samples for 100% perforation, bacteria disintegrated by sonication prior to the assay.

**Protein analyses.** Total protein concentration was determined using a modified Bradford method (Roti-Nanoquant, Roth) (Zor & Selinger, 1996). Recombinant azurocidin was analyzed by SDS/PAGE and Western blotting using anti-polyHis antibodies conjugated with horseradish peroxidase (Sigma), as described previously (Olczak & Olczak, 2006).

## RESULTS AND DISCUSSION

### Secretion of azurocidin glycosylation variants by insect cells

N-glycosylation is acquired in the endoplasmic reticulum and Golgi apparatus, both of which form part of the secretory pathway. Therefore, understanding the importance of individual N-linked sites with respect to azurocidin secretion would be advantageous. It has been shown that recombinant azurocidin expressed in baculovirus-infected insect cells possesses similar oligosaccharide structures as the native neutrophil-derived protein (Almeida *et al.*, 1996; Rasmussen *et al.*, 1996; Karlsen *et al.*, 1998). Recently, we have reported on a directly transfected insect cell system suitable for analysis of multiple azurocidin variants, including glycosylation mutants (Olczak & Olczak, 2006). We showed that recombinant azurocidin containing its native signal peptide and a fusion protein at the C-terminus was effectively expressed in transiently transfected insect cells and secreted into the culture medium at high levels (Olczak & Olczak, 2006). In this study we used site-directed mutagenesis to remove, either singly or in combination, the N-glycosylation sites by replacing the Asn residue from the consensus glycosylation sequon (Asn-X-Thr/Ser) by a Ser residue (Table 1). To determine whether azurocidin affected in the glycosylation sites can be effectively secreted, the respective protein mutants were expressed in transiently transfected insect cells and analyzed by SDS/PAGE and Western blotting. A clear decrease in the molecular mass of the mutated variants of azurocidin was observed after detection with Coomassie Brilliant Blue G-250 or anti-His antibodies (Fig. 1). Each successive mutation reduced the apparent molecular mass of the recombinant mutant protein, suggesting that the mutations prevented the addition of oligosaccharides. Our results showed that secretion of single glycosylation mutants was comparable with that observed for the wild-type re-

**Table 1. Description of azurocidin mutants altered in N-glycosylation sites**

Azurocidin variant	Expression plasmid	Occupancy of N-glycosylation sites by oligosaccharide chains <sup>a</sup>			Protein secretion <sup>b</sup>
		Asn100	Asn114	Asn145	
Wild-type	pAzuWT	•	•	•	++++
N100S	pAzu1		•	•	++++
N114S	pAzu2	•		•	++++
N145S	pAzu3	•	•		++++
N100S/N114S	pAzu1-2			•	++
N100S/N145S	pAzu1-3		•		++
N114S/N145S	pAzu2-3	•			++
N100S/N114S/N145S	pAzu1-2-3				+

<sup>a</sup>The presence of oligosaccharide chains (•) was checked experimentally by determination of electrophoretic mobility after SDS/PAGE of secreted azurocidin. <sup>b</sup>Recombinant protein secretion was examined by Western blotting.

combinant protein, secretion of double glycosylation mutants was slightly decreased, whereas in the case of the triple glycosylation mutant the decrease in secretion was pronounced (Fig. 1). This effect could be explained by the function of Asn-linked oligosaccharides in the overall protein stability and/or folding. Iversen *et al.* (1999) showed that the lack of glycosylation did not affect the folding of azurocidin, as the secondary and tertiary structures of non-glycosylated recombinant azurocidin and wild-type recombinant azurocidin were nearly identical. The surface loops to which some glycans are attached were also unaffected by the removal of the glycosylation. In contrast,

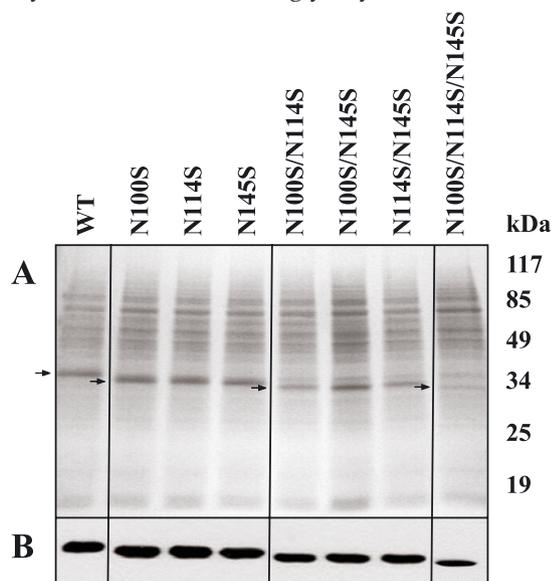
Almeida *et al.* (1996) demonstrated that recombinant azurocidin produced in tunicamycin-treated insect cells was found exclusively in a cell-associated form, suggesting that glycosylation may be important for azurocidin stability during secretion process. In addition, all attempts to produce azurocidin in *E. coli* cells have failed (Almeida *et al.*, 1996). Based on those data and our results presented in this study one may suggest that N-linked glycans might be important for proper azurocidin folding and subsequent secretion.

#### Susceptibility of azurocidin glycosylation variants to tryptic digestion

It is generally accepted that glycosylation is required to stabilize the protein structure. However, results concerning the susceptibility of glycoproteins to proteolysis are not convincing (van Berkel *et al.*, 1995; van Heen *et al.*, 2004; Valmu *et al.*, 2005). In this study we were interested whether N-linked glycosylation is important for azurocidin stability as measured by susceptibility to proteolysis. Recombinant azurocidin glycosylation variants were examined in culture medium without purification. Proteins were digested with trypsin, separated by SDS/PAGE, and visualized by anti-His antibodies. The digestion was repeated three times under the same conditions giving similar patterns. As shown in Fig. 2, the decrease of glycosylation of recombinant azurocidin was correlated with an enhanced susceptibility to degradation. These results indicated that N-linked glycosylation does contribute to azurocidin stability by protecting it from proteolysis.

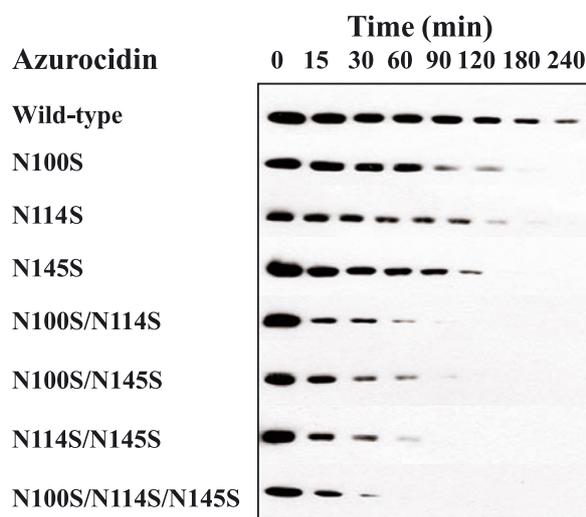
#### Antibacterial activity of azurocidin glycosylation variants

It has been reported that the N-terminal part of azurocidin may be important for its antimicrobial activity. A synthetic peptide corresponding to az-



**Figure 1. Secretion of recombinant azurocidin glycosylation variants by insect cells.**

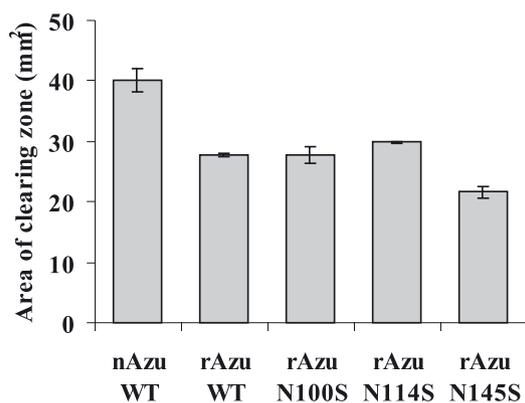
Protein secretion was examined by SDS/PAGE and Western blotting. Secreted azurocidin (shown by arrows) was visualized in culture media by Coomassie Brilliant G-250 (A) or anti-His antibodies (B) staining. The lack of individual N-glycans was evidenced by the decrease in the molecular mass of the mutant proteins. At least three clones were examined, for each mutation, giving similar secretion levels; the results are shown for representative clones.



**Figure 2. Susceptibility of recombinant azurocidin glycosylation variants to proteolysis.**

Culture media containing azurocidin were adjusted to contain the same concentrations of the recombinant protein. The molar trypsin:protein ratio was 1:500. Samples were removed at the indicated time points and analyzed by SDS/PAGE and Western blotting and visualized with anti-His antibodies. Three independent experiments were performed; the results are shown for one representative.

urocidin amino-acid residues 20 to 44 mimicked the bactericidal activity of the protein against a range of bacteria (Pereira *et al.*, 1993; Polikandritou *et al.*, 1997). This peptide also exhibited a capacity to neutralize endotoxin activity *in vitro* and *in vivo* (Brackett *et al.*, 1997). A recent report showed that binding of



**Figure 3. Antimicrobial activity of recombinant azurocidin glycosylation variants.**

Antibacterial activity against *E. coli* was determined using radial diffusion assay. The area (mm<sup>2</sup>) of the clearing zone surrounding the wells was measured and results are shown as means  $\pm$  S.D. for three independent experiments performed in duplicates, using 2.5  $\mu$ g of azurocidin per well. nAzu WT, native neutrophil-derived azurocidin; rAzu WT, recombinant wild-type azurocidin; rAzu N100S, rAzu N114S, and rAzu N145S, recombinant azurocidin glycosylation mutants.

heparin, which occurred at a highly cationic region located within loops 3 and 4 of azurocidin, inhibited the azurocidin antimicrobial activity (McCabe *et al.*, 2002). However, removal of the four positively charged amino-acid residues from the 20–44 azurocidin sequence did not affect the activity against bacteria and *C. albicans* (McCabe *et al.*, 2002). Additional mutations generated within this region also did not lead to significant changes of the bactericidal activity of azurocidin (Kastrup *et al.*, 2001).

Based on these data one may suggest that other regions or components, such as N-linked glycans, may contribute to the antibacterial function of azurocidin. To date, no reports have been published on the importance of individual N-linked glycans of human azurocidin for its antibacterial activity. To answer this question, the antibacterial activity of native neutrophil-derived and recombinant wild-type azurocidin against *E. coli*, as determined by radial diffusion assay, was first compared. The dose dependence of the activity of recombinant azurocidin was similar compared with that observed for the native neutrophil-derived protein (not shown), which is in agreement with other studies (Almeida *et al.*, 1996). As shown in Fig. 3, neutrophil-derived azurocidin showed a higher antibacterial activity compared with wild-type recombinant azurocidin. This may be explained by the presence of the C-terminal fusion protein attached to the recombinant protein or by different purification methods used. When the results of a radial diffusion experiment performed with wild-type recombinant azurocidin were compared with those for single glycosylation mutants it was clear that only the N145S azurocidin mutant exhibited a slightly decreased antibacterial activity (Fig. 3). It has been shown previously that a complete lack of glycosylation strongly reduced the biological activity of azurocidin, since the non-glycosylated protein mediated a limited stimulation of LPS-induced cytokine release from monocytes (Iversen *et al.*, 1999). Although the lack of N-glycosylation at individual sites did not significantly influence the antibacterial activity, all these data support the hypothesis that the oligosaccharide moiety of human azurocidin may play a role in its biological function.

Several investigators have suggested that the bacterial killing mechanism is initiated by the binding of azurocidin to bacterial LPS/lipid A (Pereira *et al.*, 1993; Iversen *et al.*, 1997; Brackett *et al.*, 1997; Polikandritou *et al.*, 1997). Upon binding to the lipid A component of LPS, azurocidin may induce leakage of the bacterial membrane. However our results showed that azurocidin, neutrophil-derived or recombinant, did not show membrane permeabilizing effect on *E. coli* cells under conditions used (not shown).

## CONCLUDING REMARKS

In this study, site-directed mutagenesis was employed to generate various azurocidin mutant proteins lacking individual N-glycosylation sites. Our results showed that although the lack of single N-linked glycans did not influence azurocidin secretion, the absence of two or three N-linked glycans might play a role in azurocidin folding and subsequent secretion by insect cells. Azurocidin lacking N-linked glycans, either singly or in combination, appeared to be more susceptible to trypsin digestion than its fully glycosylated form. The lack of occupancy of single N-glycosylation sites did not significantly influence the azurocidin antibacterial activity. We also found that azurocidin did not exhibit a membrane permeabilizing effect on *E. coli* cells. Based on the literature data and the results presented in this study it seems that further characterization of this important inflammatory mediator is likely to be critical to understanding the pathogenesis of various diseases and the design of effective therapeutics.

## Acknowledgements

This work was supported by grant No. 2 P05A 116 26 from the Department of Scientific Research, Ministry of Science and Higher Education (MNiSW, Poland).

*E. coli* K12 Row (PCM 1862 strain) was obtained from the Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy, PAS, Wrocław, Poland). *E. coli* JM83 was kindly provided by Adam Dubin and Pawel Mak (Jagiellonian University, Kraków, Poland). The stereomicroscope was used by courtesy of Piotr Kierzkowski (Zoological Institute, University of Wrocław, Poland).

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