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Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b

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1. SUMMARY

In culture supernatants of both *Listeria ivanovii* and *Listeria monocytogenes* Sv4b, for the first time a hemolysin of molecular weight 58 kDa was identified, which had all the characteristics of an SH-activated cytolysin, and which was therefore identified as listeriolysin O (LLO). In the case of *L. ivanovii* a second major supernatant protein of molecular weight 24 kDa co-purified with LLO. However, the function of this protein has to be determined. In culture supernatants of *L. ivanovii* a sphingomyelinase and a lecithinase activity could be detected, both enzymatic activities together contributing to the pronounced hemolysis caused by *L. ivanovii*. The N-terminal amino acid sequences of LLO and the 24 kDa from *L. ivanovii* are shown.

2. INTRODUCTION

The genus *Listeria* comprises a group of ubiquitous Gram-positive bacteria, some of which can cause severe infections (listeriosis) in animals and man [1], mostly in immunocompromised hosts. Pathogenic *Listeria* use professional phagocytic cells (monocytes and macrophages) of the infected host as the predominant site of multiplication. In normal cases infecting *Listeria* are eliminated upon activation of macrophages [2,3], but this crucial step is impaired in the immunocompromised host.

All virulent *Listeria* (mostly *L. monocytogenes*) are hemolytic [4], whereas non-hemolytic *Listeria* are avirulent. This observation led rather early to the assumption that hemolysin production is an important virulence factor in *Listeria* infections.

It has recently been shown that *L. monocytogenes* Sv1/2a strain EGD produces a listeriolysin which belongs to the group of SH-activated cytolysins [6] (for a review see [5]), the prototype of which is streptolysin O (SLO) [7]. It could also be demonstrated that listeriolysin-negative transposon mutants of *L. monocytogenes* are virtually avirulent [8,15] and that listeriolysin probably

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plays a key role in the escape of *Listeria* from the phagolysosomes of infected cells [9].

In the experimental mouse model system [10] the slightly hemolytic *L. monocytogenes* strains are quite virulent, whereas the extremely hemolytic *L. ivanovii* (formerly *L. monocytogenes* Sv5 or *L. bulgarica*) [11], is only moderately virulent.

3. MATERIALS AND METHODS

3.1. Bacterial strains

L. monocytogenes Sv4b (NCTC 10527), *L. ivanovii* (ATCC 19119, SLCC 23799), *L. monocytogenes* Sv1/2a (Mackness, SLCC 5764), *L. innocua* Sv6a, and *Rhodococcus equi* (NCTC 1621) were obtained from the strain collection of the Institute of Hygiene and Microbiology, University of Würzburg. *L. monocytogenes* Sv1/2a (EGD) was from S.H.E. Kaufmann (Ulm) and *Staphylococcus aureus* was donated by R. Lütticken (Cologne).

3.2. Media and growth conditions

Brain-heart infusion broth (BHI) (Gibco) or a defined *Listeria* medium (SLM) [12] were used and cultures were incubated at 37°C.

3.3. Purification of listeriolysin

L. monocytogenes or *L. ivanovii*, respectively, were grown in BHI or SLM for 18 h. The culture supernatants were cleared by centrifugation, concentrated about 50-fold by ultrafiltration (Amicon HPL10-20 or Millipore Pellicon PTGC) and subjected to thioldisulfide exchange affinity chromatography on thiopropyl-Sepharose 6B (Pharmacia) essentially as described by Geoffroy and Alouf [13]. In a further purification step gel filtration on Biogel P100 (Biorad) was used.

3.4. Hemolysin assay

Hemolytic activity of supernatants was determined in microtiter plates after serial two-fold dilution, and hemolytic activity of column fractions was determined in microliter tubes, using phosphate buffered saline (PBS), pH 6.0 as a diluent. After 10 min preincubation with 10 mM Dithioerythritol (DTE) sheep erythrocytes were added to a final concentration of 1%. After 30 min

at 37°C the release of hemoglobin was measured spectrophotometrically at 540 nm. For the determination of the pH optimum, 5% (w/v) sorbitol was added as stabilizer. In microtiter assays one hemolytic unit was defined as the reciprocal of the lowest dilution which gave complete hemolysis.

3.5. Production of rabbit antiserum

0.25 mg of purified 58/24 kDa proteins in incomplete Freund's adjuvant were injected subcutaneously. The same amount of antigen was injected again 2, 4 and 5 weeks after the first immunization.

3.6. SDS-polyacrylamide gel electrophoresis and immunoblotting

These were performed in 12.5% polyacrylamide slab gels, proteins were transferred to nitrocellulose filters by semi-dry blotting [14] and immunoreactive bands were visualized as previously described [15].

3.7. Determination of N-terminal amino acid sequence

This was performed on a 470A gas phase sequencer (Applied Biosystems). The phenylthiohydantoin derivatives were determined by HPLC as previously described [16].

3.8. Sphingomyelinase activity

This was measured by the method described by Gatt et al. [17] using trinitro-phenyl-aminolauryl-(TNPAL)-sphingomyelin as a substrate. The concentration of the product was measured spectrophotometrically at 330 nm. One unit was defined as hydrolysis of 1 μ M substrate/min at 37°C, pH 7.4. As positive control commercially available sphingomyelinase from *S. aureus* (Sigma) was used.

3.9. Phospholipase C activity

This was determined by a modification of a previously described method [18] using *p*-nitrophenylphosphorylcholine as a substrate. PBS (pH 7.0) was used in most experiments instead of 0.25 M Tris-Cl (pH 7.2) as no stabilization of *Listeria* phospholipase C by the latter buffer was

noted. The enzymatic reaction results in the release of *p*-nitrophenol, which can be measured spectrophotometrically at 410 nm. As positive control phospholipase C from *B. cereus* (Sigma) was used.

4. RESULTS

Hemolysin production. In order to study the influence of growth media and growth phase on hemolysin synthesis, fresh BHI or the defined SLM-medium was inoculated with washed overnight cultures (BHI, 37°C) of *L. ivanovii* or *L. monocytogenes* Sv4b. The cultures were shaken at 37°C, and samples were assayed at different times for cell density and hemolytic activity.

In both media hemolysin concentration peaked at 18 h and, in the case of *L. ivanovii*, the concentration of iron in SLM-medium had no significant effect on hemolysin concentration within the range of 1–10 µM ferric iron (data not shown).

Purification of hemolysins. Bacteria were grown at 37°C for 18 h in BHI. The purification protocol is outlined in MATERIALS AND METHODS. Supernatant proteins with reactive SH-groups were bound by thiol-disulfide exchange affinity chromatography to thiopropyl-Sepharose 6B [13]. Freshly regenerated Sepharose gave better results than the freeze-dried material obtained from the supplier, but in any case this step resulted in a significant loss of protein, as has also been noted by others [6]; a further purification step was performed by gel filtration on Biogel P-100 [13]. After each purification step the proteins contained in the hemolytic peak fractions were analyzed by SDS-PAGE. After the final step the hemolytic material of *L. monocytogenes* Sv4b consisted of a single, homogeneous protein of M_r 58 kDa (Fig. 1, lane a). Hemolytic material from *L. ivanovii* contained two proteins of M_r 58 kDa and 24 kDa (Fig. 1, lane b). The 24 kDa protein could only very incompletely be separated from the 58 kDa protein by gel filtration on Biogel.

Identification of hemolysins. The hemolytic activity of fully reduced cytotoxin preparations was severely inhibited by minor amounts of free cholesterol (final concentration much below the

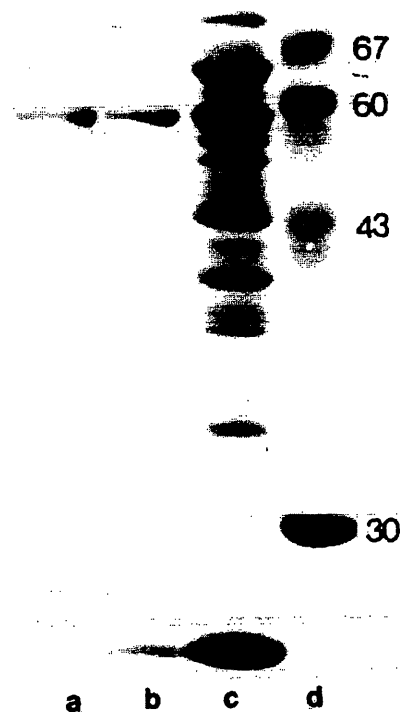


Fig. 1. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide, Coomassie blue staining). a, *L. monocytogenes* Sv4b, purified; b, *L. ivanovii*, purified; c, crude concentrated supernatant from *L. ivanovii*; d, molecular weight markers, figures on the right indicate kDa.

limits of solubility). Inhibition by cholesterol was linear between 0–0.15 µM and at 0.15 µM inhibition was 90–95%. Activation of the purified toxin preparations described above by DTE was linear in the range of 0–4 mM and saturation was reached at 10 mM. Preincubation with 10 mM DTE was used in all hemolysin assays. Stimulation by SH-reducing agents and inhibition by cholesterol is typical for SH-activated cytotoxins of the SLO-type. In order to confirm this classification we performed immunoblots of these proteins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b using anti-SLO-serum. TCA-precipitated supernatant proteins from other *Listeria* strains and from *S. pyogenes* have been included in this ex-

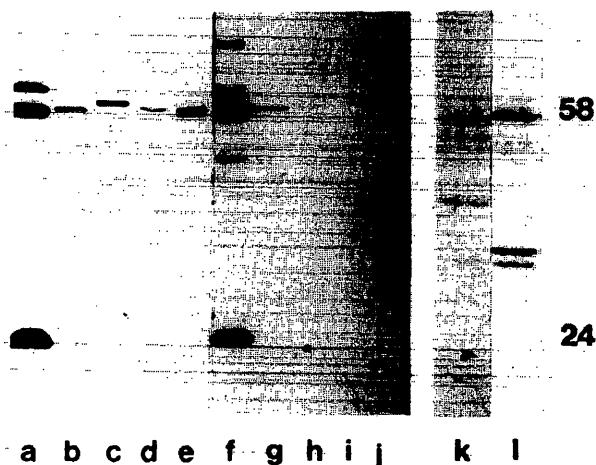


Fig. 2. Immunoblot of supernatant proteins after electrophoresis on 12.5% SDS-polyacrylamide gels. (I) Reaction with anti-LLO-serum; (II) reaction with anti-SLO-serum. a, f *L. ivanovii*; b, g *L. monocytogenes* Sv4b; c, h *L. monocytogenes* SV1/2a (EGD); d, i *L. innocua*; e, j *L. monocytogenes* 1/2a (Mackness); k, l *S. pyogenes* (SLO-producer). Figures on the right indicate the molecular weight in kDa.

periment. Replicas of the immunoblots first probed with equine anti-SLO were subsequently tested with rabbit antiserum raised against the two-component hemolytic protein (58/24 kDa) preparation from *Listeria ivanovii*. Both sera, anti-SLO and anti-*Listeria ivanovii*-hemolysin (anti-LLO), reacted in an almost identical fashion with the supernatant protein preparations from *L. ivanovii* (Fig. 2, lanes a, f). Also, in the culture supernatants from *Listeria monocytogenes* Sv1/2a

(Mackness and EGD) and Sv4b cross-reacting proteins of M_r 58 kDa clearly could be detected with both antisera (lanes b-e, g-j). Surprisingly the 24 kDa protein from *L. ivanovii* not only reacted, as expected, with anti-LLO but also with anti-SLO. Both antisera reacted with streptolysin O from *S. pyogenes* (Fig. 2, k-l). Finally, rabbit antiserum against the 24/58 kDa proteins strongly inhibited hemolysis of sheep erythrocytes by concentrated culture supernatants from *Listeria ivanovii* in standard hemolysin assays (Hemolytic titers without anti-LLO or with 10 μ l pre-immune serum were 32768 U/50 μ l, after addition of 10 μ l immune serum the titer dropped to 64 U/50 μ l).

N-terminal amino acid sequences. We have determined the N-terminal amino acid sequences of both the 58 kDa protein (listeriolysin, 22 residues determined) and the 24 kDa protein (19 residues determined) from *L. ivanovii* by microsequencing in a gas phase sequenator (Fig. 3).

Further biochemical and functional characterization. By isoelectric focussing the isoelectric points of the 58 kDa protein (listeriolysin) and of the 24 kDa protein from *L. ivanovii* were determined as 7.2 and 4.9, respectively. The pH-optima for the hemolytic activity of the purified cytolysins from *L. monocytogenes* Sv4b and *L. ivanovii* were determined as pH 5.0 and pH 5.5, respectively.

The classical way to distinguish between *L. monocytogenes* and *L. ivanovii* is their behaviour in the so-called 'CAMP'-test [19,20]: Beta-toxin (sphingomyelinase) producing *S. aureus* or on the

LLO <i>L. ivanovii</i>	Asp	Ala	Ser	Val	Tyr	Ser	Tyr	Gln	Gly	Ile	Ile	X(Ser)	His	Met	Ala
LLO <i>L. monocyt.</i>	Asp	Ala	Ser	Ala	Phe	Asn	Lys	Glu	Asn	Ser	Ile	Ser	Ser	Met	Ala
LLO <i>L. ivanovii</i>	Pro	Pro	Ala	X(Ser)	Pro	Pro	Ala	Ser							
LLO <i>L. monocyt.</i>	Pro	Pro	Ala	Ser	Pro	Pro	Ala	Lys							
24 KDa	Ala	Asp	Ile	Leu	X	Pro	Met	Pro	Ile	Asn	Gln	Ile	Phe	Pro	Asp
	Pro/Leu	Asp	Leu	Ala											

Fig. 3. N-terminal amino acid sequence of 58 kDa protein (LLO) and 24 kDa protein, both from *L. ivanovii*. An 'X' means an amino acid which could not be identified (most presumably serine). Below the the amino acid sequence for LLO from *L. ivanovii*, the amino acid sequence for LLO from *L. monocytogenes* as deduced from the DNA sequence [21] is shown.

other hand *Rhodococcus equi* cross-streaked with *Listeria* show zones of enhanced hemolysis at the intersection. *L. monocytogenes* (all serovars) reacts strongly with *S. aureus* and *L. ivanovii* reacts strongly with *R. equi*. Diffusion/reaction tests on blood agar plates using unfractionated, concentrated culture supernatants from (i) *L. ivanovii*, (ii) *S. aureus* and (iii) *R. equi* showed that in addition to the clear circular hemolytic zones surrounding the wells with supernatants from *L. ivanovii* or *S. aureus* (caused by alpha-toxin or listeriolysin, respectively), a lens-shaped hemolysis was visible in the region where diffusible products from *L. ivanovii* and *R. equi* or *S. aureus* overlapped and reacted with erythrocytes simultaneously or subsequently (Fig. 4). Enzymatic tests with 20-fold concentrated BHI culture supernatants from *L. ivanovii* clearly showed a significant phospholipase C activity and a pronounced

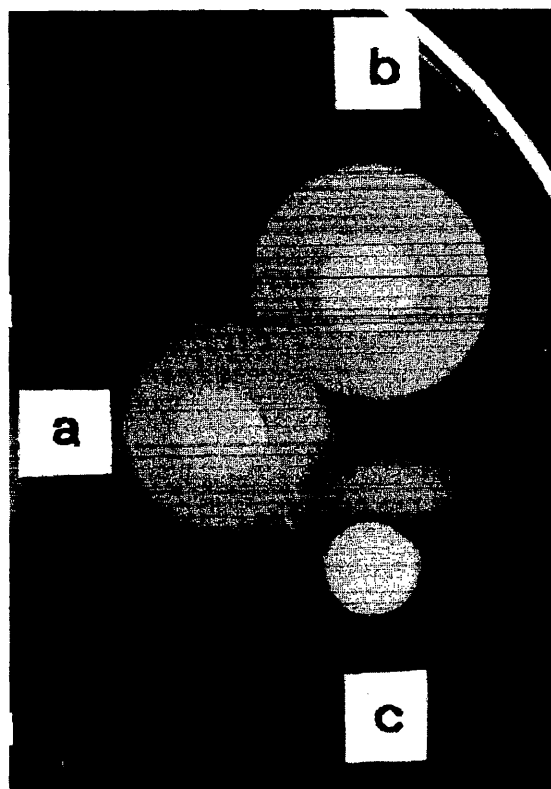


Fig. 4. Plate diffusion test on sheep blood agar plates. 50 μ l of 20-fold concentrated culture supernatants (18 h, 37°C in BHI) were applied per well. Incubation was overnight at 37°C. a, *L. ivanovii*; b, *S. aureus*; c, *R. equi*.

Table 1

	Sphingomyelinase C U/ml	Lecithinase Δ OD ₄₁₀ /16 h
Control (BHI)	0	0
<i>L. ivanovii</i>	360	0.81
ATCC 19 119		

10-fold concentrated supernatants of an 18 h culture were used

sphingomyelinase activity (Table 1). The enzymatic reaction to demonstrate phospholipase C activity in culture supernatants was slow, only after 16 h of incubation was a measurable activity detected. The value for the negative control remained zero even after this prolonged incubation. As a consequence of the long reaction time it did not seem reasonable to define the activity as units/ml, rather the difference in optical density is shown.

5. DISCUSSION

The results described above showed that relatively large amounts of a hemolytic protein with a molecular weight of 58 kDa could be purified from culture supernatants of *L. ivanovii* and also *L. monocytogenes* Sv4b, grown in BHI for 18 h at 37°C. These 58 kDa proteins fulfilled all the requirements for SH-activated cytotoxins of the SLO-type and were therefore identified as listeriolysin O (LLO): (a) Hemolytic activity was stimulated by an SH-reducing agent (DTE), (b) hemolytic activity was inhibited by cholesterol added in micromolar amounts and, (c) they cross-reacted immunologically with streptolysin O. Antiserum raised against *L. ivanovii* LLO cross-reacted with LLOs from other *Listeria* strains and with streptolysin O. Such a 'classical' listeriolysin O has not been described for *L. monocytogenes* Sv4b so far. The pH-optimal for hemolytic activity of the toxins from both *Listeria* species were in the acidic range, which is in good agreement with the findings for LLO from *L. monocytogenes* Sv1/2a [6]. This further supports the assumption that LLO may play a key role in the escape of virulent *Listeria* from the acidic phagolysosomal environment. The N-terminal amino acid se-

quence of LLO from *L. ivanovii* was determined and shows good homology (at least 11 out of 22 amino acids, the first three amino acids are identical) to the deduced amino acid sequence of LLO from *L. monocytogenes* Sv1/2a [21].

The 24 kDa exoprotein from *L. ivanovii* exhibited some unusual properties. It always copurified with LLO, but we could not determine if it bound to thiopropyl-Sepharose directly (via disulfide bonds) or via its association to LLO. In concentrated supernatants this protein apparently formed large complexes which could be dissociated only by SDS. So far we could not attribute any function to this protein. Plate diffusion tests also indicated that *L. ivanovii* secretes a factor which is phenotypically comparable to the CAMP-factor from *Streptococcus agalactiae*. Furthermore, both lecithinase and sphingomyelinase could be detected in *L. ivanovii* cultures supernatants. The lecithinase activity was rather weak, but the difference in activity between the culture supernatant sample and the blank was significant. This suggests that in *L. ivanovii* a bipartite hemolytic/cytolytic system is expressed, in addition to LLO. It has already been demonstrated that the combined action of sphingomyelinase and lecithinase can lyse mammalian cell membranes [22]. This second hemolytic system should contribute to the exceptionally high hemolytic activity and bizonal hemolysis, which is typically exhibited by *L. ivanovii* colonies on blood agar plates containing red blood cells with sphingomyelin-rich membranes, such as sheep erythrocytes.

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