3. Materials and Methods

3.1. Bacterial assays

3.1.1. Bacterial strains

All experiments were performed using either a capsule or toxin producing *B. anthracis* strain and performed in a BSL-3 facility. The Pasteur strain lacks the virulence plasmid XO1, which encodes the anthrax toxin, but expresses the poly-D glutamic acid capsule encoded on pXO2. The Sterne strain can express the toxin but not the capsule. Both strains were a kind gift of Dr. Wolfgang Beyer, Institut für Umwelt und Tierhygiene, Universität Hohenheim.

3.1.2. Bacterial growth conditions

To ensure toxin expression, the Sterne strain was grown on brain heart infusion (BHI) agar plates supplemented with 0.8% sodium bicarbonate (Sigma-Aldrich, St. Louise, USA) at 37°C and 5% CO_2. A single colony was sub-cultured overnight in 0.8% sodium bicarbonate supplemented BHI. The capsule producing strain was grown on 0.8% sodium bicarbonate supplemented BHI plates for 2 days at 37°C and 20% CO_2 and a single colony was sub-cultured overnight in RPMI supplemented with 10% BHI, 10 mM HEPES and 0.8% sodium bicarbonate. An OD_{600} of 1 corresponded to 7 x 10^6 bacteria/ml.

3.1.3. Preparation of *B. anthracis* spore suspensions

Both strains were grown in BHI (Bacto, Liverpool, Australia) overnight at 37°C before plating 1ml of the suspension on meat yeast agar (MYA) (10 g meat extract (Fluka, St. Louise, USA), 2 g yeast extract (Bacto, Liverpool, Australia), 0.04 g MnCl (Merck, Darmstadt, Germany), 15 g agar (Bacto, Liverpool, Australia), ad 1000 ml water), discarding excess fluid. The plates were incubated for 10 days at 37°C and 5% CO_2 in a plastic bag to prevent drying of plates. The spores were scraped off, washed with PBS three times and kept in PBS at 4°C until use. Sporulation was confirmed by microscopy and by heat treatment. Approximately 1 x 10^8/ml spores were generated. The spores were washed with PBS (Gibco, New York, USA) once a week to discard cell debris and potentially germinated spores.

3.1.4. Heat treatment of *B. anthracis* spores

Spores were diluted to 1 x 10^6/ml in PBS (Gibco, New York, USA) and heated at 60°C for 45 min. Under these conditions the vegetative cells die yet spores survive. The heated and a non-heated sample were plated in serial dilutions. The spores, which germinate on BHI agar (15 g agar, 37 g BHI *ad* 1000 ml water), were counted after 12 h at 37°C.

3.1.5. Toxin expression

Lethal and oedema factor as well as protective antigen expression were monitored by Western Blot analysis. 5 ml of an overnight culture were spun for 5 min at 5000 g and the supernatant subjected to TCA precipitation. The precipitate was dissolved in 2 x SDS Page sample buffer (62.5 mM Tris-HCL pH 6.8 (Sigma-Aldrich, St. Louise, USA), 500 µl ß-mercaptoethanol (Sigma-Aldrich, St. Louise, USA), 3 ml 10% sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louise, USA), 2 mg bromphenolblue (Sigma-Aldrich, St. Louise, USA), 0.5 M disodium EDTA (Serva, Heidelberg, Germany), 2 ml glycerine (Sigma-Aldrich, St. Louise, USA), *ad* 10 ml water).

3.1.6. Capsule expression

The γ-D-glutamic acid capsule expression was examined by negative staining with India ink (Pelican, Hannover, Germany). Bacteria of an overnight culture were mixed with India ink at a ratio of 1:1 and examined under the microscope. Capsule expressing bacteria showed a halo-like structure surrounding the bacterial body.

3.1.7. *Staphylococcus aureus* and *Shigella flexneri* growth conditions

S. aureus: *Staphylococcus* were streaked onto BHI agar plates and incubated overnight. A single colony was picked and incubated overnight in BHI at 37°C, shaking at 200rpm. A 1:100 subculture was made and bacteria were grown to mid log phase. An OD_{600} of 0.1 corresponds to 4 x 10^7 bacteria/ml.

Shigella flexneri: *Shigella* were streaked onto congo red agar plates (20 g tryptic soy agar (BD, New Jersey, USA), 0.05 g congo red (Sigma –Aldrich, St. Louise, USA), *ad* 500ml water, adjust pH to 7.3) and incubated overnight. A single colony was picked and incubated overnight in TSB (tryptic soy broth), 37°C, shaking at 200 rpm. A 1:100 subculture was made and bacteria were grown to mid log phase. An OD_{600} of 0.1 corresponds to 3 x 10^7 bacteria/ml.

Bacteria were incubated with different hNGE concentrations as described in 2. 10.

3.2. Cell based assays

3.2.1. Human neutrophil isolation.

Neutrophils (>95% pure) were isolated using a dextran-sedimentation protocol by Boyum (Boyum, 1968). Venous blood was obtained after informed consent of healthy volunteers and heparinized (Heparin-Natrium 25.000 ratiopharm® at 10 units/ ml) (Ratiopharm, Ulm, Germany). 60 ml blood was mixed with a 3% cold dextran (ICN Biochemicals, Eschwege, Germany) solution at a ratio of 2:1 and left at room temperature for 30 min. This isolates the erythrocytes from the leucocytes. The upper layer containing the leucocytes was carefully layered on a Ficoll-Paque ^{TM} Plus gradient (Amersham Biosciences, Pittsburgh, USA) (1:1) and centrifuged for 30 min at 400 g. The density of granulocytes is sufficient to allow them to migrate through the Ficoll-Paque layer, the lymphocytes, monocytes and platelets with their lower densities are found at the interface between the plasma and the Ficoll-Paque. The monocyte and lymphocyte containing supernatant were discarded and the neutrophil pellet re-suspended in HBSS- (Gibco, New York, USA). The cells were centrifuged for 10 min at 250 g and the supernatant discarded. The remaining erythrocytes were lysed with ultra-pure water (Biochrom AG, Berlin, Germany) for 30s and cells washed in HBSS- and centrifuged for 10 min at 250 g. The cell pellet was diluted in RPMI (Gibco, New York, USA) / 10 mM HEPES (Gibco, New York, USA) and 2% human serum albumin (Grifols, Barcelona, Spain). The cells obtained from this procedure were counted in a Neubauer chamber. With this method the neutrophils suspension show >95% purity, contaminating cells are eosinophils, lymphocytes and erythrocytes. From 60 ml bloods approximately 1-2 x 10^7 neutrophils were obtained.

3.2.2. Antimicrobial activity of neutrophils

Human neutrophils were isolated as described above. The cells of a 500 µl, 2 x 10^6/ml neutrophil suspension were allowed to adhere to plastic cell culture plates for 15 min and the cells were activated with 25 nM (or 5 nM) phorbol 12-myristate 13-acetate PMA (Sigma-Aldrich, St. Louise, USA) for 30 min. PMA activates the protein kinase C, which is responsible for the earliest stages of particle internalisation, for

cytokine production and the respiratory burst (Underhill and Ozinsky, 2002). In some experiments the cells were activated with 10 ng/ml IL-8 (BioCat, Heidelberg, Germany). IL-8 is a CXC chemokine, which induces neutrophil activation and recruitment in vivo (Baggiolini et al., 1989). Bacterial suspensions (vegetative cells or spores) were centrifuged onto the neutrophils (1250 rpm**,** 10 min) to synchronize the infection and incubated for varying time points and varying MOI at 37°C. The cells were scraped off, vigorously mixed with a pipette and serially diluted in PBS. These suspensions were plated on BHI agar plates and colonies were counted after 12 h incubation at 37°C, 5% CO_2. Bacterial killing was measured as percentages of control values (bacteria incubated alone in media without neutrophils).

3.2.3. LDH release assay

To monitor neutrophil viability, the lactate dehydrogenase activity was measured with the Cytox 96 Kit from Promega. LDH is a stable cytoplasmatic enzyme present in all cells, which is rapidly released into the cell culture supernatant upon damage of the plasma membrane (Weyermann et al., 2005). Released LDH in conjunction with diaphorase results in the conversion of a tetrazolium salt into a red formazan product. The amount of colour is proportional to the number of lyzed cells (Promega, Madison, USA). At every neutrophil/ bacteria incubation time point 50 µl of the undisturbed culture supernatant were transferred to a 96 well plate and 50 µl substrate mix added. The samples were incubated at room temperature in the dark for 30 min. 50 µl 6% acetic acid (Merck, Darmstadt, Germany) were added as a stop solution and the absorbance measured at 490 nm. Positive control was neutrophils lysed with 1% Triton X (Sigma-Aldrich, St. Louise, USA) solution, negative sample was the culture media.

3.2.4. Killing of vegetative *B. anthracis* in the presence of serum

Essentially the procedure was the same as described in 2.5. Difference was that the media contained 10% foetal calf serum (FCS) (Biochrom AG, Berlin, Germany) either complement inactivated at 55°C for 30min or left untreated.

3.2.5. NADPH oxidase inhibition

To measure antimicrobial activity in the absence of reactive oxygen species (ROS), neutrophils were isolated and 2 x 10^6 cells were left to adhere in 24 well plates for 15 min at 37°C. The cells were pre-incubated with 10 µM of the irreversible NADPH oxidase inhibitor DPI (Sigma-Aldrich, St. Louise, USA) (Cross and Jones, 1986) for 30 min at 37°C. The cells were then activated with 25 nM PMA for 30 min and infected with *B. anthracis* spores or vegetative cells.

3.2.6. Enhanced chemiluminescence

To control for absence of reactive oxygen species (ROS), neutrophils were isolated and 2 x 10^5 cells were left to adhere in 96 well plates for 15 min at 37°C. The cells were pre-incubated with 10 µM of the irreversible NADPH oxidase inhibitor DPI (Cross and Jones, 1986) for 30 min at 37°C. Inhibition of the respiratory burst was confirmed by enhanced chemiluminescence (Dahlgren and Karlsson, 1999). The cells were activated with 25 nM PMA and simultaneously 10 µM 5-amino-2,3-dihydr-1,4-phthalazinedione (luminol) (Sigma-Aldrich, St. Louise, USA) was added. This sensitive dye is excited by reactive oxygen species produced by the NADPH oxidase and releases energy in form of light (chemiluminescence). The samples were read in a BD Pharmagen TM, Monolight 3096 microplate luminometer, measurement was taken every 10 min over 2 h. Controls were neutrophils without PMA activation.

3.2.7. Cytochalsin D assay

Neutrophils were isolated as described above and seeded into 24 well plates at a density of 2 x 106 cells/ ml and left to adhere for 15 min at 37°C. Cells were activated with 25 nM PMA for 30 min at 37°C, cells were spun at 1000 rpm for 5 min and the media aspirated carefully. 500 µl media containing 10 µg/ml cytochalasin D (Sigma-Aldrich, St. Louise, USA) was added and the cells incubated for 20 min at 37°C. Cytochalasin D induces depolarization of the actin skeleton and thus inhibits phagocytosis. *B. anthracis* were added at an MOI of 1:1, spun onto the cells (1200 rpm, 10 min) and incubated for 30min at 37°C. The cells were scraped off, vigorously mixed with a pipette and serially diluted in PBS. These suspensions were plated on BHI agar plates and colonies were counted after 12 h incubation at 37°C, 5% CO_2. Bacterial killing was measured as percentages of control values (bacteria incubated alone in media without neutrophils).

3.2.8. Human neutrophil granule extract preparation

Neutrophils (>95% pure) were isolated from buffy coats obtained from the Deutsche Rote Kreuz using the dextran-sedimentation protocol by Boyum (1968) as described above. After lysing the contaminating erythrocytes, the neutrophils were washed in HBSS-, centrifuged for 10 min at 250 g and re-suspended in 5 ml HBSS without calcium and magnesium The cells were counted in a Neubauer chamber. One buffy coat results in the isolation of approximately 5-8 x 10^8 neutrophils. The cells were pooled in 5 x 10^8 cell batches, centrifuged for 5 min at 250 x g and the pellet submitted to a freeze thaw cycle. If stored for longer time periods cell pellets were kept at -20°C. 1 ml packed cells corresponded to approximately 1 x 10^9 neutrophils. Thawed cells were diluted to 3 x 10^8 cell/ml with cold pyrogen-free water. The cells were mixed and sonicated for 2 x 30 s pulses at 25% power with a Bandeli Sonoplus HD 2070. After the sonication the samples were milky white and homogenous and the cells did not settle upon standing. The purpose of the sonication was to release the granules and destroy the membranes. A final concentration of 0.16 N sulphuric acid (Merck, Darmstadt, Germany) with pyrogen free water was then added to the homogenized sample and incubated for 30 min at 4°C and vortexed every 5 min. The samples were transferred to siliconized (Eppendorf, Hamburg, Germany) tubes and spun for 20 min at 4°C at 14000 rpm. The supernatant was dialysed in Pierce Slide-A-Lyzer (Pierce, Chicago, USA) dialysis cassettes with a cut off of 3.5 kDa against 2l 20 mM sodium acetate buffer pH4 (820 ml 0.2 M acetic acid, 180 ml 0.2 M sodium acetate (Sigma-Aldrich, St. Louise, USA)) for 2 days at 4°C with two buffer changes.

3.2.9. Bactericidal activity of hNGE and α-defensins

hNGE was prepared as described by (Weiss et al., 1978). The bactericidal activity of hNGE, HPLC fractions or synthetic human α-defensin 2 (American Peptide Company, Vista, USA) was determined by incubating them with Pasteur or Sterne strain vegetative cells (10^7/ml) in a casamino buffer (0.3% casamino acids (Sigma-Aldrich, St. Louise, USA), HBSS-, 10 mM HEPES pH7.4) at 37°C, shaking for varying time periods. The casamino acids are good inducers of germination, reducing spore contamination. Survivors were counted in serial dilutions. Bacterial killing was measured as percentages of control values (bacteria incubated in media).
Spores were incubated in PBS supplemented with hNGE or α-defensins.

3.2.10. HPLC fraction killing

HPLC fractions were lyophilised with Amsco/ Finn-Aqua Yovac GT2-Typ2. Fractions were resuspended in 50 µl sodium acetate buffer (pH 4) and used for examination of anti-*B. anthracis* activity.

3.3. Analytical assays

3.3.1. TCA precipitation

To the bacterial supernatants 10% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) was added and incubated on ice for 30 min. The samples were centrifuged at 15000 g for 30 min and the pellet dissolved in acetone, to reduce TCA contamination. After a 10 min centrifugation step at 15000 g the acetone was removed and the sample left to dry (Rehm, 2002).

3.3.2. SDS Page Gel

Denaturing gel electrophoresis can resolve complex protein mixtures into hundreds of bands on a gel. The position of a protein along the separation lane gives a good approximation of its size, and, after staining, the band intensity is a rough indicator of the amount present in the sample. The discontinuous Laemmli system (Laemmli, 1970) is the most widely used system for research protein electrophoresis today. The resolution in a Laemmli gel is increased as the treated peptides are concentrated in a stacking zone before entering the separating gel.

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations, migration is determined not by intrinsic electric charge of polypeptides but by molecular weight. Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone. For almost all proteins, SDS binds at a ratio of approximately 1.4 g SDS per gram of protein, thus conferring a net negative charge to the polypeptide in proportion to its length. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and substantially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures. The proteins can be totally unfolded when a reducing agent such as ß-mercaptoethanol is employed. ß-mercaptoehthanol cleaves any disulphide bonds between cysteine residues. The

SDS-denatured and reduced polypeptides are flexible rods with uniform negative charge per unit length. Because molecular weight is essentially a linear function of the peptide chain length, the proteins separate by molecular weight.

3.3.3. Western Blot

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for Western Blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE. All sites on the membrane which do not contain blotted protein from the gel are non-specifically "blocked" so that antibody will not non-specifically bind to them, causing a false positive result.

To detect the antigen blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane. If there are any antibodies present which are directed against one or more of the blotted antigens, those antibodies will bind to the protein(s) while other antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies coupled to a reporter group such as horseradish peroxide are added. Finally after excess secondary antibody is washed free of the blot, a substrate is added, which precipitates upon reaction with the conjugate, resulting in a visible band where the primary antibody bound to the protein.

All Western blots were performed with the Mini-PROTEAN® 3 system from BioRad. The anthrax toxin samples were run on 12% acrylamide SDS gels (Resolving gel: 1.6 ml water, 2 ml 30% acrylamide (BioRad, Hamburg, Germany), 1.3 ml 1.5 M Tris (Sigma-Aldrich, St. Louise, USA) (pH 8.8), 0.05 ml 10% SDS, 0.05 ml 10% ammoniumpersulfate (BioRad, Hamburg, Germany), 0.002 ml TEMED (BioRad, Hamburg, Germany). Stacking gel: 2.1 ml water, 0.5% 30% acrylamide, 0.38%ml 1 M Tris (pH 6.8), 0.03 ml 10% SDS, 0.03 ml 10% ammoniumpersulfate, 0,003 ml TEMED. Running buffer: 800 ml destilled water, 30 g Tris Base (Sigma-Aldrich, St. Louise, USA), 144 g Glycin (Roth, Karlsruhe, Germany), 10 g SDS, calibrate to pH 8.3, *ad* 1000ml destilled water). For details of different gel concentrations refer to Sambrook and Russel, (2001). The transfer was made onto a nitrocellulose membrane (Amersham Biosciences, Pittsburg, USA) with the Polymehr system. The membranes were blocked with 3% bovine serum albumine (Gerbu, Gaiberg,

Germany) with 0.1% Tween (Sigma-Aldrich, St. Louise, USA) and the first and second antibody washed off in PBS with 1% Tween three times for 10 min. All secondary antibodies were horseradish-peroxide (HRP) labelled and bands were made visible by the ECL Western blotting detection reagents (Amersham Pharmacio Biotech, Pittsburg, USA). HRP catalyses oxidation of luminol, which in its excited state emits light (chemiluminescence). Enhanced chemiluminescence is achieved by the addition of chemical enhancers such as phenols, which increases the light output approximately 1000 fold. The antibodies used were monoclonal antibodies from Abcam (Cambridge, UK): anti-protective antigen (ab8241), anti-lethal factor (ab8242) and anti-oedema factor (ProSci Incorporated, Poway, USA, order number 3419).

3.3.4. Isoelectric focusing (IEF)

IEF is an electrophoretic method that separates proteins according to their isoelectric point (pI). The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino and carboxy terminals. The isoelectric point is the specific pH were the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI.

In IEF a pH gradient is produced and under the influence of an electric field, a protein will move to the position in the gradient were its net charge is zero. If a protein begins migrating away from its pI, it immediately gains charge and migrates back to its zero net charge point. This is the focusing effect.

The pH gradient is generated with the help of carrier ampholytes (they have both positive and negative charge). Commercially available mixtures contain hundreds of species with their pIs spanning a specific pH range. If an electric current is applied, the ampholyte molecules line up according to their pI thus creating a pH gradient. Once a sample has been separated according to its components isolelectric point, this 1°dimension gel can then be put on a SDS Page gel and the samples further separated by their molecular weight as described in 2.15.

3.3.5. Silver stain

Silver staining is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels. Its detection limit lies at 0.1–0.5 µg of protein. In silver staining, the gel is impregnated with soluble silver ions and developed by treatment

with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by protein.

The SDS gels were fixed for 2 h at room temperature in 500 ml ethanol (Merck, Darmstadt, Germany), 100 ml acetic acid (Merck, Darmstadt, Germany) and 400 ml deionized water. They were then incubated for 2 h at room temperature in 300 ml ethanol, 41 g sodium acetate (Sigma-Aldrich, St. Louise, USA), 20 ml 25% glutaraldehyde (Merck, Darmstadt, Germany), 2 g sodiumthiosulfate (Merck, Darmstadt, Germany) *ad* 1000 ml deionized water. The gels were washed 3 times for 10 min in deionized water and incubated for 30 min in a silver solution (1g silver nitrate (Merck, Darmstadt, Germany), 288 µl 35% formaldehyde, *ad* 1000 ml deionized water). The stain was developed for 1-5 min or until bands appear (25 g sodiumcarbonate (Merck, Darmstadt, Germany), 12 mg sodiumthiosulfate, 288 µl 35% formaldehyde *ad* 1000 ml deionized water and adjusted to pH 11.3 with 400 mg sodium hydrogen carbonate (Merck, Darmstadt, Germany). Development was stopped with 18.6 g Tritriplex (EDTA) (Merck, Darmstadt, Germany), 200 mg Thimerosal (Merck, Darmstadt, Germany), *ad* 1000ml deionized water.

3.3.6. High performance liquid chromatography (HPLC)

High performance liquid chromatography is a method, which fractionates complex biological samples based on their different properties. HPLC utilizes a mobile and a stationary phase to separate the components of a mixture. The components are dissolved in a solvent and then forced through a chromatographic column under high pressure. The sample interacts with both the mobile and the stationary phase and this interaction can be manipulated by the choice of these.

Ion exchange chromatography is a form of adsorption chromatography, which separates molecules on the basis of their charge. Stationary bound charged groups reversibly adsorb oppositely charged sample molecules. Desorption is brought about either by a change in the pH or by an increase in the salt concentration of the mobile phase. Proteins carry charged amino acids on their surface and can thus be adsorbed to ion exchangers. Proteins with net negative charges adsorb to anion exchangers, while those with net positive charges adsorb to cation exchangers. The more cationic a protein, the higher the salt concentration of the mobile phase must be to block the charges on the ion exchanger and the later the sample component is eluted. Altering the pH of the mobile phase induces change of the net charge of the

proteins in the sample and thus its adsorption properties. Therefore a neutral salt is usually used (e.g. NaCl) as it has little effect on the pH.

Reverse phase HPLC is a technique that separates molecules according to hydrophobicity. The stationary phase is highly substituted with hydrocarbon chains making it very hydrophobic (C4 equals four C atoms, C8 equals eight C atoms C18 equals eighteen C atoms). Proteins and peptides adsorb to the hydrocarbon chains even in pure water. The technique is preferred for peptide separation. As the adsorption in this technique is very strong, a strong organic mobile phase is needed to desorb the sample molecules. This can result in the denaturing of the tertiary structure of the proteins, resulting in loss of function.

hNGE was fractionated with a C4 RP-HPLC column (Vydac Protein C4 Column, 3,9 x 250 mm, 5 µm). Proteins were eluted with a gradient of increasing concentrations of acetonitril (Merck, Darmstadt, Germany) containing 0.1% (v/v) trifluoroacetic acid (Fluka) (flow rate 1 ml/min). Fractions were lyophilized, dissolved in 20 mM sodium acetate buffer and tested for antimicrobial activity. The antimicrobial activity was further purified in a second C4 column, (mobile phase actonitril gradient, constant 100 mM ammonium acetate (Merck, Darmstadt, Germany), pH5), followed by a C18 RP-HPLC column (X-terra RP18, 3.9 x 150 mm, 3.5 µm) with an acetonitril and 0.1% (v/v) trifluoroacetic acid gradient (flow rate 1ml/min). Separation was performed in a Waters 626 LC System with a Water 996 Photodiode Array Detector.

3.3.7. Immunoprecipitation

Immunoprecipitation is a technique that permits the purification of specific proteins from a complex mixture. A primary antibody, which is raised against the protein to be isolated, is usually bound to agarose or protein A beads, so that the formed antibody-antigen complex can physically be separate from the remaining sample. The matrix in this case was a polyacrylamide polymer, which is reported to have a low unspecific binding to proteins. This was extremely important as the hNGE encompasses many cationic components.

The UltraLink® Immunobilization Kit from Pierce (Chicago, USA) was used. The beads and antibody were used in a concentration of 5 mg beads / 50µg α-defensin 1-3 antibody (HyCult Biotechnology HM 2058, Uden The Netherlands). 160 µl antibody in coupling buffer (supplied in kit) was added to 5 mg dry acrylamide beads, vortexed

and incubated for 3 h at room temperature with constant mixing. The beads were washed four times with PBS and 160µl quenching buffer (supplied in kit) added for 2.5 h at room temperature with constant mixing. The beads were then washed twice with washing solution (supplied in kit) and twice in storage buffer (PBS + 20% ethanol (Merck, Darmstadt, Germany)) and stored at 4°C. Before the IP was performed the antibody-coupled beads were washed twice with PBS and re-suspended in 50µl PBS. 5µl of the beads were incubated for 1 h at room temperature with 5% BSA or 5% fish gelatine to block unspecific binding. The beads were washed in PBS twice and incubated with 20µl hNGE for 2.5 h at 4°C. The beads were spun down (5 min, 6000 rpm) and the supernatant lyophilised and tested for anti-*B. anthracis* activity after reconstitution in 20µl sodium acetate. As a control the experiment was done simultaneously with an unspecific antibody (protective antigen, Abcam, Cambridge, UK).

3.3.8. Mass spectrometry

Mass spectrometry provides the ability to accurately measure the mass of almost any molecule that can be ionized to the gas phase. For bio-molecules the molecular weight can be measured to within an accuracy of 0.015% of the total molecular weight. This allows for minor mass changes such as substitution of an amino acid to be detected. For small organic molecules the molecular weight can be so accurately measured that molecular formulas can be confirmed.

Mass spectrometers can be divided in three fundamental parts: The ionization source, the analyser and the detector. An ion source converts molecules into gas-phase ions. The ions are separated in the mass analyser according to their mass to charge ratio. The separated ions are detected and the data are recorded as spectra, which display ion intensities vs. their mass to charge ratio.

3.3.9. Ionisation methods

3.3.9.1. Matrix assisted laser desorption ionisation (MALDI)

Samples are first mixed with a highly absorbing matrix compound and sample/matrix crystals are formed. A laser is fired onto these crystals and the matrix transforms the laser energy into excitation energy for the sample. The sample molecules go into ionised gas phase. In the MALDI ionisation process singly charged molecules are

mainly formed by protonation of basic residues oft the side chains of cationic amino acids. The formed ions are accelerated into the mass analyzer.

3.3.9.2. Electrospray Ionisation (ESI)

The sample is dissolved in a polar, volatile solvent and pumped through a narrow, stainless steel capillary. A high voltage is applied to the tip of the capillary and as a consequence of the strong electric field, the sample is dispersed into highly charged droplets. The sample molecules can be multiply charged. A nebulising gas aids this process, a drying gas assists the solvent evaporation. Eventually, charged sample ions, free from solvent, are released from the droplets and pass into the analyser of the mass spectrometer.
The above described techniques are the most commonly used.

3.3.10. Mass analysis

3.3.10.1. Time of flight

The time of flight (TOF) mass spectrometer measures the time it takes ions of different masses to move from the ion source to the detector. The TOF tube is under a high vacuum. The higher the mass the longer it takes the ion to reach the detector. Usually MALDI is coupled to a time-of-flight (TOF) tube for mass analysis.

3.3.10.2. Ion trap

In this mass analyzer an oscillating electric potential is applied to a ring electrode; the ions are focused toward the centre of the trap. The ions absorb energy from the electric field, which causes them to become unstable and ejected from the trap. The ion can then be detected.

3.3.11. Peptide mass fingerprinting (PMF)

Peptide Mass Fingerprinting is a technique used to identify proteins by matching their peptide masses to the theoretical peptide masses generated from a protein database. The first step in PMF is that an intact, unknown protein is cleaved with a proteolytic enzyme to generate peptides. Most commonly this is done with trypsin. Trypsin cuts directly downstream of the two basic amino acids lysine (K) and arginine (R), which are fairly common residues. As different proteases cleave at different amino acid residues the PMF of a protein depends on the protease used, but will

always be the same for each one. This means that every unique protein will have a unique set of peptides and hence unique peptide masses. Identification is accomplished by matching the observed peptide masses to the theoretical masses derived from a sequence database.

In this study the identity and purity of the antimicrobial component was analyzed by MALDI mass spectrometry (Proteomics 4700 workstation, Applied Biosystems), peptide mass fingerprinting (PMF), MS/MS analysis and mass analysis of the uncleaved protein. The lyophilized sample was digested with 50 mM NH_4HCO_3, 5% acetonitrile, 2% (w/v) trypsin (Sequencing grade modified Trypsin, Promega, Madison, USA) and 0.15 M DTT (BioMol, Ilvesheim, Germany) for 4 h at 37°C. The reaction was stopped with 0.2% TFA and mixed with matrix alpha-Cyano-4-hydroxycinnamic acid (CHCA) solubilised in 50% acetonitrile 0.3% TFA with a concentration of 5 mg/ml.
Analysis of PMFs were obtained with the following parameters: reflectron mode, 20kV accelerating voltage, and a low mass gate of 800Da. MS/MS spectra were obtained without collision gas. Parameter for database searches (MASCOT, http://www.matrixscience.com) were: 30 ppm peptide mass tolerance for peptide mass fingerprints and 0.3 Da for MS/MS spectra. The uncleaved protein was analysed in linear mode with CHCA as matrix with an internal marker (Mr 2465.21).

3.3.12. Electron microscopy

For transmission electron microscopy, cells were fixed with 2.5% glutaraldehyde, postfixed with 1% osmiumtetroxide, contrasted with uranylacetate and tannic acid, dehydrated and embedded in Spurr's (Ted Pella, Redding, USA). After polymerization, specimens were cut at 60 nm and contrasted with lead citrate. Specimens were analyzed in a Leo 906E transmission electron microscope.
For scanning electron microscopy cells were fixed with 2.5% glutaraldehyde, postfixed using repeated incubations with 1% osmium tetroxid / 1% tannic acid, dehydrated with a graded ethanol series, critical-point dried and coated with 2 nm platinum. After dehydration and critical-point drying, the specimens were coated with 5 nm Platinum/Carbon and analyzed in a Leo 1550 scanning electron microscope.