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Two-step fast protein liquid chromatographic purification of the *Serratia marcescens* hemolysin and peptide mapping with mass spectrometry

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Abstract

The pore forming toxin of *Serratia marcescens* (ShlA) is secreted and activated by an outer membrane protein (ShlB). Activation of inactive ShlA (termed ShlA*) by ShlB is dependent on phosphatidylethanolamine (PE). Activation may be a covalent modification of ShlA. To test this hypothesis, the responsible activation domain (in the N-terminal 255 amino acids of ShlA) was isolated from whole bacteria with 8 *M* urea in an inactive form (ShlA-255*) and from the culture supernatant in an active form (ShlA-255), followed by a two-step purification by anion-exchange chromatography and gel permeation chromatography. Comparison of a tryptic peptide map of both forms with subsequent electrospray mass spectrometry (ES-MS) and sequencing by tandem ES-MS revealed no modification. These data imply that ShlB presumably imposes a conformation on ShlA-255 that triggers activity.

Keywords: Purification; *Serratia marcescens*; Peptide mapping; Hemolysin

1. Introduction

Almost all strains of *Serratia marcescens* secrete a cytotoxin [1–5] that causes hemolysis of human and animal erythrocytes and the release of inflammatory mediators from leukocytes [6]. Hemolysin production increases uropathogenicity of *Escherichia coli* 563/21 transformed with the *S. marcescens* hemolysin determinant in an experimental rat urinary tract infection model [7]. *E. coli* K-12 transformed with the *S. marcescens* hemolysin determinants displays the same hemolytic properties as the *S. marcescens* parent [8,9].

Hemolytic activity is determined by two proteins, ShlA and ShlB. The hemolysin ShlA is secreted with the aid of ShlB, which is located in the outer membrane [9,10]. Cells lacking ShlB contain inactive ShlA (termed ShlA*) in the periplasm [10]. ShlB catalyses secretion of ShlA* across the outer membrane and conversion of ShlA* to an active hemolysin (then termed ShlA). Mutant analyses have revealed a tight coupling between activation and secretion since no mutated ShlB has been found that activates ShlA* without secretion, or that secretes inactive ShlA* for a long period. New results presented the uncoupling in vivo [11]. Mutants of ShlB have been found which are able to secrete inactive ShlA* into the supernatant and other ShlB mutants which activates ShlA without secretion. In vitro, secretion can be uncoupled from activation

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since lysates of cells that synthesise only ShlB activate ShlA* in lysates of cells that synthesise only ShlA* [12]. The in vitro activation of highly purified ShlA* by ShlB is dependent on phosphatidylethanolamine (PE), but PE is not covalently linked to ShlA [13]. This activation by ShlB is irreversible in contrast to complementation of ShlA* by ShlA-255 which is reversible since removal of ShlA-255 from ShlA* by gel permeation chromatography results in inactive ShlA* [12]. However, functional analysis of inactive ShlA* polypeptides is facilitated by complementation to hemolytic ShlA by an N-terminal fragment that contains 255 residues (ShlA-255) [12]. This N-terminal fragment is secreted by ShlB and converted into a form that complements ShlA* to hemolytic active ShlA, which suggests that it is modified by ShlB in the same way as ShlA* when it is converted to ShlA. Hence, PE-dependent activation of ShlA and ShlA-255 appeared to be a modification through ShlB.

The large ShlA protein (1578 AA) is not accessible to detailed biochemical examination because its half life in aqueous solution is about 3 min due to aggregation and precipitation. In contrast, the activated and secreted N-terminus (ShlA-255) is soluble, stable and small enough (relative molecular mass about 28 000) to represent the activation site of ShlA in further biochemical studies. To examine the putative modification in ShlA-255 as compared to the inactive, unmodified N-terminus ShlA-255*, ShlA-255* was genetically engineered. Without ShlB, ShlA-255* is not secreted and activated and remains in the periplasm. In vitro, inactive ShlA-255* could be activated by ShlB similar to ShlA*. Successful activation of isolated ShlA-255* by ShlB in vitro could not be achieved within a single tube test by complementation with ShlA* because the ShlA* would also be activated by ShlB. Therefore, a deletion mutant of ShlA, lacking the amino acids from position 4-96 (termed ShlA-56), is no longer activated by ShlB but still complemented by ShlA-255 [10]. Thus, the activation of ShlA-255* by ShlB could be easily determined by complementation with ShlA-56 in a one tube assay. Isolation of inactive ShlA-255* from the bacterial periplasm was achieved by sonic disruption of the bacteria in the presence of 8 *M* urea. A purification protocol, consisting of only two steps, was developed using a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) with anion-exchange chromatography and gel permeation chromatography. To isolate the activated ShlA-255, the protein was precipitated from the bacterial culture supernatant and purified by a single anion-exchange chromatography step [13]. Active and inactive forms of ShlA-255 were characterised by electrospray mass spectrometry (ES-MS). In addition, both proteins were digested with trypsin and the resulting peptides were separated by reversed-phase chromatography (RPC) on a FPLC system and sequenced by subjecting them to tandem ES-MS.

2. Materials and methods

2.1. Chemicals

Ammonium acetate, acetonitrile, ammonium sulphate, sodium phosphate, sodium chloride, urea, HEPES [*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonate] were of analytical grade and purchased from Merck, Darmstadt, Germany. Phosphate-buffered saline (PBS) consisted of 0.8% NaCl, 0.02% NaH_2PO_4 and 0.12% $Na_2HPO_4 \cdot 2H_2O$. Bacteria were cultured in LB-medium (Difco Labs., Detroit, MI, USA). Ampicillin was purchased from Sigma, Deisenhofen, Germany. Isopropyl-β-D-thiogalactopyranoside (IPTG) for induction of expression vectors was from Roche Diagnostics, Mannheim, Germany. Prepacked columns for FPLC of HiPrep 26/10 Desalting, Resource Q, 6 ml, Mono Q HR 5/5, Superdex 75 HR 10/30, RPC-6 were purchased from Pharmacia, Uppsala, Sweden. Water used for FPLC was purified by reverse osmosis. Buffers were filtered through a 0.2-μm sterile filter and degassed prior to use.

2.2. Fast protein liquid chromatography

Protein purification was performed using a Pharmacia FPLC system, consisting of a LCC-501 plus controller, two pumps P-500 with a 0.6-ml mixer (24 VAC), motor valve MV-7 and a FRAC-200 fraction collector. A variable-wavelength monitor VWM 2141 and a Pharmacia conductivity monitor connected in series served to monitor the column eluent.

The FPLC system was interfaced to a Compaq Deskpro XE466 and controlled by the FPLC director software (Pharmacia). Chromatograms were generated using a HP DeskJet 560C printer (Hewlett-Packard, Boise, ID, USA).

2.3. Electrospray mass spectrometry and tandem mass spectrometry

Electrospray mass spectra were recorded on a API III triple-quadrupole mass spectrometer (Perkin-Elmer Sciex, Thornhill, Canada) with a mass range of m/z 10–2400 equipped with a nebulizer-assisted electrospray (ion spray) interface [14]. The mass spectrometer was operated in positive ion mode under conditions of unit mass resolution for all determinations. Profile spectra were obtained by acquiring data points every 0.5 relative molecular mass with a dwell time of 0.5 ms or 1 ms. The potential of the spray needle was held at +4.8 kV; orifice voltages were +140 V. For mass calibration, a solution of CsI (0.5 mg of CsI in 0.5 ml methanol–water, 1:4, v/v) was used, yielding cluster ions of caesium iodide $(Cs_{n+1}J_n)+$ with $n=0$–8 at m/z 132.9, 392.7, 652.5, 912.3, 1172.2, 1432.0, 1691.8, 1951.6 and 2211.4. Sample solutions (proteins and peptides) in sample buffer were flow-injected with an autosampler. The ''HyperMasses'' of the proteins were calculated with the computer program MacSpec 3.2 (Sciex).

ShlA-255 was desalted on a fast desalting HiTrap column (Pharmacia) in 20 m*M* ammonium acetate, pH 8.4, prior to MS. ShlA-255*-containing fractions after gel permeation were flow injected. Peptides containing peak fractions from reversed-phase chromatography were flow injected for MS and sequencing by MS–MS.

The tandem mass spectrometer consisted of an arrangement of three consecutive quadrupole systems in which the second one served as the collision region [15]. MS–MS (collision-induced dissociation [16] was performed with argon as collision gas [with the same apparatus as above (API III, Sciex)] and collision energies ranging from 30 to 60 eV.

2.4. Fermentation of recombinant bacteria

The bacterial strains and plasmids used in this study are listed in Table 1. An overnight culture (400 ml) of *E. coli* BL21 (pTA4 *shlA255*) or *E. coli* BL21 (pRO2 *shlB, shlA255*) was used to inoculate 9.6 l of TY medium, consisting of 0.8% tryptone (Difco Labs.), 0.5% yeast extract, 0.5% NaCl, pH 7.0, supplemented with ampicillin (50 mg/l). Cells were grown in a 10-l fermenter (Biostat E, B. Braun, Melsungen, Germany) at 37°C with stirring at 200 rpm until the culture reached an absorbance of 0.5 at 578 nm ($O.D._{578}$). IPTG was added to a final concentration of 1 m*M*, and the fermentation was continued for 2 h ($O.D._{578}$ 2.0–2.2, 3.5 g/l bacterial wet mass).

2.5. Isolation of proteins from the supernatant (ShlA-255) and periplasm (ShlA-255* and ShlA-56)

ShlA-255 was obtained from the culture supernatant of IPTG-induced *E. coli* BL21(pRO2 *shlA255*

Table 1
E. coli strains and plasmids used

Strain/plasmid	Relevant genotype	Source/Ref.
E. coli		
BL21 (DE3)	Lysogenic for phage λ carrying the phage T7 RNA polymerase gene under control of the *lacUV5* promoter	[26]
Plasmids		
pES56	pT7-6 *shlB shlA*(Δ4-97)	[10]
pRO2	pT7-6 *shlB shlA*(Δ256-1578)	[24]
pTA4	pT7-7 *shlA*(Δ256-1578)	This work
pT7-6		[21]
pT7-7		[21]

shlB) by precipitation with 55% ammonium sulphate. Then, 3 *M* urea was added and incubated at 4°C for 18 h without stirring. Proteins floating on the surface were collected with a pipette which reduced the volume from 15 l to 200 ml and facilitated subsequent centrifugation (45 min at 30 000 *g*). The precipitate was solubilised in 6 ml 20 m*M* ammonium acetate buffer, pH 8.4. Insoluble material was removed by centrifugation.

ShlA-255* and ShlA-56 were obtained from a whole cell extract. Cells were harvested by centrifugation. Cells (4 g) suspended in 8 ml of 20 m*M* HEPES, 8 *M* urea, pH 4.0, were treated with a Branson sonifier (Emerson Technologies, Dietzenbach, Germany), using a 3 mm microtip at the maximal amplitude for 2 min and then stored overnight at 4°C. The precipitated material and cell debris was removed by centrifugation (15 min at 60 000 *g*), and the supernatant was used for chromatography.

2.6. Purification of proteins by FPLC

The crude extract containing ShlA-255* was desalted and the solvent replaced by 20 m*M* ammonium acetate buffer, pH 8.4 on a HiPrep 26/10 Desalting column (Pharmacia, Freiburg, Germany). Desalted fractions were pooled and loaded on a 6-ml Resource Q column via a 50-ml superloop. Proteins were eluted in 1-ml fractions, using a continuous gradient in 20 m*M* ammonium acetate, pH 8.4, from 0 to 0.3 *M* NaCl within 30 min. Elution of proteins was monitored by UV monitoring at 280 nm. The gradient was monitored by conductance. The elution of ShlA-255* was determined by in vitro activation as described. Active peak fractions (6–8 ml) were collected, pooled and reduced in volume to 500–600 μl by lyophilisation. The concentrated proteins were loaded on a Superdex 75 gel permeation column and eluted with 20 m*M* ammonium acetate, pH 8.4.

2.7. Tryptic peptide map of ShlA-255 and ShlA-255* by RPC

A 100-μg volume of highly purified protein was treated with 1 μg trypsin in 100 μl ammonium acetate buffer, pH 7.8 at 37°C for 3 h. The protein digest was loaded on a 3 ml Resource RPC column with 50 m*M* ammonium acetate, pH 4.0. Peptides were eluted with a continuous gradient from 0 to 80% with 50% acetonitrile in 50 m*M* ammonium acetate, pH 4.0 (v/v) in 18 min at a flow-rate of 1 ml/min. Samples were collected in 0.5-ml fractions for further examination.

2.8. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis was carried out on a BRL (Gaithersburg, MD, USA) vertical gel electrophoresis system with 20×20 cm glass plates. SDS electrophoresis gels (0.75 mm thickness) were prepared with 11% and 16% acrylamide in the running gel and performed as described by Lugtenberg et al. [17]. Proteins were applied in sample buffer according to Laemmli [18].

2.9. Determination of the activity of isolated proteins

For the determination of hemolysis in liquid culture outdated human erythrocyte concentrate obtained from the Blood Centre of the University of Tübingen was used. Washed erythrocytes were pelleted by centrifugation in a 50-ml Falcon tube, 2000 *g* for 10 min and suspended to a final concentration of 8% (v/v) in PBS. Hemolytic activities are presented either as the percentage of the total erythrocytes lysed (percent hemolysis) or as hemolytic units (HU). Hemolytic units were determined as described [19]. Serial dilutions of hemolytic samples were prepared with PBS or 50 m*M* HEPES, 6 *M* urea, pH 8.0. A 100-μl aliquot of each dilution was incubated with 1 ml of erythrocyte suspension for 15 min at 22°C and then centrifuged for 1 min in a microcentrifuge. The absorbance at 405 nm (A_{405}) of released hemoglobin was determined spectroscopically. The hemolytic activity is defined as:

$$\text{hemolytic activity (\%)} = \frac{A_{405}(\text{sample with hemolysin}) - A_{405}(\text{control without hemolysin})}{A_{405}(\text{total lysis caused by SDS}) - A_{405}(\text{control})} \times 100$$

One hemolytic unit (HU) defines the activity which releases 50% of the total hemoglobin of 1 ml, 8% erythrocyte suspension within 15 min at 22°C.

The activity of ShlA-255 was determined after complementation with ShlA*. Samples (10 μl) of peak fractions, stored on ice, were mixed with 10 μl of a ShlA* crude extract [13], incubated 5 min at 22°C after which 200 μl of an 8% suspension of human erythrocytes in PBS was added. Hemolysis was determined spectroscopically at 405 nm after 15-min incubation, as described above. For in vitro activation, 10 μl of peak fractions, stored on ice, containing inactive ShlA-255* were mixed with 10 μl of a ShlB crude extract [13] and incubated for 10 min at 22°C. Activation was determined by adding 10 μl of a ShlA-56 crude extract for complementation. Hemolytic activity was determined as described above.

*2.10. Genetic construction of ShlA-255**

Plasmid pTA4 was constructed by introduction of an NdeI cleavage site at the starting ATG-codon of ShlA-255 by ''site directed mutagenesis'' via polymerase chain reaction (PCR) [20]. The PCR product was cleaved with NdeI, SacI and ligated in a pT7-7 vector digested with NdeI, SacI [21]. The introduction of NdeI cleavage site and correct location in pTA4 was characterised by sequencing (A.L.F., Pharmacia, according to the instructions of the manufacturer).

2.11. Protein analytical procedures

Protein concentrations were determined as described [22,23].

3. Results and discussion

3.1. Purification of ShlA-255 from the periplasm*

ShlA-255* was overproduced in *E. coli* BL21(DE3) (pTA4) that encodes the T7 RNA polymerase on the chromosome and the N-terminus of the *shlA* gene cloned downstream of the gene 10 promoter of phage T7 on pTA4. Transcription of *shlA255* was initiated by adding IPTG which induces transcription of the T7 polymerase which in turn transcribes *shlA255*. ShlA-255* was extracted with 8 *M* urea at pH 4 which maintains ShlA-255* in solution (Fig. 3, lane 1). Despite the harsh extraction procedure, ShlA-255* was activated by ShlB (see below). The crude extract of ShlA-255* was desalted and applied on the strong anion-exchange column Resource Q. The bound proteins were eluted using a NaCl gradient in ammonium acetate buffer. The ShlA-255*-containing peak fractions eluted between 0.175 and 0.245 *M* NaCl, were pooled (Fig. 1 and Fig. 3, lane 2) and were concentrated to about 500 μl by lyophilisation. Ammonium acetate evaporated and no denaturation of proteins occurred due to increasing buffer salt concentrations. After this step, ShlA-255* was rather pure and therefore the hydrophobic interaction chromatography (HIC) step could be omitted. The sample was directly chromatographed on a Superdex 75 column (separation range between 3000 and 70 000 relative molecular mass). ShlA-255* had a retention time of about 8.7 min at a 1 ml/min flow (Fig. 2). This purification protocol resulted in an electrophoretically homogeneous ShlA-255* protein (Fig. 3, lane 3). Larger and minor contaminating proteins could be clearly separated from ShlA-255* with gel permeation chromatography. Using the urea treatment together with the high purification capacities of Resource and Superdex material, purification of ShlA-255* from a whole cell extract was achieved with only two chromatography steps. This method may also be suitable to isolate recombinant proteins from inclusion bodies. Using FPLC, the procedure was finished within one day and rapid purification of large amounts of ShlA-255*, suitable for MS, were achieved.

3.2. Purification of ShlA-255 from bacterial culture supernatants

ShlA-255 was overproduced in *E. coli* BL21(DE3) (pRO2) that encodes the T7 RNA polymerase on the chromosome, ShlB and the N-terminus of the *shlA* gene cloned downstream of the gene 10 promoter of phage T7 on pRO2. Transcription was initiated by adding IPTG, which induces transcription of the T7 polymerase and in turn transcription of *shlB shlA255*. After centrifugation of the bacteria, the proteins in the culture supernatant were precipitated by ammonium sulphate and isolated by centrifugation. In this step, 3 *M* urea was added to adapt the buffer to the one used for the

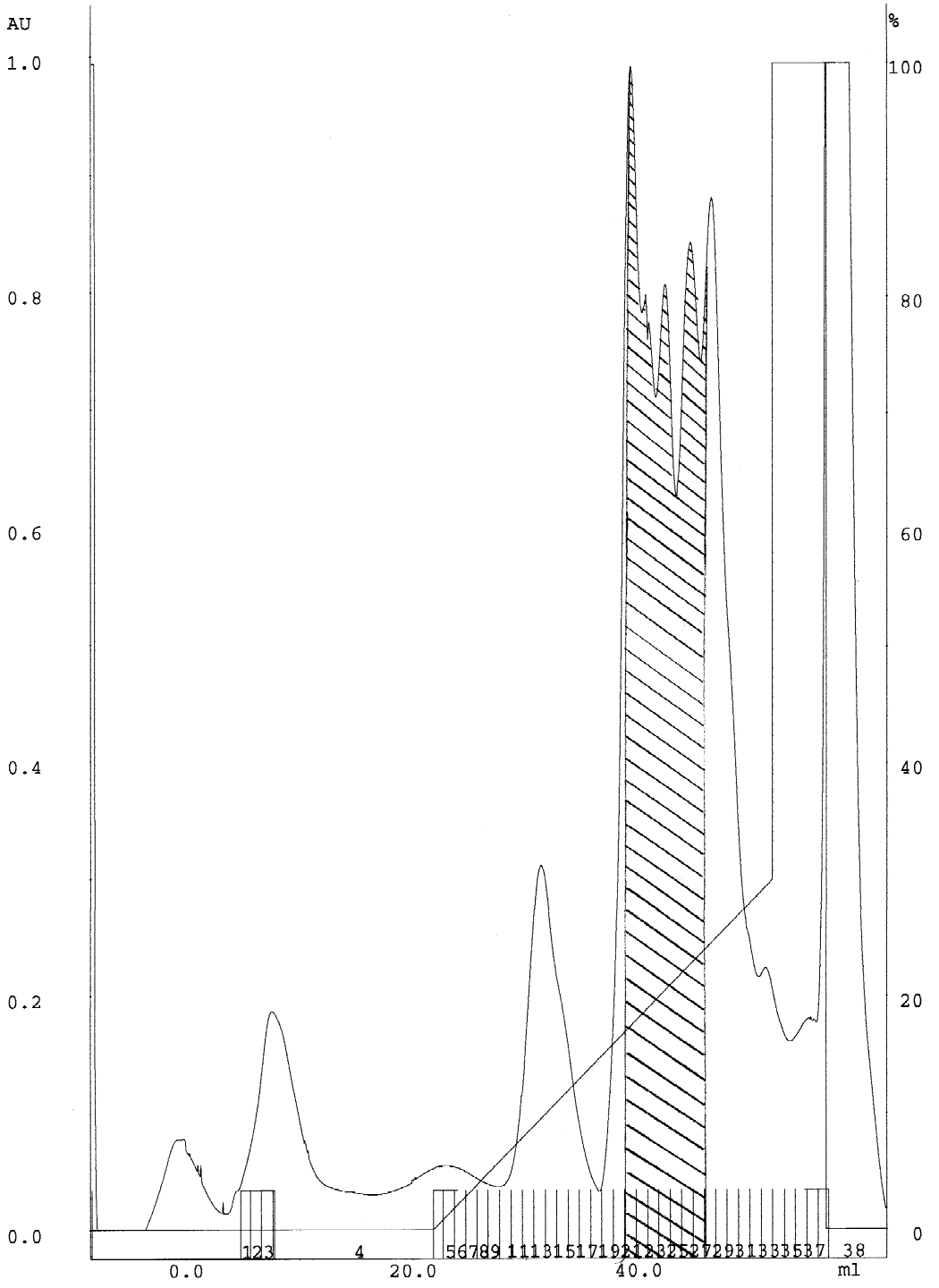

Fig. 1. Chromatogram of ShlA-255* on Resource Q. The desalted crude extract of approximately 0.8 g bacterial wet mass was loaded on the column in two 3-ml portions. Fractions containing complementation activity are hatched (fractions 21–27). These fractions were pooled.

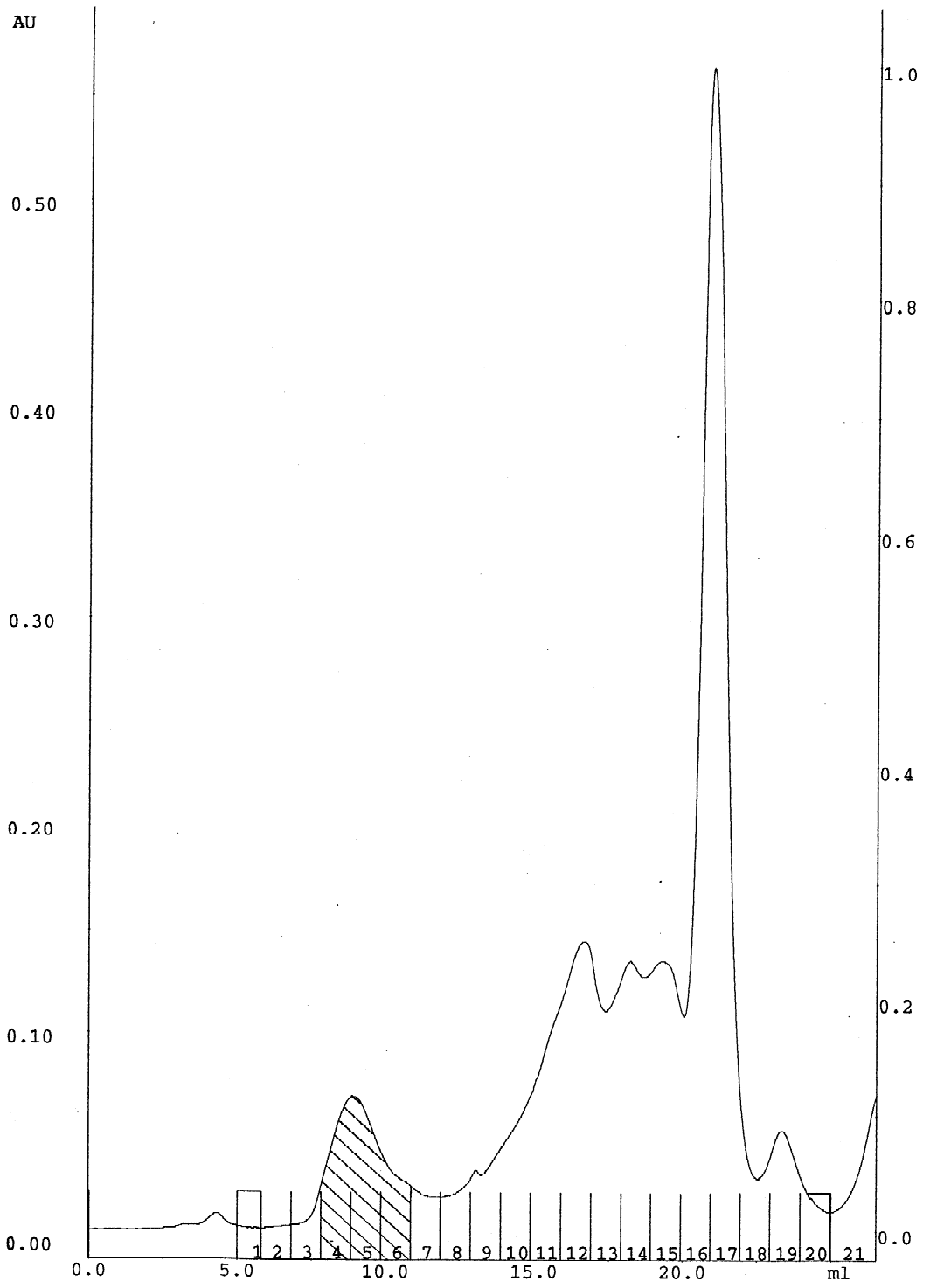

Fig. 2. Chromatogram of ShlA-255* on Superdex 75. Lyophilised active fractions pool of Fig. 1 (about 500 μl) was loaded. Fraction No. 5 is shown in Fig. 3. Complementation activity containing fractions are hatched.

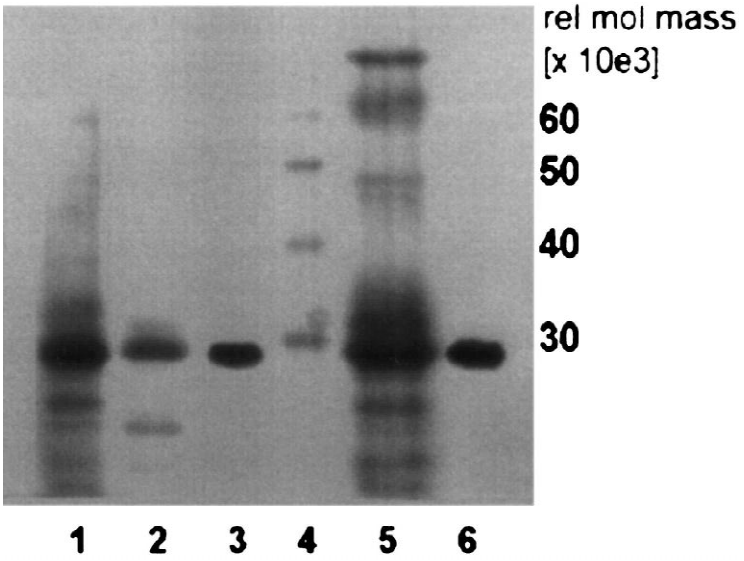

Fig. 3. SDS–PAGE (11%) of samples during purification of ShlA-255* and ShlA-255. Crude urea extract of *E. coli* BL21 transformed with plasmid pTA4 *shlA255* was applied to lane 1. Lane 2: activity peak fraction after anion-exchange chromatography. Lane 3: activity peak fraction No. 5 of gel permeation chromatography. Standard proteins and their relative molecular masses are in lane 4. Lane 5: crude extract of the culture supernatant of *E. coli* BL21 transformed with plasmid pRO2 *shlB shlA255*. Lane 6 contains the peak fraction after anion-exchange chromatography on Mono Q HR 5/5. The proteins were visualised by staining with Coomassie brilliant blue.

isolation procedure as with ShlA-255*. The complementing activity in the crude extract was not affected by the addition of urea. Due to the fact that ShlA-255 was solely secreted by this recombinant *E. coli* strain, the solubilised crude extract was highly enriched in ShlA-255 (Fig. 3, lane 5). Only a single purification step on a Mono Q or Resource Q anion-exchange column was required to yield highly purified of ShlA-255 (Fig. 3, lane 6).

3.3. Complementation of ShlA-255 and ShlA-255*

A 10-μl volume of ShlA-255* containing peak fractions from the anion-exchange separation step, gel permeation chromatography or crude extract were activated by adding 10 μl of a ShlB crude extract. Activation of ShlA-255* was determined by complementation with ShlA-56 crude extract, 10 μl, which became hemolytic. Hemolytic activity depended on the concentration of ShlA-255*. According to this assay about 2000 hemolytic units (HU) per gram of bacterial wet mass were isolated (specific activity 0.5±0.1 HU/mg protein). After anion-exchange chromatography the specific activity of the peak fractions was 336±18 HU/mg and further purification by gel permeation chromatography yielded a specific hemolytic activity of 527±9 HU/mg. With only two chromatographic steps a 1000-fold enrichment of native ShlA-255* was achieved.

In contrast, ShlA-255, activated and secreted by ShlB into the culture supernatant, is already rather pure because only few proteins in low amounts are secreted by the recombinant *E. coli* strains used for ShlA-255 synthesis. After ammonium sulphate precipitation from the supernatant, the solubilized sample displayed about 590±14 HU/mg. After anion-exchange chromatography a 20-fold purification (1340±27 HU/mg) was achieved. This data underline the high purity of the crude extract. The higher hemolytic activity of ShlA-255 as compared to ShlA-255* originates from the in vitro activation.

ShlA-255* has first to be activated in vitro by ShlB and then be complemented with ShlA-56 whereas ShlA-255 is activated and secreted in vivo and complementation with ShlA* could be directly examined. Complementation with ShlA-56 is about 34% less effective than with ShlA* (tested with similar amounts of active ShlA-255, unpublished

observation). It is also possible that a portion of ShlA-255* remained inactivated by ShlB. But even with activated ShlA-255, the activity could only be detected by complementation of ShlA*, provided in excess. Therefore, the discrimination between native and denatured protein during the chromatographic procedures by testing the activity was not possible. The specific complementation activity of ShlA-255 was estimated to be 1.3 HU/μg in this experiment for the first time and therefore stated to be complete native. Native PAGE of purified ShlA-255 and ShlA-255* done under non-denaturing conditions gave single bands (data not shown) suggesting that the proteins existed in a single form (presumably native). The renaturing conditions during or after purification were not examined in detail. However, the purified proteins active after the chromatographic steps could be examined by biochemical and biophysical methods.

3.4. Mass spectrometry and peptide mapping

Purified active ShlA-255 was subjected to MS. The relative molecular mass of ShlA-255, deduced from the signals of multi-charged ions, was 27 690±4.2 and thus very close to the theoretical relative molecular mass of 27 672 as deduced from the amino acid sequence (Fig. 4). These data exclude a covalent modification of ShlA-255 by lipids (ranging about 200 relative molecular mass), phospholipids (ranging about 630) or phosphate (80).

To determine whether a single amino acid was chemically modified, the proteins were digested with trypsin. The resulting peptides were separated on a RPC column via FPLC (Fig. 5) and subjected to MS–MS sequencing. All peptides isolated exhibited the theoretically expected amino acid sequence, as indicated in Fig. 5 with one exception. Peptide 1923 showed a gap within the ANPN motif. This could

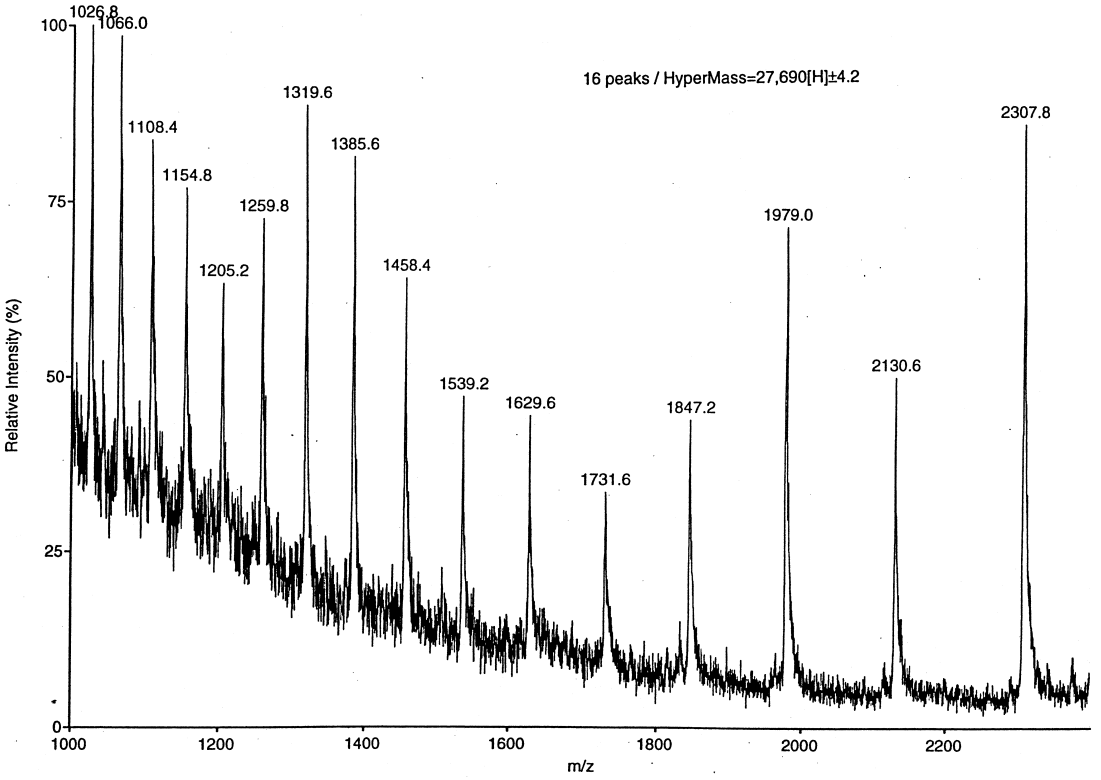

Fig. 4. Determination of the molecular mass of ShlA-255 by electrospray mass spectrometry. From the series of multiply charged ions, an average molecular mass of 27 690±4.2 was calculated for ShlA-255.

have indicated an unknown modification but synthesis of this peptide with subsequent examination by MS–MS revealed the same gap in the sequence. Obviously, this was an artefact by the method used and not a modification. It is known that there are problems to crack proline–asparagine residues with the MS–MS technique. After tryptic digestion of ShlA-255, only 40% of peptides which should be released theoretically, were detected. The detection range was from 10 to 30 amino acids (AA) whereas peptides below 10 AA and above 30 AA could not be isolated from the RPC column chromatography. The main peptide lacking (62 AA, relative molecular mass 6525) is the sequence containing the ANPN motif at position 109. It is known that active ShlA is rather resistant to trypsin in contrast to inactive ShlA* which is completely digested [10]. This is also true for the isolated N-terminus [24,25]. However, extended tryptic digestion of inactive ShlA-255* showed a similar peptide pattern as active ShlA-255 and therefore offered no hint upon differences between the activated and inactive forms.

Summarising these results, previous data implied a conformational difference between the active and inactive form of ShlA which render potential cleavage sites inaccessible for trypsin in the activated form. Therefore, MS and peptide sequencing provided no evidence for a covalent modification of ShlA during activation by ShlB and support the conformational change hypothesis as deduced previously from temperature-dependent differences in the mobility on SDS–PAGE between inactive and active

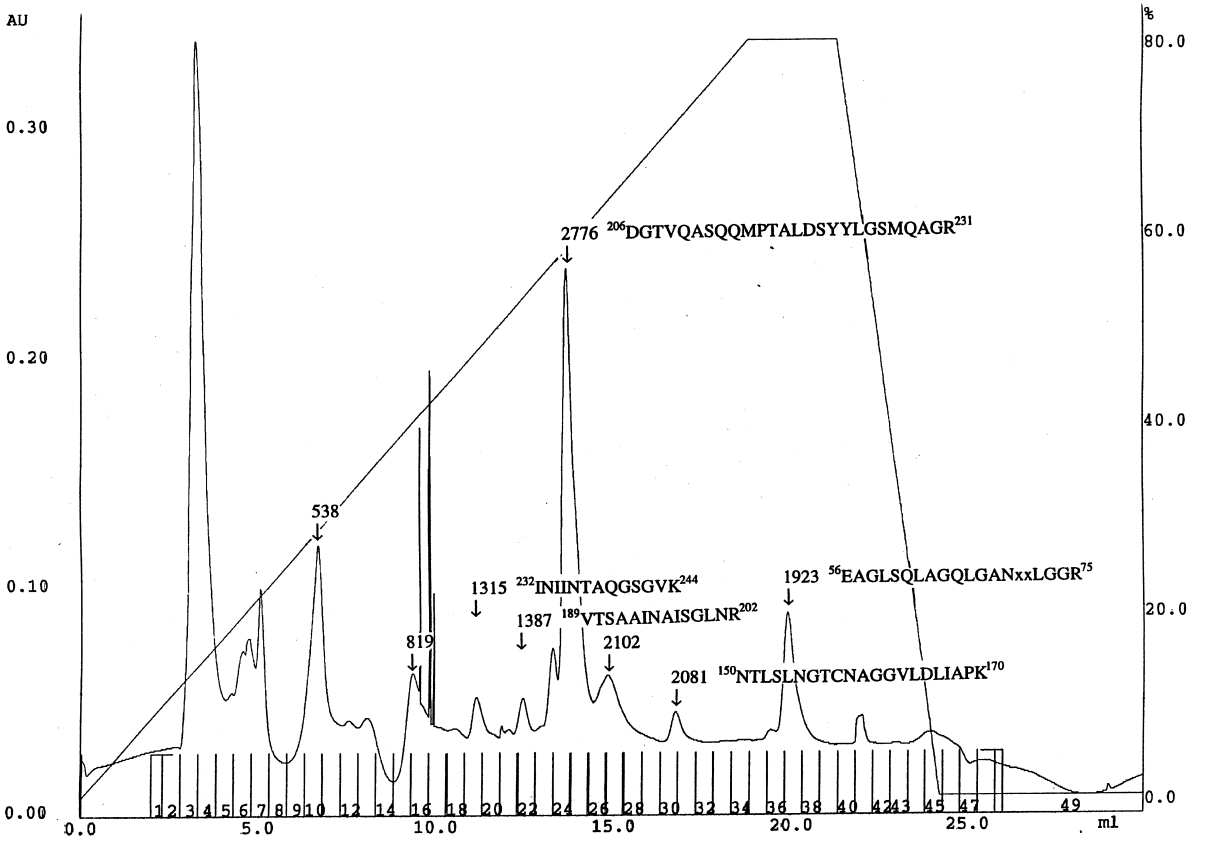

Fig. 5. Chromatogram of a tryptic digest of ShlA-255 on Resource RPC. A 100-μl volume (1 μg/ml) was loaded. Sequenced peptides are indicated by their sequence. Numbers indicate the total relative molecular mass of the peptides. Superscripts indicate the amino acid position of the peptides in the protein sequence of ShlA.

ShlA [10]. It is still unclear how ShlB imposes this conformational change on the ShlA protein during secretion.

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References

[1] V. Braun, R. Schoenherr, S. Hobbie, Trends. Microbiol. 1 (1993) 211.
[2] V. Braun, S. Hobbie, R. Ondraczek, FEMS Microbiol. Lett. 100 (1992) 299.
[3] V. Braun, T. Focareta, Crit. Rev. Microbiol. 18 (1991) 115.
[4] G.V. Carbonell, M.C. Vidotto, J. Med. Biol. Res. 25 (1992) 1.
[5] Y. Ruan, V. Braun, Arch. Micobiol. 154 (1990) 221.
[6] W. König, Y. Faltin, J. Scheffer, H. Schöffler, V. Braun, Infect. Immun. 55 (1987) 2554.
[7] R. Marre, J. Hacker, V. Braun, Microb. Pathogen. 7 (1989) 153.
[8] V. Braun, B. Neuss, Y. Ruan, E. Schiebel, H. Schoffler, G. Jander, J. Bacteriol. 169 (1987) 2113.
[9] K. Poole, E. Schiebel, V. Braun, J. Bacteriol. 170 (1988) 3177.
[10] E. Schiebel, H. Schwarz, V. Braun, J. Biol. Chem. 264 (1989) 16311.
[11] F.-L. Yang, S. Hobbie, V. Braun, 1999, unpublished.
[12] R. Ondraczek, S. Hobbie, V. Braun, J. Bacteriol. 174 (1992) 5086.
[13] R. Hertle, S. Brutsche, W. Groeger, S. Hobbie, W. Koch, U. Könninger, V. Braun, Mol. Microbiol. 26 (1997) 853.
[14] T.R. Covey, R.F. Bonner, B.I. Shushan, J. Henion, Rapid. Commun. Mass Spectrom. 2 (1988) 249.
[15] R.A. Yost, R.K. Boyd, Methods Enzymol. 193 (1990) 154.
[16] R.N. Hayes, M.L. Gross, Methods Enzymol. 193 (1990) 237.
[17] B. Lugtenberg, J. Meijers, R. Peters, P. van der Hoek, L. Van Alphen, FEBS Lett. 58 (1975) 254.
[18] U.K. Laemmli, Nature 227 (1970) 680.
[19] A.W. Bernheimer, Methods Enzymol. 165 (1988) 213.
[20] G. Laufer, 1998, unpublished.
[21] S. Tabor, C.C. Richardson, Proc. Natl. Acad. Sci. USA 82 (1985) 1074.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[24] R. Schoenherr, R. Tsolis, T. Focareta, V. Braun, Mol. Microbiol. 9 (1993) 1229.
[25] G. Walker, F.-L. Yang, R. Hertle, V. Braun, Conference Proceeding, GBU, Biomem., Jena, April 1999, p. 155.
[26] F.W. Studier, B.A. Moffatt, J. Mol. Biol. 189 (1986) 113.