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Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr

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Abstract

The maturation of the peptide antibiotic (lantibiotic) subtilin in *Bacillus subtilis* ATCC 6633 includes posttranslational modifications of the propeptide and proteolytic cleavage of the leader peptide. To identify subtilin processing activities, we used antimicrobial inactive subtilin precursors consisting of the leader peptide which was still attached to the fully matured propeptide. Two extracellular *B. subtilis* proteases were able to activate subtilin precursors, the commercially available serine protease prototype subtilisin (AprE) and WprA. The latter was isolated from *B. subtilis* WB600, a strain deficient in six extracellular proteases. Surprisingly, the *aprE wprA* double mutant of the ATCC 6633 strain was still able to produce active subtilin, however, with a reduced production rate. No subtilin processing was found within the culture supernatant of the WB800 strain, which is deficient in eight extracellular proteases. Vpr was identified as the third protease capable to process subtilin.

Keywords: *Bacillus subtilis*; Lantibiotic; Subtilin; Processing; Serine protease

The peptide antibiotic (lantibiotic) subtilin is produced by the Gram-positive bacterium *Bacillus subtilis* ATCC 6633 [1]. Lantibiotics contain characteristic interresidual thioether bridges formed by meso-lanthionine and 3-methyl-lanthionine and the unusual amino acids di-dehydroalanine and di-dehydrobutyrine [for recent reviews, see [2–4]]. Subtilin belongs to type-A lantibiotics like nisin [5–8] from *Lactococcus lactis*, epidermin [9,10] and pep5 [11,12] from *Staphylococcus epidermidis*, and ericin from *B. subtilis* A 1/3 [13]. Type-B lantibiotics exhibit globular structures like cinnamycin (Ro 09-0198) [14–16], duramycin [17], and ancovenin [18]. The action of type-A lantibiotics is based on pore formation into the cytoplasmatic membrane of Gram-positive bacteria [19], which is combined with specific binding to the membrane anchored cell wall precursor lipid II [20,21].

Generally, lantibiotics are ribosomally synthesized as precursor peptides, which consist of a N-terminal leader peptide followed by a C-terminal propeptide moiety which undergoes extensive posttranslational modifications. After dehydration of serine- and threonine residues the lanthionine and 3-methyl-lanthionine residues are formed by stereospecific additions of neighboring cysteine thiol groups. These reactions are possibly catalyzed by LanB and LanC proteins [22,23]. For nisin and subtilin membrane associated complexes of LanBC proteins with the transporter LanT protein have been shown [24,25]. Lantibiotic maturation is completed after proteolytic cleavage of the leader peptide.

In the subtilin gene cluster, enzymes for modification [26,27], immunity [28], and regulation [29,30] are conserved, but no processing protease, suggesting that an intrinsic *B. subtilis* protease is able to process immature subtilin. The addition of PMSF to growing *B. subtilis* ATCC 6633 cultures provoked the accumulation of subtilin precursors species in the culture supernatant [31]. This indicates that subtilin processing is catalyzed by extracellular proteolytic activities.

In this work we used the tool of antimicrobial inactive subtilin precursors as biological relevant substrate to identify the proteolytic activities from *B. subtilis*

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involved in subtilin maturation. We demonstrate that at least three extracellular serine proteases are capable to process subtilin precursors.

Materials and methods

Bacterial strains, plasmids, media, and growth conditions. B. subtilis cells were grown in Difco sporulation medium (DSM, Difco). 1 l TY broth contained 8 g tryptone (Difco, Heidelberg, Germany), 5 g select yeast extract (Invitrogen, Karlsruhe, Germany), and 2.5 g NaCl (Roth, Karlsruhe, Germany). Rich Medium was prepared according to [32]. Where necessary, media were supplemented with 50 μg/ml ampicillin (Amp; Roth) for *Escherichia coli*, 8 μg/ml kanamycin (Kan), and 5 μg/ml chloramphenicol (Cam) for *B. subtilis* (both from Serva, Mannheim, Germany).

Molecular biology techniques. Established protocols were followed for molecular biology techniques [33]. DNA cleavage was performed according to the conditions recommended by the supplier of restriction enzymes (Promega, Mannheim, Germany). DNA fragments were purified with the QIAquick PCR purification kit (Quiagen, Hilden, Germany). *E. coli* plasmids were isolated with the alkaline extraction procedure [34]. PCR was carried out following standard procedures [33] in a Hybaid combi-thermal-reactor R2, using Taq DNA-polymerase (Promega).

Construction of plasmids and disruption of proteases in B. subtilis ATCC 6633. A 1187 bp *aprE* containing fragment was PCR-amplified with oligonucleotides aprE1 (5′-GTTAATCTCGAGGATGGCGTTCAGC-3′) and apre2 (5′-GATCATGGAACGAATTCAACATGC-3′) and chromosomal *B. subtilis* 168 DNA as a template, cleaved with *Xho*I and *Eco*RI, and cloned in pBSK$^-$ (pSD10). The Cam-cassette was excised from pCE26 [26] with *Hin*cII and cloned into the *Hin*cII linearized pSD10 (pSD11). pSD11 was used for integration into the chromosome of *B. subtilis*. Transformations were carried out as described in [26]. Inactivation of *aprE* was verified by PCR using primers aprEpX2_1 (5′-GGGACTAGTGTGAGAAGCAAAAAATTGTGG-3′) and aprEpX2_2 (5′-CTTTTTAAGATCTTTGTGCAGCTGCTTGTACG-3′). *B. subtilis* ATCC 6633 wild-type and Δ*aprE* strains were transformed with chromosomal DNA of *B. subtilis* 168WA to delete *wprA*. *WprA* inactivation was verified by PCR using primers CC7wprA1 (5′-GCGGCCGCCATGGTCGATCAGGCGGC-3′) and CCdelwprA2 (5′-GCGGCCGCCATGGTCGATC AGGCGGC-3′).

Purification of WprA. The purification was performed according to [32] with modifications. *B. subtilis* WB600 deficient in six extracellular proteases (*trpC2*, Δ*nprE*, Δ*aprE*, Δ*epr*, Δ*bpf*, Δ*mpr*, and Δ*nprB*) [35] was grown in 1 L cultures (37 °C, 125 rpm), the cells were harvested at an A_{600} of 8.0. 2 l of the clarified supernatant was adjusted to pH 7.5, using 1 M NaOH and loaded onto a 24 ml SP Sepharose Fast Flow (Amersham Biosciences, Freiburg, Germany) column equilibrated in 50 mM Hepes, pH 7.5 and 25 mM NaCl (buffer 1). Proteins were eluted over 7 column volumes with a linear gradient of 50 mM Hepes, pH 7.5 and 1.5 M NaCl (buffer 2), and a flow rate of 4 ml/min. Fractions (12 ml) were collected. For further purification active fractions were pooled and diluted 1:3 with buffer 1. Samples were loaded onto a MonoS (HR5/5) high resolution column (Amersham) equilibrated in the same buffer. Proteins were eluted with a linear gradient of 40 ml buffer 2 and a flow rate of 1 ml/min. Fractions (1 ml) were collected. Eluting proteins were monitored by measuring their absorbance at 280 nm. The subtilin processing activity of each fraction was assayed as described below.

Activation of subtilin precursors. The activation of subtilin precursor peptides by proteolytic activities of culture supernatants or column fractions was analyzed by an agar-based diffusion assay using *Micrococcus luteus* ATCC 9341 as the indicator strain [26]. After incubation of 1 μl test solution with 9 μl of 50 mM Hepes, pH 7.5, 5 μl of the subtilin precursor peptide (0.5 mg/ml) [31], and 5 μl of 0.1 M calcium acetate at 37 °C for 15 min, the mixture was poured on sterile 3 M Whatman filter paper, placed onto *M. luteus* plates, and incubated at 37 °C for 24 h.

Quantification of subtilin. B. subtilis cultures were centrifuged at 9000*g* for 10 min. The culture supernatant was centrifuged at 20,000*g* for 20 min to remove impurities. The subtilin production rate was determined by quantitative RP-HPLC as described previously [36].

SDS–PAGE. Chromatography was performed using 12% polyacrylamid gels in Tris–glycine buffer under denaturating/reducing conditions [37]. Staining was performed with Coomassie blue and/or silver (Invitrogen silver staining kit).

Mass spectrometry. Delayed extraction (DE) MALDI-TOF mass spectra were recorded on a Voyager RP-DE instrument (PerSeptive Biosystems, Framingham, MA, USA), using a 337-nm nitrogen laser for desorption and ionization. Linear or reflector modes were used. External calibration was performed using angiotensin III, bovine insulin, and ubiquitin (all from Sigma–Aldrich, Munich, Germany). The total acceleration voltage was 20 kV, 11.6 kV was used on the first grid. The delay time was 375 ns. 0.7 μl of the sample was spotted with 0.7 μl matrix solution [38] directly on the sample plate and dried in ambient air. Between 128 and 256 single scans were accumulated for each mass spectrum.

Results

For identification and characterization of subtilin processing activities, antimicrobial inactive subtilin precursor species encompassing the leader peptide sequence attached to the completely posttranslational modified propeptide moiety were used [31].

Subtilisin (AprE) activates subtilin precursor molecules

Most lantibiotic processing proteases belong to the large class of subtilisin-like serine proteases. The strong influence of the serine protease inhibitor PMSF on subtilin processing in *B. subtilis* ATCC 6633 [31] suggested that a serine protease is involved in this process. Subtilisin processing resulted in the activation of subtilin precursors as indicated by antimicrobial growth assays (Fig. 1A) and confirmed after MALDI-TOFMS analyses (Fig. 1B). The signal at *m*/*z* 3320.8 exactly corresponds to the calculated molecular mass of matured subtilin. Two additional peaks, L9-Sub* (*m*/*z* 4347) and L7Sub* (*m*/*z* 4090), correspond to partially processed subtilin precursors with different chain lengths.

To analyze the function of AprE in subtilin processing, we sequenced *aprE* within the subtilin producing *B. subtilis* ATCC 6633 strain. AprE of the 6633 (Accession No. AJ539133) and 168 strain showed 95% identity. Disruption of *aprE* in the ATCC 6633 strain resulted in a subtilin production rate comparable to the wild-type strain, suggesting the presence of additional subtilin processing activities.

The cell wall-attached serine protease WprA activates subtilin precursor molecules

Subtilin processing was obtained after incubation with the culture supernatant of *B. subtilis* 168, a strain

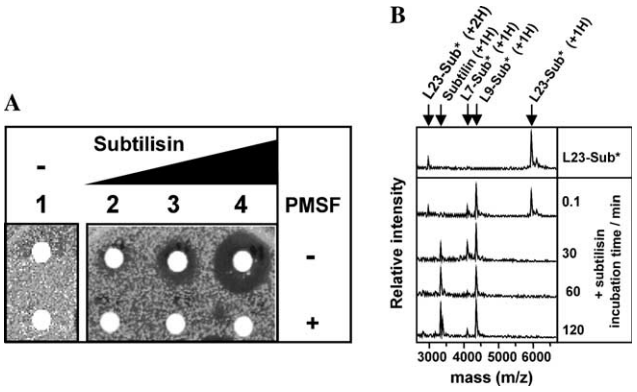

Fig. 1. MALDI-TOFMS analysis of subtilin precursor activation with subtilisin. The processing of the subtilin precursor peptide L23-Sub* by subtilisin is monitored antimicrobial growth assays (A) and MALDI-TOFMS analysis (linear mode) (B) as indicated under Materials and methods. L23-, L9-, and L7-Sub*; subtilin precursor molecules encompassing leader peptides with indicated numbers of amino acid residues attached to the fully matured propeptide moiety [31].

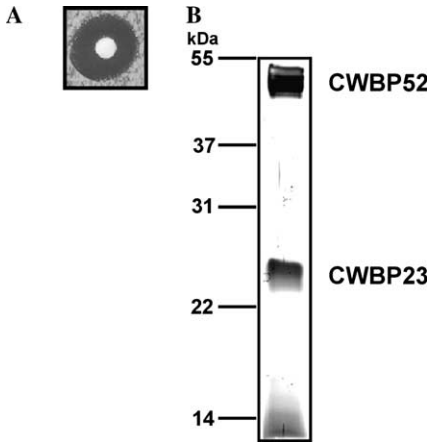

Fig. 3. Subtilin precursor activation by WprA. Twenty microliter of a MonoS fraction containing the main subtilin processing activity (A) was silver stained after SDS–PAGE (B). The protein bands were identified by tryptic peptide mass fingerprinting and identified as CWBP 52 and 23, both subunits of WprA.

without subtilin genes [31] (Fig. 2). We tested the subtilin processing activity of the WB600 strain, which is deficient in six extracellular protease genes, including *aprE*, and showed only 0.32% proteolytic activity within the supernatant as compared to the wild-type 168 strain [35]. Surprisingly, the supernatant of the WB600 strain exhibited subtilin processing activity comparable to the supernatant of the 168 strain (Fig. 2).

We decided to characterize the subtilin processing activity within the WB600 supernatant. After two cation-exchange chromatographic steps, the most active fraction contained only two protein bands in a silver stained SDS–PAGE (52 and 23 kDa; Fig. 3). Both bands could be unambiguously identified as subunits of the cell-wall bound protease WprA after tryptic peptide mass fingerprinting. These results are in good agreement with the WprA characterization reported by Babé and Schmidt [32]. Expectedly, subtilin processing activity was inhibited by PMSF in a dose-dependent fashion (not shown).

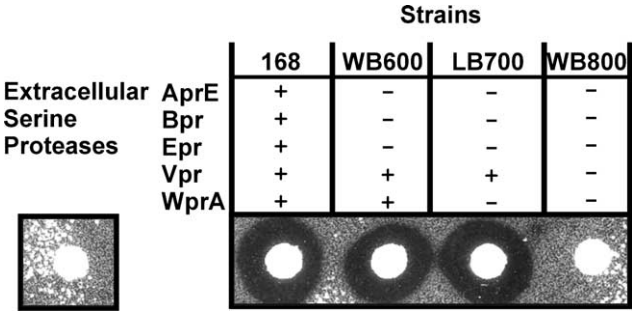

Fig. 2. Activation of subtilin precursors by culture supernatants of different *B. subtilis* strains. The upper table summarizes extracellular serine proteases of different *B. subtilis* strains. Below, subtilin precursor processing after incubation with *B. subtilis* culture supernatants is monitored by antimicrobial growth inhibition. As a control, the subtilin precursor was incubated with TY medium (left).

We sequenced *wprA* within *B. subtilis* ATCC 6633. WprA from the 6633 strain (Accession No. AJ539134) exhibited 95% identity as compared to WprA of *B. subtilis* 168. In order to analyze the influence of WprA on subtilin processing, the *wprA* gene was disrupted in the wild-type and in the *aprE* disruption strain. Both *wprA* deficient strains were still able to produce antimicrobial active subtilin, however, for the double disruptant *wprA aprE* a significantly reduced production rate was obtained as quantified analyzed by quantitative RP-HPLC (not shown). Samples taken from growing cultures of the *wprA* lacking strains at the beginning of the stationary phase revealed a series of signals (*m*/*z* 4092, 4349, and 4535), which correspond to subtilin precursor molecules with 7, 9, and 11 amino acid residues in their leader peptides (Fig. 4). They fit with subtilin precursors which have been already observed in PMSF-treated *B. subtilis* ATCC 6633 cultures [31].

A third serine protease, Vpr, is capable to activate subtilin precursors

Within the LB700 strain the *wprA* gene of *B. subtilis* WB600 was deleted [39]. The supernatant of this strain was still able to activate subtilin precursors. This indicates that more than two serine proteases are involved in subtilin processing, suggesting that the remaining extracellular serine protease Vpr is also capable to process subtilin precursor molecules.

By disruption of the extracellular proteases Vpr and WprA within *B. subtilis* WB600, the WB800 strain was constructed [66]. The culture supernatant of this strain shows no more subtilin processing activity (Fig. 2). This is in full agreement with our observations that both proteases, WprA and Vpr, are involved in subtilin processing.

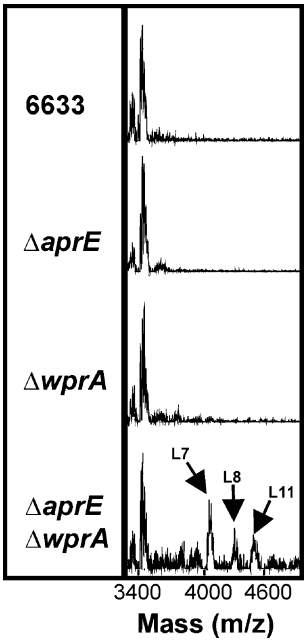

Fig. 4. MALDI-TOFMS analysis of subtilin precursor production in protease deficient strains. Subtilin precursor production in protease deficient 6633 strains is monitored by MALDI-TOFMS (reflector mode) as indicated under Materials and methods. Five milliliter of culture supernatant was passed over 10 μl packed POROS 50 R2 material (PerSeptive Biosystems, Framingham, MA, USA) washed with 1 ml of 20% acetonitrile 0.1% TFA (v/v), eluted with 5 μl of 70% acetonitrile 0.1% TFA (v/v) and 0.5 μl were spotted. Main signals were obtained at 3322 for subtilin and 3422 for succinylated subtilin [61]. L7-, L9-, and L11-Sub* correspond to signals at *m/z* 4092, 4348, and 4534 encompassing truncated leader peptides with indicated numbers of amino acid residues attached to the fully matured propeptide moiety [31].

Discussion

Subtilin processing was obtained after incubation with the culture supernatant of *B. subtilis* 168, a strain that contains no subtilin genes [31]. This observation, together with the lack of a protease within the subtilin gene cluster in *B. subtilis* ATCC 6633 suggested that no specific protease is dedicated to process subtilin. Because the genome of *B. subtilis* encodes a great number of proteases [40] which obviously can take over subtilin processing, no selection pressure was on the conservation of a specific subtilin processing protease within the 6633 strain. Although the pre-lantibiotic processing proteases LanP are closely related (Fig. 5A) the localization of the processing reaction differs. The mature lantibiotic can be generated prior to, concomitantly with, or after its secretion. PepP is located intracellular [22,41], for NisP an attachment to the outside of the cytoplasmatic membrane was demonstrated [42,43], and EpiP is an soluble exo-protease [44]. The presence of chimeric proteins consisting of transporter and proteolytic domains as found for lactocin DR (LctT) [45] and cytolysin (CylT) [46], imply that the processing reaction occurs concomitantly with the secretion of the peptide antibiotic. Interestingly, also the mersacidin producer *Bacillus* ssp. (HIL Y–85,54728) contains a chimeric transporter-protease protein (MrsT) [47]. Obviously, specific processing during the transport catalyzed by the protease domain of MrsT cannot be taken over by unspecific *Bacillus* proteases.

Since the serine protease inhibitor PMSF blocks subtilin processing, we focused on the characterization of extracellular serine proteases. *B. subtilis* secretes, as one of two the major types of proteases, five extracellular serine proteases into the culture medium [48].

With subtilin precursor processing we could determine a novel function for *B. subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr. However, we cannot rule out a function for Bpr and Epr in this process, since their high similarity to the identified proteases. For *B. subtilis* extracellular serine proteases AprE (28.5 kDa), which is controlled by several sporulation

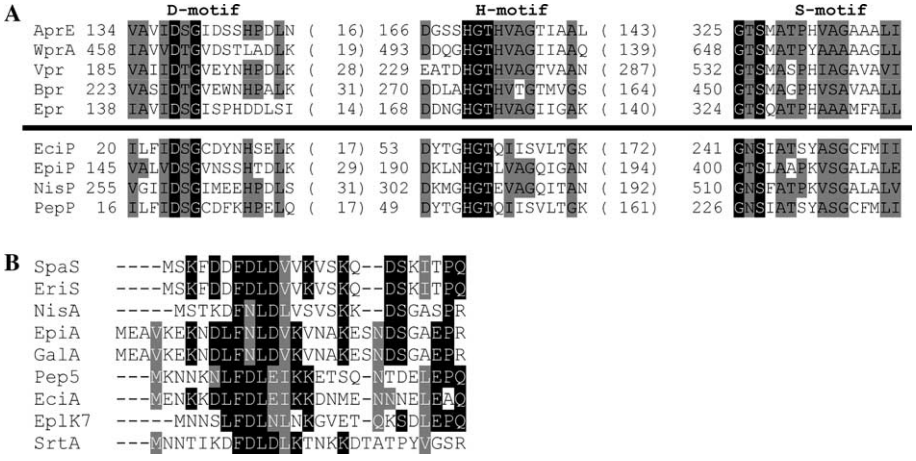

Fig. 5. Amino acid alignments. (A) Active site regions of extracellular *B. subtilis* serine proteases compared to corresponding regions of subtilisin-like proteases from lantibiotic producing strains. (B) FNDL-type leader sequences of type-A lantibiotics first described for nisin by van der Meer et al. [43]. Lan corresponds to Eci, epicidin 280 [62]; Epi, epidermin; Nis, nisin; Pep, pep5; Spa, subtilin; Eri, ericin; Gal, gallidermin [63]; EplK7, epilancin [64]; and Srt, streptin [65].

genes [49,50], WprA (52 kDa), Vpr (28.5 kDa) [51], and Bpr (also referred to as bacillopeptidase f; 48 and 50 kDa) [52,53] pH-optima from 7 to 9 have been determined. Epr (34–40 kDa) has been characterized as a minor extracellular protease [54], which is involved in *B. subtilis* swarming [55]. The catalytic centers of these enzymes, the triad of serine, aspartate, and histidine are highly conserved (Fig. 4A), suggesting that their genes have been developed from a common ancestor by divergent evolution [56]. All these proteases are synthesized in the end of the logarithmic and beginning of the stationary growth-phase [57–59], which is in good agreement with both, transcriptional [30] and translational expression of the subtilin biosynthetic genes [27,30].

Babe et al. [32] used artificial peptides coupled to methylcoumarylamide to reveal the substrate specificity of the extracellular serine proteases WprA and AprE. Both showed comparable specificities, with preferred cleavage sites after the hydrophilic amino acids M, K, D, R, Q, as well as F and A. This is in good agreement with the predominant cleavage sites within the subtilin leader peptide observed in PMSF-treated cultures [31]. In accord, subtilin precursor species with corresponding cleavage sites (L7-, L9-, and L11Sub*) accumulate in the culture supernatants of the *wprA* and the *apre wprA* disruption strains.

Several properties were found to be highly conserved among the leader peptides of type-I lantibiotics: The consensus motif F-(N/D)-L-(D/E)-(L/V/I), the processing site P-(Q/R)-|X, (Fig. 5B), a high percentage of hydrophilic amino acids with a net negative charge, and a helical structure in hydrophobic solvents [60]. Although the subtilin and ericin gene clusters contain no protease encoding gene, their leader peptides are perfectly conserved. Most probably, the conserved leader peptides bear structural prerequisites for molecular interactions with the modification machinery LanBC and/or the lantibiotic transporter LanT. For example a hairpin-like structure of the leader peptide interacting with the propeptide part is conceivable as proposed earlier [60].

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