Serine Protease EpiP from Staphylococcus epidermidis Catalyzes the Processing of the Epidermin Precursor Peptide

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The function of serine protease EpiP in epidermin biosynthesis was investigated. Epidermin is synthesized as a 52-amino-acid precursor peptide, EpiA, which is posttranslationally modified and processed to the mature 22-amino-acid peptide antibiotic. epiP was expressed in Staphylococcus carnosus with xylose-regulated expression vector pCX15. The cleavage of the unmodified EpiA precursor peptide to leader peptide and proepidermin by EpiP-containing culture filtrates of S. carnosus (pCX15epiP) was followed by reversed-phase chromatography and subsequent electrospray mass spectrometry.

Lantibiotics such as epidermin, nisin, Pep5, and subtilin are ribosomally synthesized as prepeptides and posttranslationally converted into the mature peptide (reviewed in reference 28). They are characterized by the occurrence of thioether amino acids such as didehydroalanine (10), didehydrolaevlanthionine. In addition to the thioether amino acids, other unusual amino acids such as didehydroalanine (10), didehydrobutyryl and d-alanine (36) are present in some of the lantibiotics. Proposed modification reactions involved in lantibiotic biosynthesis are dehydration of serine and threonine residues and sulfide bridge formation.

Epidermin is synthesized as 52-amino-acid prepeptide EpiA (N-terminal leader peptide with amino acids 30 to 1 and C-terminal proepidermin with amino acids 1 to 22), which is posttranslationally modified and processed to the mature tetracyclic 22-amino-acid peptide antibiotic, as was elucidated by the sequencing of structural gene epiA (33). epiA is flanked by epiB, epiC, and epiD, whose gene products are involved in modification of prepeptide EpiA (1, 2, 32). Two additional genes, epiQ and epiP, are coded for in the opposite direction downstream of epiD (24, 32). With plasmid pTepi14 containing the two transcription units epiABCD and epiPQ, heterologous host Staphylococcus carnosus synthesizes epidermin (1, 32).

Presently, we are investigating the function of enzymes EpiB, EpiC, EpiD, and EpiP in epidermin biosynthesis. epiD encodes a flavoprotein (21) that catalyzes the oxidative decarboxylation of the C-terminal cysteine residue of precursor peptide EpiA (18, 19) and consequently is involved in the formation of the modified C-terminal S-[Z]-2-aminovinyl]-d-cysteine of epidermin.

The final modification step in lantibiotic biosynthesis is the removal of the leader peptide. The sequences of the leader peptides of the type A lantibiotics differ from sec-dependent protein export signal sequences. The following motifs are characteristic for the leader peptides of epidermin (33), nisin (5), subtilin (3), epilancin K7 (39), and Pep5 (16): F-N-D-L/D/E and the processing site P^2-Q/R^1][X]^-1. The sequence at the cleavage site of the epidermin precursor peptide is AEPR^1][I]^-1[ASK. The influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by Lactococcus lactis has recently been investigated (41). The leader peptide is not necessary for oxidative decarboxylation of the epidermin precursor peptide catalyzed by EpiD (18, 19). The leader sequences of lantibiotics streptecoccin A-FF22 (13), lacticin 481 (25), and salivaricin A (27) and of cytolysin from Enterococcus faecalis (8) are similar to those of the leader peptides of the group II nonlantibiotic bacteriocins of lactic acid bacteria (4) and actinomycetales (21). The processing site G^-2[GA^-1][X]^-1. It has been postulated that ABC transporters containing an N-terminal proteolytic domain are involved in the cleavage of these so-called double-glycine-type leader peptides (12). Mersacidin (4) and cinnamycin (15) have long leader peptides with 48 and 59 amino acid residues, respectively, which share no sequence homologies to leader sequences of the nisin-type lantibiotics.

Because of the sequence similarities of EpiP to such serine proteases as subtilisin (32), it has been proposed that EpiP is the processing enzyme that cleaves mature epidermin from its leader peptide. The proposed catalytic domain of EpiP is most similar to that of protease NisP, which is coded for in the gene cluster for biosynthesis of nisin in L. lactis (40). Here, we report the expression of epiP in S. carnosus, the determination of the amino-terminal amino acid sequence of the exoenzyme, and the cleavage of lantibiotic precursor peptide EpiA by EpiP. This cleavage was monitored by electrospray mass spectrometry. For the investigation of the specificity of EpiP, mutated peptide EpiAR-IQ was used.

Construction and purification of MBP-EpiP fusion proteins. To obtain antiserum against EpiP, we used maltose-binding protein (MBP)-EpiP fusion protein as an antigen. Since in the beginning of our studies, the size of mature EpiP was not clear, we fused epiP (the gene encoding the primary translation product) to MBP. The fusion was made as described previously for epiD (21). epiP was amplified by PCR (23) with pTepi14 (2) being used as a template and the following primers: 5’-[A(8379)]TGAACAAATTTAAATTTTTCATTG(8355)]-3’ and 5’-[A(6979)]TCATATTTATCCCTTCAATTAG(7005)]-3’. The numbers within parentheses refer to the published nucleotide sequence (32). The PCR fragment was inserted into the polylinker StuI site of MBP vector pH902 (2). Plasmid pH902epiP was transformed into Escherichia coli TB1 (14) by electroporation (7). The correct fusion site of malE-epiP was verified by double-stranded DNA sequencing (6) by the dideoxy procedure (29), using the Pharmacia AutoRead sequencing kit and the A.L.F. DNA sequencer from Pharmacia LKB. The epiP start codon immediately followed...
FIG. 1. Detection of EpiP in the culture supernatant of induced S. carnosus (pCX15epiP) cells by SDS-PAGE (10% acrylamide) and Coomassie staining (A) and by Western blotting (immunoblotting) with anti-MBP-EpiP antibody (B) (several cross-reactions of the antiserum used are observed). Molecular size markers (Bio-Rad Laboratories) are shown in lane S (in kilodaltons). Lanes: 1, induced S. carnosus (pCX25) (control; lipase expression of pCX25 is strongly reduced by mutagenesis of the ribosomal binding site); 2, noninduced S. carnosus (pCX15epiP); 3, induced S. carnosus (pCX15epiP) (20 h after induction with 0.5% xylose) EpiP (identified also by Edman degradation of the blotted protein) with an apparent molecular mass of 46 kDa was visible in induced cultures of S. carnosus (pCX15epiP). In some cases, a weak immunoreactive band at 58 kDa was detected in the culture supernatant of induced S. carnosus (pCX15epiP) cells; this band was not detected in the controls.

Expression of epiP in S. carnosus with the xylose-inducible pCX15 vector. Since it was not guaranteed that EpiP is processed correctly in E. coli, we expressed epiP in S. carnosus with xylose-inducible vector pCX15 (43). S. carnosus TM300 had no detectable proteolytic activity on skim milk agar, rendering this strain an ideal host for exoprotease cloning and expression (37).

For cloning in pCX15, epiP was amplified by PCR (23) with pCUEpiQ (2.48-kb HpaII fragment of pEpi14 cloned in pCU1 [2]) being used as a template and the following primers: (i) 5′-d[C(8514)TCGCTGTATCAGATCTTGATACCTCAGC(8487)]-3′, introducing a BglII site (underlined); and (ii) 5′-d[C(6938)ATAAAGATAGAATTTCCACCTCACCAGC(6965)]-3′, introducing an EcoRI site (underlined). The numbers within parentheses refer to the published epiP nucleotide sequence (32). The nucleotides in boldface are introduced mutations for the generation of the mentioned restriction sites. The 1.55-kb BglII-EcoRI epiP fragment was cloned in pCX15 restricted with BglII and EcoRI (the lipase gene of pCX15 was deleted by this procedure), and S. carnosus was transformed with protoplasts (9). The correct sequence of epiP cloned in pCX15 was determined by DNA sequencing with appropriate primers.

After induction of an S. carnosus (pCX15epiP) culture (grown to an optical density at 578 nm of 0.5 in B broth without glucose (10 g of casein hydrolysate 140 [Gibco], 5 g of yeast extract [Difco], 5 g of NaCl, and 1 g of K2HPO4 per liter [pH 7.3]) with 0.5% xylose, EpiP was detected by SDS-PAGE and immunoblotting (38) (Fig. 1). For immunoblot analysis, the nitrocellulose membrane (BA83; Schleicher & Schuell) was incubated with anti-MBP-EpiP antiserum for 4 h, and this incubation was followed by incubation for 1 h with anti-rabbit immunoglobulin G biotin conjugate (1:1,500; Sigma) and for 20 min with streptavidin-biotinylated horseradish peroxidase complex (1:3,000; Amersham). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL system; Amersham). The molecular mass of exoenzyme EpiP was estimated to be 46 kDa. EpiP was already visible 4 h after induction, and with increasing incubation time after induction, the amount of EpiP increased. No proteolytic degradation of EpiP...
was observed. By immunoblot analysis, no EpiP was detected in the crude extract of induced *S. carnosus* (pCX15epiP) cells.

Partial purification and N-terminal amino acid sequence of EpiP. The protease was isolated from 5 liters of culture supernatant of xylose-induced *S. carnosus* (pCX15epiP). For partial purification, EpiP was precipitated with ammonium sulfate (60%) after induction, dialyzed against 20 mM Tris-HCl (pH 8.0) containing 1 mM CaCl₂, and subjected to HiTrapQ (Pharmacia) chromatography. EpiP eluted at 0.8 M NaCl. The EpiP-containing HiTrapQ fraction was dialyzed against NH₄HCO₃ and concentrated by lyophilization. The concentrated proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore), as described by Matsudaira (22). Automated Edman degradation of blotted EpiP was performed with a model 477A pulsed-liquid protein sequencer equipped with a model 120A on-line phenylthiohydantoin-amino acid analyzer (Applied Biosystems). The N-terminal sequence was determined to be Ser-Val-(Lys or Ile)-Thr-Gly-(Lys or Ile)-Asn-Leu-Asn-Asn-(Lys or Ile)-(Lys or Ile)-Xxx-Gly-Ser-His-Asp-Xxx-Phe. This amino acid sequence matched that deduced from the nucleotide sequence of EpiP (32), starting at Ser-100.

The extracellular location of EpiP in *S. carnosus* (pCX15epiP) and the determined N terminus suggest that EpiP is synthesized as a pre-pro enzyme with a 99-amino-acid pre-pro sequence. Mature EpiP is composed of 362 amino acids with a calculated molecular mass of 40.3 kDa. Analysis of the N-terminal amino acid sequence of EpiP by the method of von Heijne (42) revealed that the pre-pro sequence is composed of a 25-amino-acid signal peptide and a 74-amino-acid pro region. EpiP may activate itself autocatalytically by removing the 74-amino-acid pro region, as has been demonstrated for several bacterial exopeptidases (26, 35). The sequence of the processing site is VVEK⁹⁹ ↓S¹⁰⁰VKT; therefore, the specificity of autocatalytic processing differs from the specificity of precursor peptide processing (cleavage after Lys instead of Arg).

Because of low expression, it was not possible to identify EpiP in the culture supernatants of *S. carnosus* (pTepiI4) or *Staphylococcus epidermidis* Tu3298. Therefore, it was not possible to compare the processing of EpiP in epidermin-producing strains with that in *S. carnosus* (pCX15epiP).

In this case of nisin precursor processing (40, 41), the protease is not found in the supernatant of the producing organism *L. lactis*. NisP is probably anchored in the cell wall, since it has the C-terminal consensus sequence LPXTG found in the anchor region of surface proteins of gram-positive bacteria (31). The different localizations of EpiP and NisP are reflected in their amino acid sequences: NisP and EpiP both have a pre-pro organization, but the C-terminal anchor sequence of NisP is lacking in EpiP. However, C-terminal processing of EpiP cannot be excluded completely at the moment. With whole cells of *L. lactis* NZ980 but not with the supernatant or with a membrane-free extract, the cleavage of posttranslationally modified nisin precursor peptide to active nisin has been demonstrated (40).

Determination of enzymatic activity of EpiP. We incubated culture supernatants of various *S. carnosus* clones with posttranslationally unmodified peptides EpiA and EpiAR-1Q. Because of low expression, posttranslationally modified EpiA (proposed to be the in vivo substrate of EpiP) was not available from *S. carnosus* pTepiABCDQ (2). EpiA and EpiAR-1Q were purified by factor Xa cleavage of MBP-fusion protein proteins (20). To ensure that no factor Xa (which cleaves EpiA to leader peptide and proepidermin) was present in the preparations, the peptides were purified by reversed-phase chromatography (20), dried with a vacuum concentrator, and dissolved to a concentration of 2 to 3 μg/μl in 20 mM Tris-HCl (pH 8.0) containing 1 mM CaCl₂. Each peptide solution (10 μl) was
incubated with 150 μl of the various culture supernatants and incubated for 15 h at 37°C. To ensure that no cells were present, the culture supernatants were filtered (0.2-μm pore size). The reaction mixture was then separated by reversed-phase chromatography with a μRPC C₆/C₁₈ SC 2.1/10 column and the SMART protein and peptide purification system from Pharmacia. Peptides were eluted with a linear gradient of 0 to 50% acetonitrile–0.1% trifluoroacetic acid in 3.8 ml with a flow rate of 200 μl/min (Fig. 2). The peptides were collected by the peak fractionation method, dried with a vacuum concentrator, stored at −70°C, dissolved in 30% acetonitrile, and analyzed by electrospray mass spectrometry as described recently (18, 19) (Fig. 3).

EpiA was cleaved to peptides with molecular masses of 3,347 and 2,296 Da only when EpiP was present. Taking the retention times and the A₁₉₄₄, A₂₆₉₉, and A₃₉₀₃ of the reaction products also into consideration (data not shown) (20), the cleavage products were identified as leader peptide (theoretical mass, 3,347.7 Da) and proepidermin (theoretical mass under oxidization conditions, 2,298.7 Da), respectively. We did not observe cleavage at the various lysine residues of the precursor peptide. When the arginine residue at the processing site was exchanged for glutamine (EpiAR-1Q), no cleavage of the precursor peptide occurred.

On the basis of homology modeling, it has been proposed that the binding of preepidermin to EpiP is dominated by electrostatic interactions of the Arg and Glu residues of processing site A′ EPR I ASK X-3 (34), indicating that modification of the preepidermin (presence of thioether rings and unsaturated amino acid residues) is not absolutely required for binding of preepidermin to EpiP. However, EpiP probably cleaves the posttranslationally modified peptide at higher reaction rates than unmodified peptide EpiA.

In the presence of disulfide reducing reagent dithiothreitol (5 mM), cleavage of EpiA by EpiP was less efficient (data not shown). When serine protease inhibitor 3,4-dichloroisocoumarin (1 mM) (11) was used in the assay, no cleavage of EpiA was observed, whereas in the presence of only 50 μM 3,4-dichloroisocoumarin, EpiA was still cleaved.

With culture supernatants of S. epidermidis or cell extracts of S. epidermidis obtained by sonication of washed cells being used, it was not possible to demonstrate the cleavage of EpiA at processing site A′ EPR I ASK. EpiA and the leader peptide of EpiA were degraded rapidly during the incubation, mainly because of an endoprotease GluC activity (data not shown).

In the future, we intend to characterize purified EpiP with respect to its stability, enzymatic properties, and especially its specificity (cleavage of postranslationally modified precursor peptides). Furthermore, we are interested in studying the localization of EpiP in S. epidermidis Tü3298, the processing of the pro form, and the function of the propeptide. We will further investigate the degradation of the leader peptide of EpiA by endoprotease GluC of S. epidermidis Tü3298.

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