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Molecular characterization of fervidolysin, a subtilisin-like serine protease from the thermophilic bacterium *Fervidobacterium pennivorans*

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Abstract The *fls* gene encoding fervidolysin, a keratin-degrading proteolytic enzyme from the thermophilic bacterium *Fervidobacterium pennivorans*, was isolated using degenerate primers combined with Southern hybridization and inverse polymerase chain reaction. Further sequence characterization demonstrated that the 2.1-kb *fls* gene encoded a 699-amino-acid preproenzyme showing high homology with the subtilisin family of the serine proteases. It was cloned into a pET9d vector, without its signal sequence, and expressed in *Escherichia coli*. The heterologously produced fervidolysin was purified by heat incubation followed by ion exchange chromatography and emerged in the soluble fraction as three distinct protein bands, as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino-terminal-sequence analysis of these bands and their comparison with that determined from biochemically purified keratinase and its predicted protein sequence, identified them as a 73-kDa fervidolysin precursor, a 58-kDa mature fervidolysin, and a 14-kDa fervidolysin propeptide. Using site-directed mutagenesis, the active-site histidine residue at position 79 was replaced by an alanine residue. The resulting fervidolysin showed a single protein band corresponding in size to the 73-kDa fervidolysin precursor, indicating that its proteolytic cleavage resulted from an autoproteolytic process. Knowledge-based modeling experiments showed a distinctive binding region for subtilases, in which binding of the propeptide could take place prior to autoproteolysis. Assays using keratin and other proteinaceous substrates did not display fervidolysin activity, perhaps because of the tight binding of the propeptide in the substrate-binding site, where it could then function as an inhibitor.

Key words Keratinase · Serine protease · Site-directed mutagenesis · *Fervidobacterium pennivorans* · Thermostable

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Introduction

Thermophilic microorganisms are adapted to thrive at temperatures above 60°C. They are a source of interesting enzymes that are both thermoactive and thermostable (Niehaus et al. 1999; Vieille et al. 1996). Thermophiles are able to grow on a range of proteinaceous substrates, such as casein, tryptone, peptone, and casamino acids. For this purpose, these organisms possess an array of enzymes that enables the degradation, uptake, and further conversion of these substrates under these extreme conditions. Within this process of proteolysis, an important role is played by extracellular proteases. Initiated by a signal peptide, these proteases are targeted across the cell membrane, where they hydrolyze protein polymers into peptides (Wandersman 1989).

The serine proteases comprise the majority of all the thermostable proteases that have been produced and characterized to date (Niehaus et al. 1999). In general, most of the serine proteases belong to the subtilisin-like serine proteases, also referred to as subtilases (Barrett and Rawlings 1995; Rawlings and Barrett 1994; Siezen et al. 1991). In total, more than 200 members of the subtilase family originating from all domains of life are known and classified (Siezen and Leunissen 1997). Up to now, only a limited number of thermophilic subtilases from archaea (Klingeberg et al. 1995; Völkl et al. 1994; Voorhorst et al. 1996, 1997) and bacteria (Choi et al. 1999; Jang et al. 1992; Terada et al. 1990) have been characterized at the biochemical and genetic level (de Vos et al. 2001).

Recently, a novel thermophilic bacterium, *Fervidobacterium pennivorans*, was isolated from a hot spring on the Azores Islands. This organism has the ability to degrade native chicken feathers at high temperatures. A cell-bound keratinolytic enzyme system capable of hydrolyzing these keratins has been purified from *F. pennivorans,* and its enzymatic properties have been described previously (Friedrich and Antranikian 1996). Until recently, keratinolytic activity was mainly attributed to actinomycetes (Böckle et al. 1995; Bressollier et al. 1999; Mukhopadhyay and Chandra 1990; Noval and Nickerson 1959), a few mesophilic fungi (Kunert 1972; Siesenop and Böhm 1995; Takiuchi et al. 1982), and some *Bacillus* species (Lin et al. 1995; Takami et al. 1989; Varela et al. 1996). Except for the thermophilic actinomycete *Thermoactinomyces candidus*, which grows at 65°C (Ignatova et al. 1999), no other thermophilic organism is reported to digest keratinaceous materials. However, no molecular features of the keratinolytic enzyme from *T. candidus* have been described.

In this study we describe the cloning, identification, and characterization of the *fls* gene encoding the protease, hereafter referred to as fervidolysin, that is responsible for keratinolysis by *F. pennivorans*. Moreover, we expressed the *fls* gene in *Escherichia coli* and showed by site-directed mutagenesis that the produced fervidolysin is subject to autoproteolytic processing, as expected from predictions based on knowledge-based modeling.

Materials and methods

Organisms, growth conditions, and plasmids

F. pennivorans was cultivated under conditions described previously (Friedrich and Antranikian 1996). The bacterial strain used for the initial cloning experiments was *Escherichia coli* TG1 [*sup*E *hsd*D5 *thi* D(*lac*$^-$ *pro*AB), F′, (*tra*D36 *pro*AB *lac*I^q *lac*ZDM15)] (Gibson 1984). *E. coli* BL21(DE3) (*hsd*S *gal* lcI*ts* 857 *ind*1 *sam*7 *nin*5 lacUV5-T7 gene 1) (Studier et al. 1990) was used for heterologous expression. Cultivation of these strains was carried out at 37°C in LB broth (Sambrook et al. 1989), supplemented with either 50 µg of kanamycin or 10 µg of ampicillin per ml. The plasmids used for recombinant work were pGEF+ (Schanstra et al. 1993), pET9d from Novagen (Madison, WI, USA), pGEM-T supplied by Promega (Madison, WI, USA), and pUC18 from Amersham Pharmacia Biotech (Roosendaal, Netherlands).

Recombinant DNA techniques

Genomic DNA of *F. pennivorans* was isolated by using an established protocol (Ramakrishnan and Adams 1995). Small-scale plasmid DNA was isolated by using the Qiagen purification kit (Qiagen, Valencia, CA, USA). DNA was digested with restriction endonucleases from Life Technologies (Invitrogen, Breda, Netherlands), used as specified by the manufacturer. Ligation was carried out with T4 DNA ligase following the manufacturer's specifications (Life Technologies). DNA fragments were purified from agarose by QiaexII or from a polymerase chain reaction (PCR) mix by using a PCR purification kit (Qiagen). Transformations of *E. coli* TG1 and BL21(DE3) were carried out by using established procedures (Sambrook et al. 1989).

Cloning of the *fls* gene encoding fervidolysin

Two oppositely oriented degenerate primers (Eurogentec, Seraing, Belgium) were designed on the basis of the conserved regions, including either the aspartic acid or the serine active-site residue from the subtilisin-like serine proteases (Fig. 1). BG95: 5′-GTIGCIGTIMTIGAYACIGG-3′ (I, inosine; M, A or C; Y, T or C). BG97: 5′-GGIGWIGSC ATISWIGTICC-3′ (I, inosine; W, A or T; S, C or G). PCR analyses were performed using 100 ng of each oligonucleotide and 500 ng *F. pennivorans* genomic DNA as template in a final volume of 100 µl. After an initial denaturation step of 5 min at 95°C, the DNA thermal cycler (PerkinElmer, Shelton, CT, USA) was programmed for a PCR of 35 cycles with *Taq* polymerase (at 1 U per reaction) as follows: 1 min at 95°C (denaturation), 2 min at 35°C (annealing), and 3 min at 72°C (extension), followed by a final extension of 7 min at 72°C. The resulting 0.65-kb fragment was cloned into a pGEM-T vector (Promega) and named pLUW754 (Fig. 1). Southern hybridization of genomic *F. pennivorans* DNA with the 0.65-kb PCR fragment was performed as described below. Hybridizing fragments were isolated from the gel and subcloned in pUC18 and pGEF+, resulting in pLUW751 and pLUW752 (Fig. 1). The 3′-end of the gene was identified and characterized by using the inverse PCR technique described below. On the basis of the overall sequence, two primers were designed to amplify the *fls* gene. The sense primer was designed excluding a putative signal sequence and adding a methionine upstream of the initial asparagine: BG361, 5′-CGCGC**TCATGA**ATCCGAGTTT TGAGCCAAGG (*Bsp*HI restriction site in boldface) The antisense primer was designed as follows (*Bam*HI restriction site in boldface): BG574, 5′-GCCGTTAAA**GGATCC** CTATCACTGT-CCG. PCR was executed by using *Pfu* polymerase and the method described above. A product with the expected size of 2.0 kb was digested with *Bsp*HI and *Bam*HI and cloned in a pET9d expression vector, resulting in pLUW761, which was introduced into *E. coli* TG1 and BL21(DE3).

Southern hybridization

Genomic DNA of *F. pennivorans* was digested with *Hin*cII/*Hin*dIII, *Bam*HI/*Nco*I, and *Pvu*I/*Hin*dIII and separated on a 1% agarose gel. Following gel electrophoresis, the DNA was transferred by capillary blotting to a Hybond N$^+$ filter (Amersham Pharmacia Biotech) (Sambrook et al. 1989). Hybridization was performed overnight at 65°C with the 0.65-kb PCR product that was [α-P^{32}]ATP-labeled (nick translation). Subsequently, the filter was washed, twice with

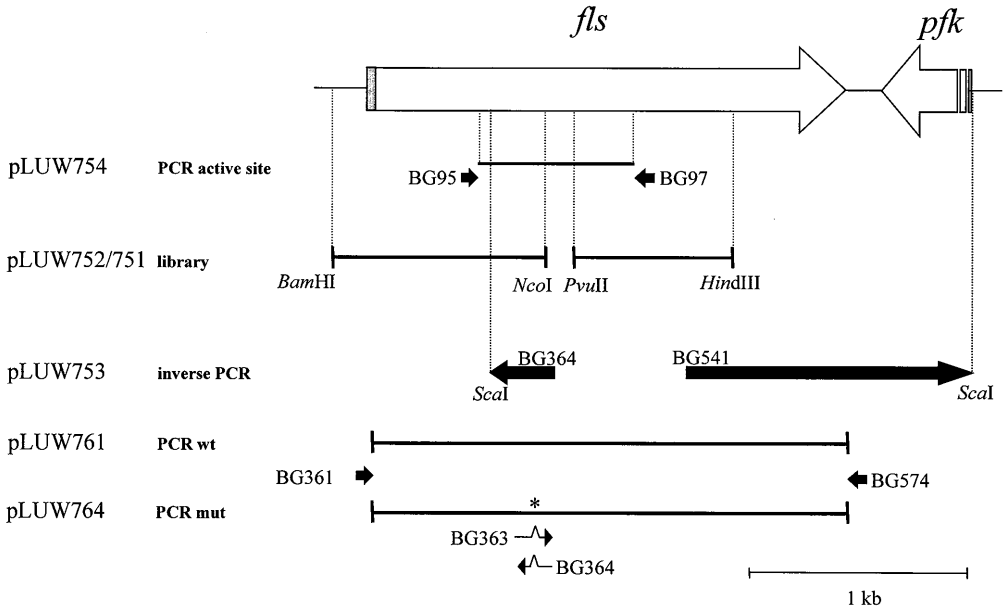

Fig. 1. Overview of the cloning strategy followed to obtain the fervidolysin (*fls*) gene (*top arrow*). The signal sequence is depicted in *gray*. Downstream from the *fls* gene in the opposite transcription direction, an open reading frame is depicted coding for a putative phosphofructokinase (*pfk*). Essential restriction enzymes are depicted and primers used are represented by their *BG number*. All constructs are represented by their assigned *pLUW number*. The expression constructs are described as *PCR wt* and *PCR mut*. The *asterisk* represents the mutation His → Ala. *PCR*, polymerase chain reaction; *wt*, wild-type; *mut*, mutant

2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) containing 1 mM ethylenediaminetetraacetate (EDTA) and twice with 2 × SSC containing 0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by overnight exposure of the filter to a phosphor screen imager.

DNA amplification by inverse PCR

Inverse PCR (Triglia et al. 1988) was carried out with 50 ng of genomic *F. pennivorans* DNA, digested with *Sca*I, and self-ligated to obtain circular DNA. Five nanograms of the ligation mix was used in a PCR with 1 unit of Expand Long Template enzyme mixture (Roche Diagnostics, Mannheim, Germany). After preheating to 94°C for 5 min, 30 PCR cycles were performed, consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and elongation at 72°C for 3 min. The primers used were BG364 and BG541. The resulting 1.2-kb PCR product, containing the 3′-end of the gene, was cloned in a pGEM-T vector and named pLUW753 (Fig. 1).

DNA sequencing

Cloned PCR products were sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977) with a Li-Cor automatic sequencing system (model 4000L, Westburg, Leusden, Netherlands). DNA and protein sequencing data were analyzed with DNASTAR software and compared to the GenBank database by BLAST (Altschul et al. 1997).

Three-dimensional protein modeling of the catalytic domain of fervidolysin

Three-dimensional modeling was carried out by using QUANTA 3.2.3 software (Molecular Simulations, Cambridge, UK) (Sali and Blundell 1993), and CHARMm 22 (Brooks et al. 1983) was run on a Silicon Graphics (Mountain View, CA, USA) 4D25TG workstation. The model is based on the known X-ray structure of subtilisin BPN′ (originating from *Bacillus amyloliquefaciens* strain N′). Insertions of more than six residues relative to subtilisin were not included. After addition of H atoms, the active-site residues (Asp32, His64, and Ser221), the Asn155 (subtilisin BPN′ numbering; Siezen et al. 1991), and the two β-sheet strand backbones eI and eIII involved in the substrate binding were constrained, and the entire molecule was subjected to energy minimization.

Overexpression of the *fls* gene and purification of fervidolysin

E. coli BL21(DE3) harboring pLUW761 was grown overnight, and cells were harvested by centrifugation. The obtained cell pellet was resuspended in 20 mM Tris buffer (pH 8.0) and sonicated using a Branson sonifier (Danbury, CT, USA). Cell debris was removed by centrifugation, and the resulting supernatant was subjected to incubation at 70°C for 20 min. Precipitated proteins were removed by an additional centrifugation step. The supernatant was loaded onto a Q Sepharose column (Amersham Pharmacia Biotech), which was equilibrated with the same buffer.

Bound proteins were eluted by a linear gradient from 0 to 1 M NaCl in 20 mM Tris buffer (pH 8.0). The pooled fractions were concentrated (Filtron Technology, Northborough, MA, USA; 30-kDa molecular weight cutoff). Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (w/v) gels by using the method of Laemmli (1970). The samples were prepared by heating for 5 min at 100°C in an equal volume of sample buffer containing 5% SDS.

Zymogram staining

For zymogram analysis, samples were loaded onto a standard SDS-PAGE gel (Laemmli 1970) after a 5-min heat-incubation step at 100°C. Following electrophoresis, the gel was washed overnight at room temperature in a 50-mM Tris–HCl buffer, pH 10, containing 10 mM $CaCl_2$ and soaked for 1 h in a 1% (w/v) Hammarsten casein solution (Serva, Heidelberg, Germany) in water, pH 10. Subsequently, the gel was incubated at 55°C for 1 h in the washing buffer. Finally, the gel was stained with Coomassie blue and destained to reveal the zones of casein hydrolysis.

N-terminal amino acid sequence analysis

The keratinolytic enzyme purified from *F. pennivorans* (Friedrich and Antranikian 1996) was digested with thermolysin before analysis of the N-terminal sequence of the generated fragments by Edman degradation. Purified *E. coli*-produced fervidolysin was separated by SDS-PAGE (Laemmli 1970) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) by electroblotting at room temperature in 10 mM 3-(cyclohexamino)-1-propanesulfonic acid (CAPS) blotting buffer (pH 11.0), containing 10% methanol (Sambrook et al. 1989). Transferred protein bands were visualized by staining with Ponceau S. The N-termini of the generated fragments of the purified keratinolytic enzyme were analyzed by Edman degradation at ChromaTec in Greifswald, Germany, and the fragments resulting from the recombinant gene were examined with an Applied Biosystems 477A Protein Sequencer (Foster City, CA, USA) at the Institute for Organic Chemistry and Biochemistry in Freiburg, Germany.

Determination of *N*-glycosylation sites

N-glycosylation was examined by using the periodic acid-Schiff (PAS) technique, as described by Zacharius and coworkers (Zacharius et al. 1969). Alternatively, the purified keratinolytic enzyme from *F. pennivorans* was incubated overnight at 37°C with 1 U of *N*-glycanase F (Roche Diagnostics). The degree of *N*-glycosylation was examined by comparing the mass of the protease to its untreated state with SDS-PAGE.

Mutagenesis of the *fls* gene

F. pennivorans DNA was used as a template for mutagenesis of the *fls* gene, in which the active-site mutation His79Ala was introduced in a two-step PCR using overlap extension (Higuchi et al. 1988) (Fig. 1). The first step involved the two primers introducing the mutation and the two flanking primers, described earlier (BG361 and BG574). An amount of 300 ng of each primer was used. The two mutagenesis primers were BG363 (5′-GGTTCA **GCCGGC**ACA CACGTTGCTGG, sense) and BG364 (5′-CGTGTGT-**GCCGGC**TGAACCACC GTAGG, antisense). In the second step, the two overlapping PCR products of the first step were used as a template (200 ng each), which, in combination with the flanking primers BG361 and BG574, resulted in the amplification of the complete *fls* gene carrying the mutation. The verification of the mutation was simplified by introducing a *Nae*I site at the mutation site (boldface in the primers). Nucleotides changed by the introduction of the *Nae*I site are underlined. After sequence analysis, the amplified DNA was cloned as previously described, and *E. coli* BL21(DE3) was transformed with the resulting plasmid (pLUW764).

Nucleotide sequence accession number

The nucleotide sequence has been submitted to the GenBank/EMBL Data Bank with the accession number AY035311.

Results

Isolation and characterization of the *fls* gene

With the degenerate primers BG95 and BG97 (Fig. 1), the active-site region of a subtilisin-like serine protease was amplified (de Vos et al. 2001; Voorhorst et al. 1997), as was confirmed by a database search. Southern blot analysis resulted in the isolation of a 1.1-kb *Nco*I/*Bam*HI fragment, which was subcloned in pGEF+ (pLUW752) and included the sequence of the 5′-end of the *fls* gene. A second hybridizing fragment of 0.7 kb (*Pvu*II/*Hin*dIII) covering a large part of the 3′-end of *fls* was subcloned in pUC18 (pLUW751)(Fig. 1). Ultimately, the 3′-end was identified by inverse PCR techniques. Nucleotide sequence analysis of the subclones (Fig. 1) revealed the complete 2,103-bp *fls* gene, encoding a 699-amino-acid protein that showed notable similarity to the subtilase family of the serine proteases (Siezen and Leunissen 1997). The gene is flanked by *cis* elements such as a consensus Shine-Dalgarno sequence and a 13-nucleotide (nt) inverted repeat (Fig. 2). No obvious promoter regions could be identified. Following the Von Heijne rules, a putative signal peptide of 21 amino acids was predicted (Fig. 3) (von Heijne 1985, 1986). Downstream of this signal peptide, a potential propeptide sequence of 128 amino acids was identified on the basis of its homology to

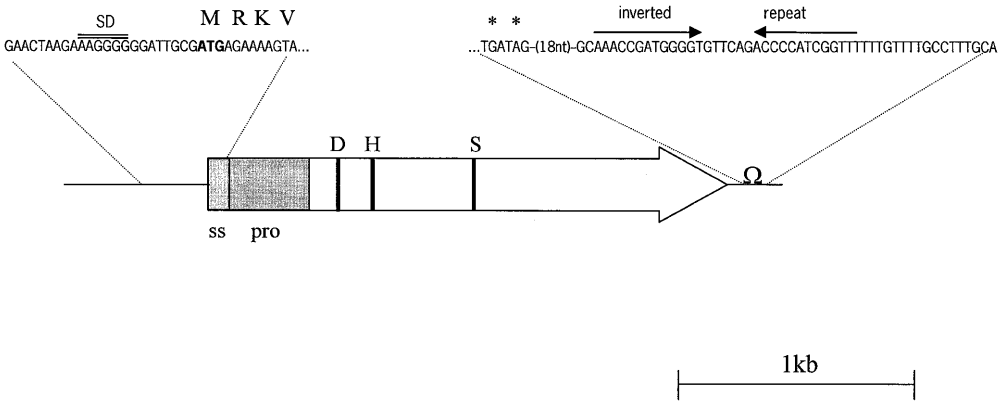

Fig. 2. Overview of the genetic organization of the *fls* gene. Upstream of the *fls* gene, a Shine-Dalgarno region (*SD*) is marked. Downstream, a possible terminator (Ω) is identified by an inverted repeat, followed by a T-stretch. The ATG start codon is in *boldface*. The first four amino acids of fervidolysin (*MRKV*) are depicted above the sequence, and *asterisks* illustrate the double stop codon (*TGATAG*). *ss*, signal sequence; *pro*, prosequence; *D*, Asp; *H*, His; *S*, Ser

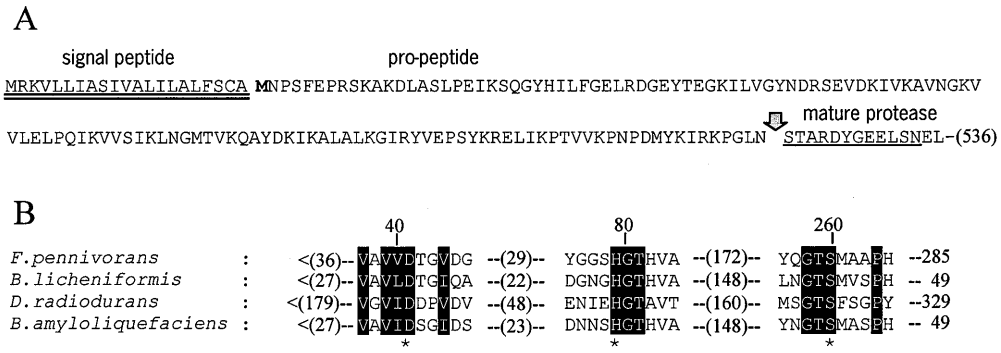

Fig. 3. **A** Overview of the N-terminal amino acid sequence of the fervidolysin precursor. The signal peptide (*double underlined*), the introduced methionine at the start of the propeptide (*boldface*), and the cleavage site between the propeptide and the mature fervidolysin (*arrow*) have been indicated. The N-terminal sequence, as determined by Edman degradation of the mature fervidolysin, is *underlined*, indicating the start of the mature protease. **B** Sequence alignment of fervidolysin with related subtilisin-like serine proteases. Partial sequences are depicted around the active-site residues Asp, His, and Ser (marked by *asterisks*). *Shading* of the residues indicates the level of homology. The depicted sequences are available under the following GenBank accession numbers: *Deinococcus radiodurans* subtilase (AAF12593); *Bacillus licheniformis* keratinase (S78160); *Bacillus amyloliquefaciens* subtilisin BPN′ (X00165)

other propeptides that include conserved hydrophobic residues (Siezen et al. 1995) and confirmed by N-terminal analysis (see below). The mature protein contains the catalytic triad Asp41, His79, and Ser260 in that order (numbering begins at the start of the mature protease), as well as a high degree of homology near these residues at the amino acid level when compared with other subtilases (Fig. 3). No hydrophobic region that could serve as a membrane anchor was identified at the C-terminus. The sequence harbors two cysteine residues, one of which, however, is positioned in the signal peptide and therefore unlikely to be involved in disulfide bridge formation. A putative cleavage site upstream of the first cysteine residue, which is characteristic of lipoproteins, was excluded by the Von Heijne rules (von Heijne 1985, 1986, 1989). The predicted molecular mass of the fervidolysin precursor is 75 kDa, and that of the predicted mature protein is 58 kDa. BLASTP analysis of the complete amino acid sequence (Altschul et al. 1997) found the greatest similarity (approximately 35% identity) with two subtilases from *Deinococcus radiodurans* (White et al. 1999). No homologues were identified in the genome sequence from the phylogenetically related bacterium *Thermotoga maritima* (Nelson et al. 1999). The 3′-end of a second open reading frame (orf) that was identified 152 nt downstream of the *fls* gene, appearing in the opposite transcription direction, showed the highest homology with a putative carbohydrate kinase (belonging to the *pfk*B family) from *Archaeoglobus fulgidus* (Fig. 1).

Expression of the *fls* gene in *E. coli* and purification of fervidolysin

The *fls* gene encoding fervidolysin was amplified by PCR, excluding its signal sequence and including an additional methionine at position –1, and cloned in a pET9d vector under control of the T7 promoter. The resulting plasmid was named pLUW761 and contained the putative catalytic domain and a likely propeptide sequence. The gene was significantly overexpressed in *E. coli* BL21(DE3), even in the

absence of isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 4A, lane 1). The produced protein was initially purified from a cell-free extract by a 20-min heat incubation step at 70°C. Fractions were analyzed on an SDS-PAGE gel, revealing three bands of approximately 73 kDa, 58 kDa, and 14 kDa (Fig. 4A). All three bands were absent in the extract of *E. coli* BL21(DE3) carrying the pET9d plasmid. No overexpression was detected when an *fls* construct devoid of the propeptide sequence was used (not shown). On the basis of the calculated mass of all three bands, the upper 73-kDa band corresponded to the pro-protease precursor, whereas the second band matched the size of the predicted mature protein. The lowest band indicated the propeptide, as shown by its size of 14 kDa, which was determined in a high-percentage polyacrylamide gel (Fig. 4B, lane 5). This heat-stable cell-free extract was further purified by anion exchange chromatography (Q Sepharose), during which the protein eluted at around 0.45 M NaCl. All three bands coeluted as one single peak during both ion exchange chromatography, and gel filtration and showed a similar heterogeneity upon SDS-PAGE (Fig. 4B, lane 5). To elucidate the identity of the three polypeptides, the N-terminal amino acid sequence of all three protein bands was determined. The N-terminal sequence of the second, 58-kDa polypeptide was as follows: X-Thr-Ala-Arg-Asp-Tyr-Gly-Glu-Glu-Leu-Ser-Asn (X represents an unidentified residue). This matched the amino acid sequence that was expected after removal of the potential 128-residue propeptide (Fig. 3). In addition, the N-termini of the 73-kDa and 14-kDa bands, corresponding to the predicted size of the propeptide precursor and the propeptide, respectively, were determined. Both propeptide proteins displayed an identical N-terminal amino acid sequence: X-Ser-Lys-Ala-Lys-Asp-Leu-Ala-Ser-Leu. The experiments, however, surprisingly revealed that the *E. coli*-produced fervidolysin lacked the first eight N-terminal amino acids of the propeptide (including the additional methionine), when compared to the Fls sequence (Fig. 3). Regarding the mature fervidolysin, exactly the same N-terminal sequence was found for the keratinolytic protease that was purified from the toga of *F. pennivorans* by detergent treatment and SDS-PAGE (Friedrich and Antranikian 1996). Since initial attempts to sequence it failed, this batch of purified protease was first cleaved with thermolysin prior to Edman degradation. To analyze and compare the molecular mass of both the *E. coli*- and the *F. pennivorans*-produced enzyme, a zymogram staining was carried out (Fig. 4C). SDS-PAGE analysis of crude cell extract from *F. pennivorans* revealed a clear casein-hydrolyzing band with an apparent molecular weight of around 90 kDa (Fig. 4C, lane 6), corresponding to the most recently purified native keratinase (Fig. 4C, lane 7). The bands in the cell-free extracts of fervidolysin expressed in *E. coli* clearly migrated faster in the gel, demonstrating an apparent difference in molecular mass of about 30 kDa. To examine whether this difference was caused by glycosylation, the purified keratinase from *F. pennivorans* was subjected to periodic acid-Schiff staining. However, no signs of glycosylation were found on the *F. pennivorans* purified keratinase. Alternatively, the keratinase was incubated with *N*-glycanase, a glycosidase that hydrolyzes the linkage between the glycan and an asparagine residue. Subsequently, its molecular mass was compared to its untreated

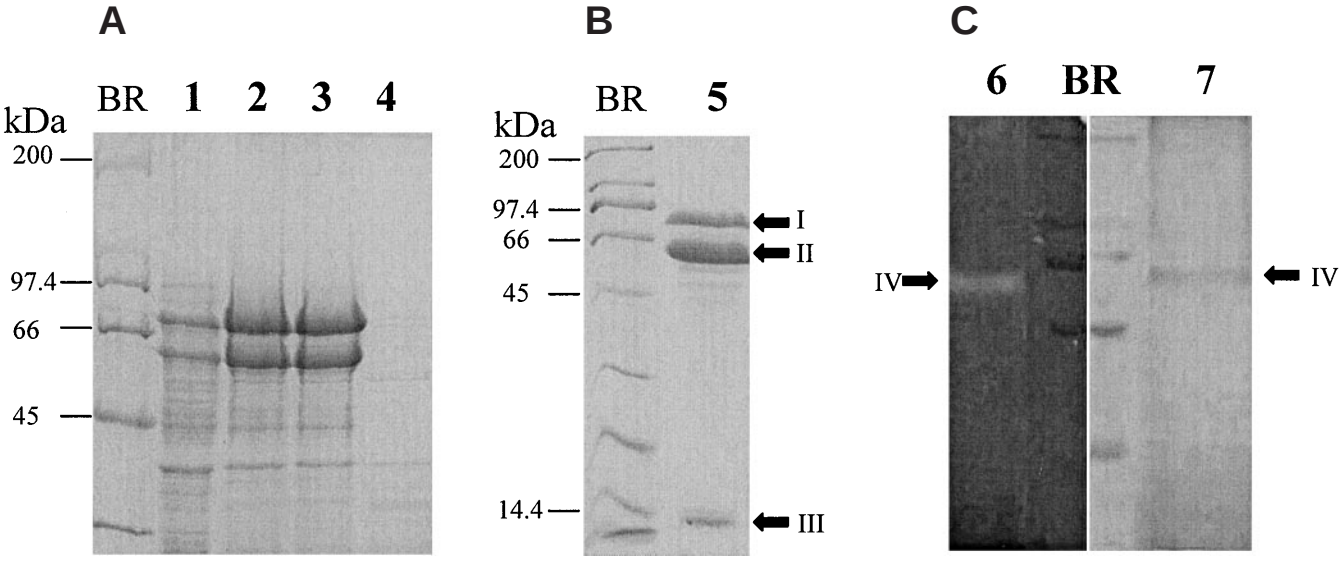

Fig. 4A–C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein extracts from *E. coli* BL21(DE3), overexpressing the *fls* gene on pLUW761, on a 10% gel (**A**) and a 12% gel (**B**). **C** SDS-PAGE analysis of purified *F. pennivorans* keratinase juxtaposed to zymogram staining of caseinolytic activity of *F. pennivorans* crude extract. *Roman numbers* adjacent to the gels indicate the type of polypeptide: *I*, fervidolysin precursor (73 kDa); *II*, mature fervidolysin (58 kDa); *III*, propeptide (14 kDa); *IV*, purified *F. pennivorans* keratinase (90 kDa). *BR*, Broad range marker; *lane 1*, crude cell extract; *lane 2*, soluble fraction; *lane 3*, soluble fraction after heat incubation; *lane 4*, *E. coli* harboring pET9d supernatant after heat incubation; *lane 5*, purified fraction, after anion exchange chromatography. *Lane 6*, crude *F. pennivorans* extract; *lane 7*, purified *F. pennivorans* keratinase

state. Although the *N*-glycosylation motif Asn-X-(Ser/Thr) (Marshall 1972) occurs seven times in the mature protease sequence, the results showed that there was no difference in mass before and after *N*-glycanase treatment, as judged from SDS-PAGE analysis (not shown).

Site-directed mutagenesis of the *F. pennivorans* fervidolysin

To determine whether the appearance of the two lowest bands on the SDS-PAGE gel was a result of either autoprocessing or proteolytic activity by the host organism, an active-site mutant was constructed in plasmid pLUW761. Following the overlap extension method, the active-site residue histidine at position 79 (Fig. 3) was changed into an alanine residue by creating a *Nae*I restriction site at this position. The construct was called pLUW764 (Fig. 1). The effect on the expression level was determined by analyzing the heat-incubated cell-free extract on an SDS-PAGE gel (Fig. 5). The 58-kDa and 14-kDa bands, which were present in the cell-free extract of the wild-type construct, were absent in the extract obtained from an *E. coli* BL21(DE3) culture containing the mutant construct.

Activity of recombinant fervidolysin

The characterization of the keratinase, isolated from a feather-degrading culture of *F. pennivorans*, has been described previously (Friedrich and Antranikian 1996). Unfortunately, we were not able to detect significant activity of the purified recombinant fervidolysin when using dye-labeled substrates (collagen, casein), chromogenic substrates, casein hydrolysate, or keratin powder (results not shown). Attempts to initiate activity by denaturing/renaturing techniques using 6 M urea were also not successful. Alternatively, we attempted to functionally express the *fls* gene in *Bacillus subtilis* BCL1050 (Dartois et al. 1994) using a customized pBCH3 vector (Dartois et al. 1994; Stanssens et al. 1989). Although the gene was cloned successfully, as was confirmed by nucleotide sequencing and restriction analysis, neither intracellular nor extracellular production of a new protease was detected, and no activity was observed with casein as a substrate (results not shown).

Homology modeling

Fervidolysin was modeled against the crystal structure of subtilisin BPN′ from *Bacillus amyloliquefaciens*, of which the structure and coordinates are known (Bott et al. 1988). A schematic model of the substrate-binding region is shown in Fig. 6. The substrate specificity is determined by the interaction between side-chain residues of the substrates and the enzyme residues at the binding sites (Grøn et al. 1992). In general, the pockets *S1* and *S4* are most distinctive in subtilases. Modeling of the cleavage site of the propeptide in these binding sites of fervidolysin revealed that the actual residues involved in this subsite interaction did not distinguish a clear substrate preference. The *S1* pocket remained charge neutral and average in size, leaving room for several amino acid residues at position P1, including the asparagine in the fervidolysin propeptide. Also, the slightly hydrophobic *S4* pocket did not discriminate specific residues at position P4, leaving space for the P4 proline in subsite *S4*. The 3-D modeling experiments resulted in the observation of a weak Ca^{2+}-binding site (result not shown).

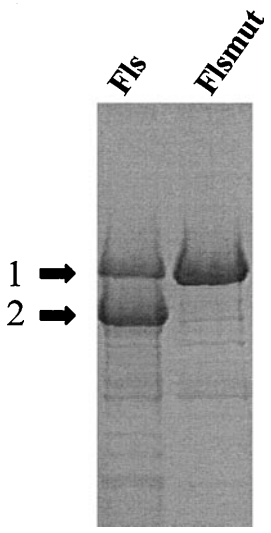

Fig. 5. SDS-PAGE of the purified recombinant fervidolysin and the active-site mutant. *Numbers* adjacent to the gel indicate the type of polypeptide: *1*, fervidolysin precursor (73 kDa); *2*, mature fervidolysin (58 kDa) (propeptide not shown in this gel)

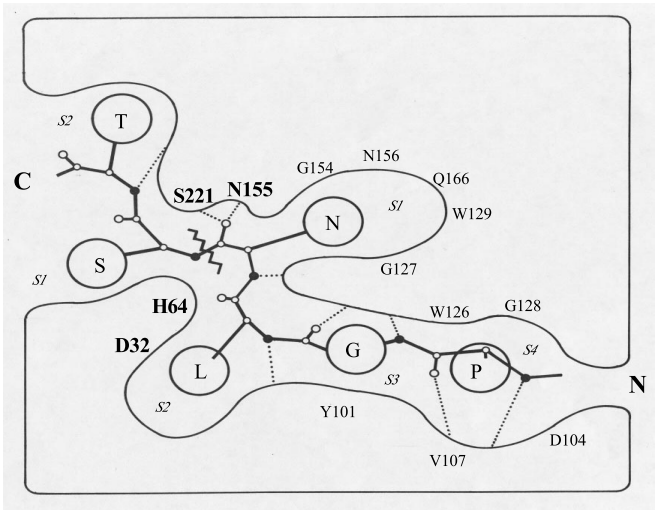

Fig. 6. Schematic representation of the substrate-binding region of fervidolysin. The catalytic residues (*D32*, *H64*, and *S221*) and the oxyanion (*N155*) are marked in *boldface* (Subtilisin BPN′ annotation). *C* and *N* denote the C-terminus and the N-terminus of the substrate, respectively. The binding subsite nomenclature *S4–S2′* is according to Schechter and Berger (Schechter and Berger 1967). The propeptide of the fervidolysin precursor is modeled in the substrate-binding site of the enzyme. A *zigzag line* indicates the cleavage site after the P1 position. Substrate backbone atoms are shown as *solid circles* (nitrogen), *small open circles* (carbon), and *large open circles* (oxygen). Potential backbone hydrogen bonds between the propeptide and fervidolysin are shown as dotted lines

Discussion

We have identified the active-site region of a subtilisin-like serine protease from *F. pennivorans* by means of a PCR analysis. Subtilases are characterized by conservation at the amino acid level near the three catalytic residues aspartic acid, histidine, and serine (Fig. 3), and an oxyanion-hole asparagine (Siezen and Leunissen 1997). Southern hybridization and inverse PCR revealed an open reading frame of 2.1 kb, flanked by regions that can be recognized as *cis* elements, such as a Shine-Dalgarno sequence and a transcription terminator.

The deduced primary sequence showed high homology with the subtilisin-like serine proteases and had the highest degree of similarity with a subtilase from *D. radiodurans* (White et al. 1999). Following the Von Heijne rules (von Heijne 1986), a signal sequence of 21 amino acids was recognized, which in vivo is essential for initiating the export of the protein toward the cell wall. The signal peptide is followed by a propeptide, which is the domain that mediates the correct folding of the protein into a biologically active state (Ikemura and Inouye 1988; Ikemura et al. 1987). The size of the propeptide was estimated to be 128 residues, judged from sequence similarities among hydrophobic regions in the propeptide (Siezen et al. 1995) and alignment comparisons (Siezen and Leunissen 1997), and from determination of the N-terminus of the mature protease.

The purified fervidolysin, heterologously produced by *E. coli*, did not show activity toward casein, collagen and keratin powder, or chromogenic substrates designed for the determination of the substrate specificity of subtilases. Additional attempts to functionally express the *fls* gene in a *B. subtilis* expression system also did not result in an active keratinase. Comparison of the N-terminal sequence of the 58-kDa polypeptide with that of the thermolysin-treated keratinase indicated that the N-terminus of the *E. coli*-produced protease was identical to the purified keratinase (Fig. 3A). As a result, it could be concluded that the *fls* gene codes for the same keratinolytic protease as described earlier (Friedrich and Antranikian 1996). Since the purified keratinase was pretreated with thermolysin, N-terminus experiments were carried out on fragmented keratinase. Given that the sequence shows complete identity with the first 12 amino acids of fervidolysin, it can be considered to be the N-terminus of the enzyme.

In addition, the N-terminal analyses of all three bands showed that the occurrence of the mature fervidolysin was a result of autoproteolytic modification of the protease precursor, present as the 73-kDa-protein band (Fig. 4B). This autoproteolytic process is necessary for proteins to fold into their active conformation; however, it is not sufficient for activation of the protease. This could imply that posttranslational modification performed by the original host organism *F. pennivorans* is required. An alternative explanation could be that the protease improperly folds in both *E. coli* and *B. subtilis*, although the protease remained in the soluble fraction when expressed in *E. coli*. The addition of crude cell extract of *F. pennivorans* to the heterologously produced enzyme did not result in a restoration of activity (unpublished data). Although this might exclude possible involvement of targeting factors, it does not rule out the involvement of chaperones or other factors that act cotranslationally.

Zymogram staining combined with SDS-PAGE analysis (Fig. 4C) revealed that the apparent molecular mass of the biochemically purified keratinase was about 30 kDa higher than its recombinant counterpart (Fig. 4B). This difference could be due to *N*-glycosylation. Although the *N*-glycosylation motif Asn-X-(Ser/Thr) (Marshall 1972) occurs seven times in the mature protease sequence, actual *N*-glycosylation could not be shown by treatment with *N*-glycanase or by periodic acid-Schiff staining. Moreover, the ultimate effect on the protein mass is difficult to estimate, since the degree of glycosylation and the length of the glycan part is hard to predict.

Extracellular proteases are able to become fully active only when their propeptide is further degraded (Ikemura and Inouye 1988). N-terminus experiments on the mature protease showed that the cleavage of the propeptide, which is the first step toward activity, had taken place. In general, the propeptide remains noncovalently bound within the catalytic domain of the subtilase after being cleaved and functions as an inhibitor. This inhibition is then relieved by internal cleavage, after which the enzyme is able to use other substrates (Shinde and Inouye 1995a, b). This phenomenon was extensively described for kexin, a subtilase from *Schizosaccharomyces pombe* (Powner and Davey 1998). In the case of fervidolysin, the propeptide remains tightly bound during the purification, as judged from SDS-PAGE analyses (Fig. 4B, lane 5). Therefore, the propeptide is likely to operate as an inhibitor. Increasing the salt concentration to weaken the propeptide/mature protease complex did not give rise to any keratinolytic activity. Fervidolysin contains two sites that may serve as a secondary cleavage site, both toward the C-terminal end of the propeptide (Siezen et al. 1995). Although the propeptide is lacking eight amino acids, this lack does not seem to be the result of the additional cleavage step, which is required for the rendering activity. Perhaps a degradation of the propeptide is instigated by external proteolytic enzymes, which might be present in vivo. This degradation process has been shown before, although only in eukaryal proteases that are processed at a vacuolar level (Hiraiwa et al. 1997; Naik and Jones 1998). In general, both the cleavage and the removal of the propeptide is an autocatalytic process (Shinde and Inouye 1995a).

Mutagenesis of the active-site residue histidine into an alanine showed that the proteolytic cleavage of the fervidolysin precursor was a result of autoproteolytic activity, and was not caused by proteolysis carried out by the expression host *E. coli*. Obviously, these results illustrate the importance of the catalytic histidine residue. Besides playing a crucial role in substrate catalysis (Perona and Craik 1995), the histidine residue is also involved in the autocatalytic cleavage process, in which the propeptide is removed via an intramolecular autoprocessing mechanism (Shinde and Inouye 1995a). Regarding fervidolysin, however, the propeptide appears to remain tightly bound to the mature subtilase. This clearly proves that the propeptide cleavage is

carried out within the profervidolysin molecule itself. Similar results were obtained by Li and coworkers in their work on subtilisin E from *B. subtilis* (Li and Inouye 1994).

The propeptide sequence plays an essential role in guiding the correct folding of the protein into an active protease (Ikemura and Inouye 1988; Ikemura et al. 1987). Examination of the N-termini of the pro-protease precursor and the propeptide sequence showed that the propeptide was lacking eight amino acids (additional methionine included) compared with the predicted sequence. This might very well be a result of proteolytic activity carried out by the host organism, since the cleavage sequence does not resemble the N-terminal sequence of the mature protease. The prosequences are generally indispensable for the proper folding of proteins (Siezen et al. 1995), and it was shown for Fls that it could not be expressed when the propeptide was omitted. The importance of an intact prosequence was mentioned earlier by Ikemura and coworkers (Ikemura and Inouye 1988; Ikemura et al. 1987), who described the role of the propeptide in the expression of subtilisin E. In addition, Lee and colleagues (Lee et al. 1991) showed that partial deletions in the N-terminal prosequence gave rise to an inactive aqualysin I, a thermophilic subtilase from *Thermus aquaticus*, and the precursor protein was found to be unstable in *E. coli*. Chang and coworkers also showed the inactivating effect of a truncated form on the Npr protease of *Streptomyces cacaoi* (Chang et al. 1994). It is, however, hard to predict whether the lack of the eight amino acids at the N-terminal end of the propeptide mainly results in an incorrect folding of fervidolysin, or whether this deletion blocks further processing.

Preliminary experiments with the biochemically purified keratinase on chromogenic substrates showed that the enzyme prefers to cut between acidic amino acids rather than between alkaline amino acids. While the substrate specificity for keratinases is well defined, not much is known about the amino acid sequence that they prefer to cleave.

Although we were not able to produce fervidolysin in an active form, it still remains an interesting industrial enzyme, considering its thermal origin and its substrate preference. Possibly, expression in a host that is phylogenetically closely related to *F. pennivorans*, such as *T. maritima* or even the original host itself, might be a better solution for producing the active form of this enzyme.

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