

Cloning and Expression of Islandisin, a New Thermostable Subtilisin from *Fervidobacterium islandicum*, in *Escherichia coli*

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Received 15 July 2004/Accepted 24 January 2005

A gene encoding a subtilisin-like protease, designated islandisin, from the extremely thermophilic bacterium *Fervidobacterium islandicum* (DSMZ 5733) was cloned and actively expressed in *Escherichia coli*. The gene was identified by PCR using degenerated primers based on conserved regions around two of the three catalytic residues (Asp, His, and Ser) of subtilisin-like serine protease-encoding genes. Using inverse PCR regions flanking the catalytic residues, the gene could be cloned. Sequencing revealed an open reading frame of 2,106 bp. The deduced amino acid sequence indicated that the enzyme is synthesized as a proenzyme with a putative signal sequence of 33 amino acids (aa) in length. The mature protein contains the three catalytic residues (Asp177, His215, and Ser391) and has a length of 668 aa. Amino acid sequence comparison and phylogenetic analysis indicated that this enzyme could be classified as a subtilisin-like serine protease in the subgroup of thermitase. The whole gene was amplified by PCR, ligated into pET-15b, and successfully expressed in *E. coli* BL21(DE3)pLysS. The recombinant islandisin was purified by heat denaturation, followed by hydroxyapatite chromatography. The enzyme is active at a broad range of temperatures (60 to 80°C) and pHs (pH 6 to 8.5) and shows optimal proteolytic activity at 80°C and pH 8.0. Islandisin is resistant to a number of detergents and solvents and shows high thermostability over a long period of time (up to 32 h) at 80°C with a half-life of 4 h at 90°C and 1.5 h at 100°C.

Hyperthermophiles that grow optimally between 80 and 110°C belong to phylogenetically distant groups. Due to the low solubility of oxygen at high temperature, biotopes of hyperthermophiles are mainly anoxic (13). The eubacterial order *Thermotogales*, including the genera *Thermotoga*, *Thermosiphon*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Thermopallium*, and *Marinitoga*, comprises the most extremely thermophilic eubacteria presently known. Phylogenetically, this order represents the deepest branch within the bacteria (39). Members of the order *Thermotogales* are able to grow on various substrates such as proteins, starch, cellulose, and xylan. They are all capable of growing at temperatures above 60°C with an optimum of about 80°C. The cells of *Thermotogales* are characteristically surrounded by a “toga,” a sheath-like envelope containing regularly arranged porin-like proteins (12).

Enzymes from thermophilic microorganisms are called thermozymes. They can potentially be used in several industrial processes, in which they replace mesophilic enzymes or chemical catalysts. The main advantages of performing processes at higher temperatures are a reduced risk of microbial contamination, lower viscosity, improved transfer rates, and improved solubility of substrates (7). To date, the mechanisms that are responsible for the thermostability of proteins are not well understood. Often it is a combination of intrinsic properties, such as increased number of Van der Waals interactions, hydrogen bonds and ion-pairs, which lead to extreme thermosta-

bility (35). Furthermore, protein chaperones and compatible solutes can act as extrinsic factors in maintaining protein integrity (8, 20).

Proteolytic enzymes catalyze the hydrolysis of proteins into amino acids and peptides. They act as processing enzymes taking part in regulatory or catabolic processes in the cell or as extracellular enzymes playing an important role in degrading proteinaceous substrates serving as carbon or energy sources. Serine endopeptidases and exopeptidases are extremely widespread in occurrence and diverse in function. The superfamily of serine proteases has been divided into clans (3), of which the clan SB comprises only one family, the family S8, which includes the subtilases. Subtilases are synthesized intracellularly as a precursor called preprosubtilisin, in which the presequence and prosequence are attached to the N terminus of the mature protein (15, 38). The presequence acts as a signal peptide that facilitates the secretion of a prosubtilisin across the cytoplasmic membrane. The prosequence acts as an intramolecular chaperone, guides correct folding of the mature protein, and is cleaved by autoproteolysis (14, 30). The mature enzymes were found to contain up to 1,775 residues, with N-terminal catalytic domains (CDs) ranging from 268 to 511 residues and signal and/or activation peptides (preprosequence) ranging from 27 to 280 residues (32). The subtilisin-like serine proteases show optimal proteolytic activity at a basic pH (7–10), due to the requirement of a deprotonated histidine (26).

Many of the *Thermotogales* strains could be identified as protease-producing bacteria. The proteases are optimally active at 80°C (9), which makes them attractive candidates for

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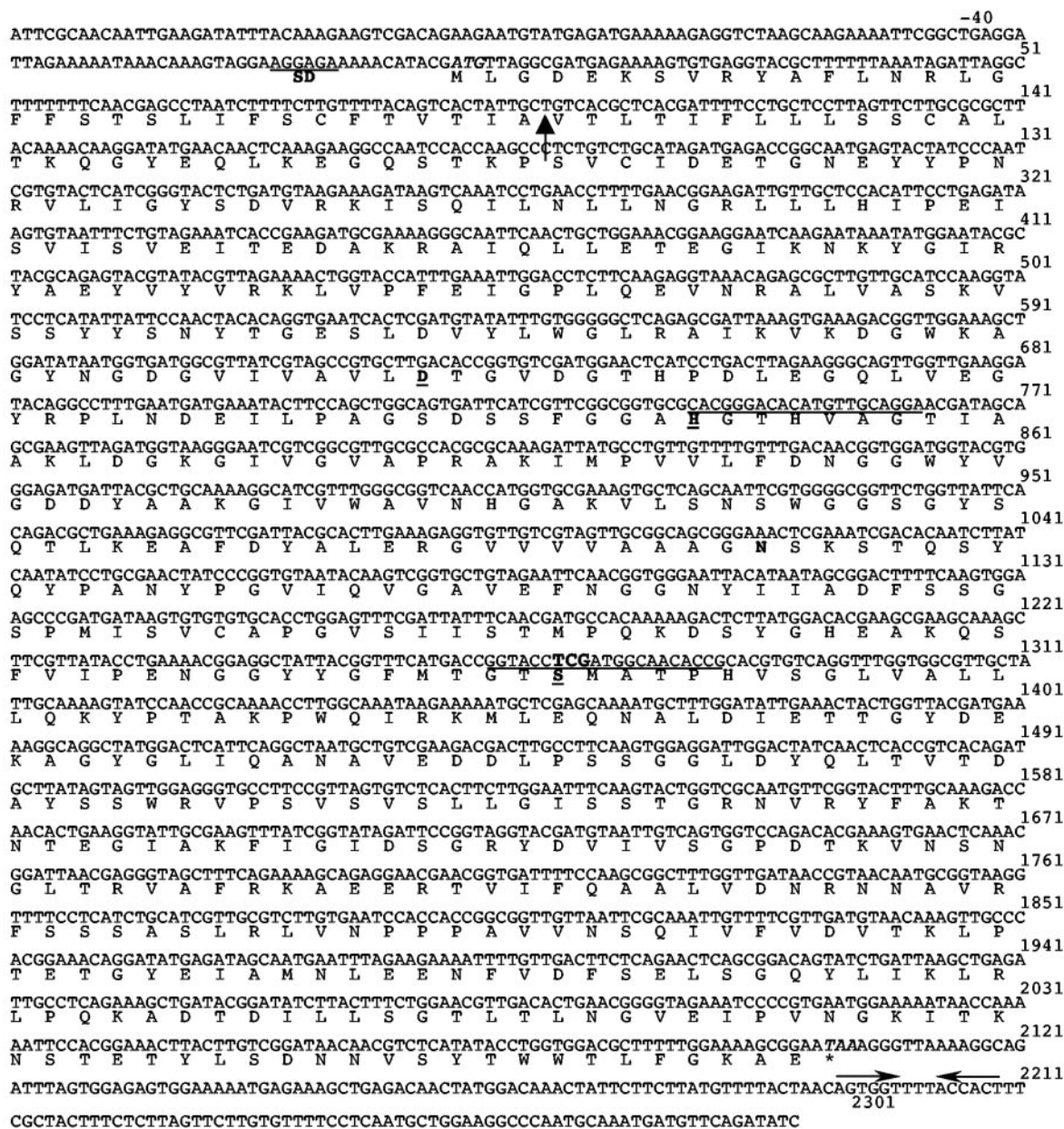


FIG. 1. Nucleotide sequence of the cloned islandisin-encoding gene and the deduced amino acid sequence. The number of nucleotides starts at the 5' terminus of the gene encoding islandisin. Typical expression signals, such as the Shine-Dalgarno sequence (SD), a transcriptional termination signal (in italics), and the binding sites of degenerated primers (underlined), are indicated. The three conserved amino acids (Asp-His-Ser) of the catalytic domain and the Asn residue of the oxyanion hole are indicated in boldface letters. The putative processing site for the signal peptide of the islandisin signal sequence is indicated by a thick vertical arrow. A putative hairpin structure for the mRNA is indicated by two convergent arrows.

several industrial applications (22). Since cultivation of *Thermotogales* strains only leads to low cell yields, the cloning and expression of protease-encoding genes in a mesophilic host are obligatory, if the enzymes are to be used in industrial applications. Until now, only a few proteases of the *Thermotogales* have been characterized in detail. A protease-encoding gene from *Fervidobacterium pennivorans* was detected, amplified by PCR and cloned in *E. coli*. The recombinant protein, however, did not show any activity (18).

In this paper we report (i) the nucleotide sequence of the

gene encoding islandisin, (ii) comparison of the deduced amino acid sequence with other subtilisin-like enzymes, (iii) the production and purification of enzymatically active recombinant islandisin in *E. coli*, and (iv) structural analysis of the enzyme by molecular modeling.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Fervidobacterium islandicum* was grown in a complex medium (TF medium). The medium contained (per liter): NH₄Cl, 0.5 g; MgSO₄ · 7H₂O, 0.16 g; K₂HPO₄, 1.6 g; NaH₂PO₄ · H₂O, 1.0 g; CaCl₂, 0.05 g;

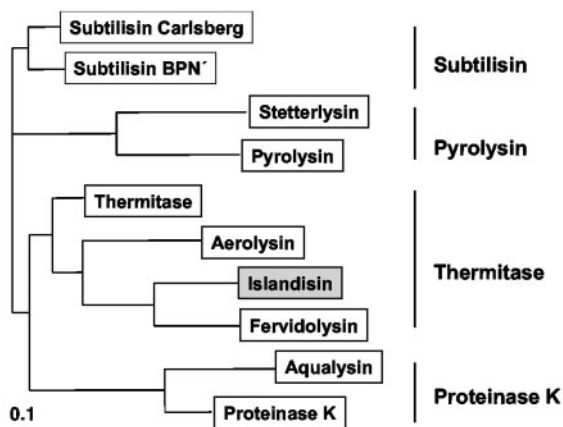


FIG. 2. Phylogenetic tree of subtilisins inferred from the amino acid sequence alignment. The scale bar corresponds to 10 amino acid changes per 100 amino acids. Islandisin, *Fervidobacterium islandicum*; fervidolysin (accession no. AAK61552), *F. pennivorans*; subtilisin Carlsberg (accession no. CAB56500), *Bacillus licheniformis*; subtilisin BPN' (accession no. P00782), *B. amyloliquefaciens*; aerolysin (accession no. AAB32719), *Pyrobaculum aerophilum*; stetterlysin (accession no. AAC68832), *Thermococcus stetteri*; proteinase K (accession no. PO6873), *Tritirachium album*; aqualysin (accession no. PO8594), *Thermus aquaticus*; pyrolysin (accession no. NP_127331), *Pyrococcus furiosus*; thermitase (accession no. SUMYTV), *Thermoactinomyces vulgaris*.

trace elemental solution (DSMZ-141) (10 fold), 1 ml; vitamin solution (DSMZ-141) (10 fold), 1 ml; trypticase, 2.0 g; yeast extract, 1.0 g; resazurin, 1 mg. The medium was reduced with Na₂S · 9H₂O at a final concentration of 0.4 g/liter and maintained under anaerobic conditions (2) with N₂ in the gas phase and then

TABLE 1. Purification scheme of the recombinant islandisin

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Enrichment (fold)	Yield (%)
Crude extract	116.1	11.2	0.1	1	100
Heat treatment	9.8	12.8	1.3	13.5	114.3
Hydroxyapatite	1.4	3.2	2.3	23.9	28.6

adjusted to pH 6.8 with 1 N NaOH. Glucose in a final concentration of 0.5% (wt/vol) was added to the medium as an additional carbon source. Incubation was performed anaerobically without shaking at 70°C.

Escherichia coli BL21(DE3)pLysS (Invitrogen, La Jolla, CA) was grown in Luria-Bertani broth (containing per liter: NaCl, 5 g; tryptone, 10 g; yeast extract, 5 g) supplemented with ampicillin (100 µg/ml). The plasmid pET-15b (Novagen, Inc., Madison, Wis.) was used for cloning and expression.

Detection of the protease-encoding gene from *F. islandicum* by PCR using degenerated primers. The sequences corresponding to mature subtilisin-like proteases from different microorganisms were aligned using the CLUSTAL X multiple sequence alignment program to determine areas of homology. The highly conserved areas around the three catalytic residues of subtilisin-like enzymes (Asp, His, and Ser) were used to design degenerated oligonucleotide primers. A degenerated forward primer, 5'-CAYGGIACICRIKIGCIGG-3', of the His-active site and a degenerated reverse primer, 5'-CAYGGIACICRIKIGCIGG-3', of the Ser-active site were designed. Standard PCR was performed using 100 ng bacterial genomic DNA in a 100 µl reaction mixture containing 1× amplification buffer (30 µg bovine serum albumin, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂) (Roche, Mannheim, Germany), 0.2 mM deoxynucleoside triphosphates, and 0.4 µM each primers with 2 U of Hifi-Polymerase (Roche, Mannheim, Germany). Amplification was performed with a thermocycler (Perkin Elmer Gene Amp System 2400) programmed for 35 cycles, each cycle consisting of 30 s at 94°C, 1 min at 35°C, and 1 min at 72°C, with a final 7-min extension at 72°C. The products of amplification were checked on 1.0% (wt/vol) agarose gels (Bio-Rad Laboratories, München, Germany). The gel-slice contain-

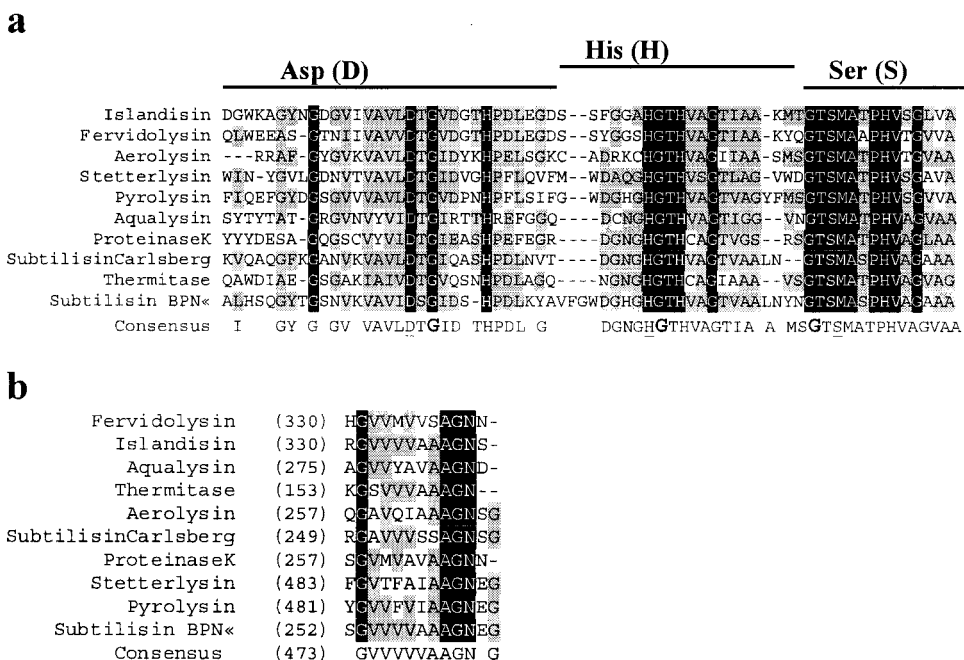


FIG. 3. Conserved regions around the three catalytic residues Asp (D)-His (H)-Ser (S) (a) and around the Asn (N) of the oxyanion hole (b). Letters in white represent the identical positions found in the 10 sequences, and letters shaded in gray represent the conservative amino acids. The numbers represents the positions of the amino acid residues starting from the N terminus of the presequence. Islandisin, *Fervidobacterium islandicum*; fervidolysin, *F. pennivorans*; subtilisin Carlsberg, *Bacillus licheniformis*; subtilisin BPN', *B. amyloliquefaciens*; aerolysin, *Pyrobaculum aerophilum*; stetterlysin, *Thermococcus stetteri*; proteinase K, *Tritirachium album*; aqualysin, *Thermus aquaticus*; pyrolysin, *Pyrococcus furiosus*; and thermitase, *Thermoactinomyces vulgaris*. Respective accession numbers are given in the legend of Fig. 2.

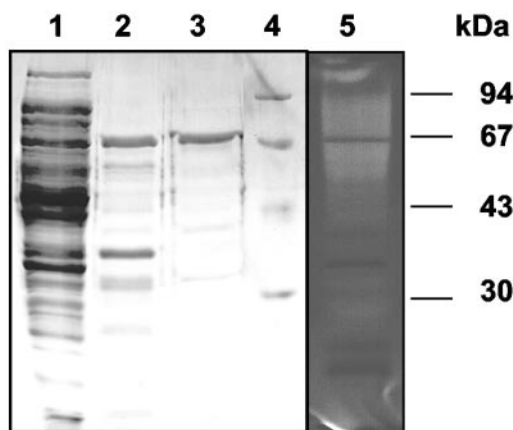


FIG. 4. Electrophoretic analysis of samples during purification using a 12% (wt/vol) SDS-polyacrylamide gel and activity staining. Samples (10 μ g of protein) were subjected to electrophoresis on a 12% (wt/vol) polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie brilliant blue. For activity staining, the gel was soaked in 1.5% (wt/vol) azocasein after electrophoresis for 30 min at 20°C and incubated at 80°C and pH 8.0 for 1 h. Clearing zones corresponding to azocasein hydrolysis were obtained after the gel was stained with Coomassie brilliant blue. Lane 1, crude extract; lane 2, heat-denatured crude extract; lane 3, protease fraction after purification on hydroxyapatite; lane 4, low-molecular-weight marker; lane 5, activity staining.

ing the amplicon was excised and DNA was extracted with a QIAquick gel extraction kit (QIAGEN, Inc., Hilden, Germany). The purified amplicon was cloned into the pCRII-TOPO plasmid vector (Invitrogen, San Diego, CA), and the insert was sequenced. The deduced amino acid sequence was compared to protein databases with BLAST and FASTA programs. The whole gene was completed by inverse PCR techniques.

Construction of the expression plasmid with *fis*. Expression of the *fis* gene in *E. coli* was carried out by standard methods (27). The full-length sequence encoding the protease of *fis* was obtained from genomic DNA of *F. islandicum* by PCR amplification. Primers were designed to include an NdeI site at the 5' end and a BamHI site at the 3' end of *fis*. The primers used were 5'-GGAGA AAAACATATGATGTTAGGC-3' (sense primer; NdeI site underlined) and 5'-CTGCCCTTTGGATCCTTATCCGC-3' (antisense primer; BamHI site underlined). Purified PCR products were ligated into NdeI-BamHI restriction sites of the pET-15b expression vector (Novagen, Madison, Wis.). *E. coli* BL21(DE3)pLysS was transformed with the ligation mixture. The resulting plasmid was designated pFiS. The complete gene was sequenced.

Expression of the *fis* gene. *E. coli* BL21(DE3)pLysS carrying recombinant plasmid pFiS was cultured overnight at 30°C in Luria-Bertani broth containing ampicillin (100 μ g/ml). The overnight culture was inoculated (1%) into the same medium and incubated at 37°C. For induction of pFiS, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture broth (optical density at 600 nm, 0.4) with 1 mM as the final concentration. Cells were harvested after 4 h of growth (5,000 \times g; 10 min at 4°C) and suspended in 20 mM potassium phosphate buffer, pH 7.2.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymogram staining. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% (wt/vol) gels (21), and protein bands were detected by Coomassie staining (37). To determine the molecular mass of the proteins, a low-molecular-weight marker (Bio-Rad, Hercules, CA) was used. For activity staining, the gel was soaked in 1.5% (wt/vol) azocasein after electrophoresis for 30 min at 20°C and incubated at 80°C, pH 8.0, for 1 h. Clearing zones corresponding to azocasein hydrolysis were obtained after the gel was stained with Coomassie brilliant blue.

Protease assay. Proteolytic activity was measured by a modified method of Kunitz (19). Enzyme fractions (10 μ l = 13.5 mU) were added to 490 μ l of 0.25% (wt/vol) casein (Hammersten; Merck, Darmstadt, Germany) in 120 mM universal buffer, pH 8 (6), to give a final volume of 500 μ l. The samples were incubated in a water bath at 80°C for 40 min. The reaction was stopped by the addition of 0.5 ml 10% (wt/vol) trichloroacetic acid, and the mixture was centrifuged at 13,000

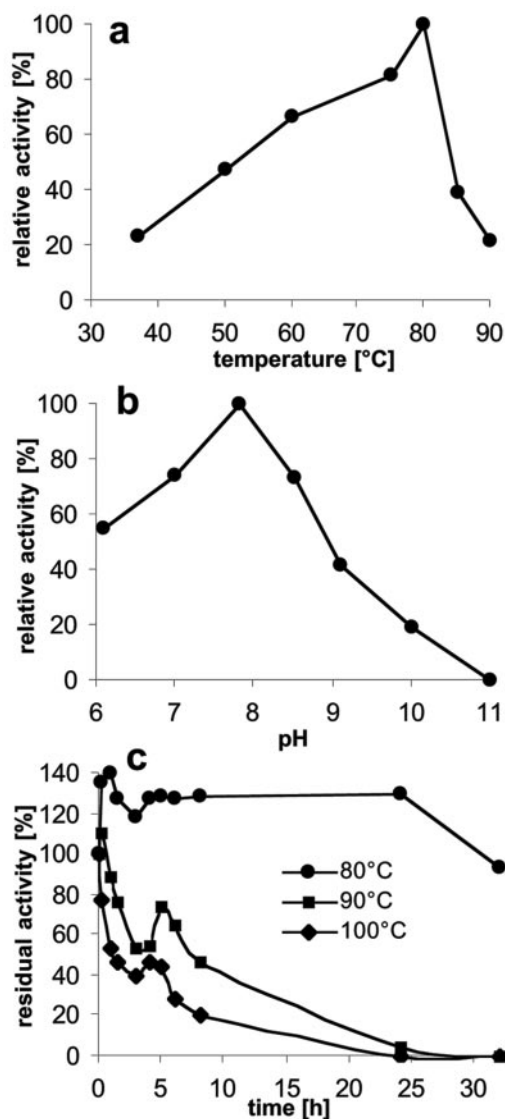


FIG. 5. Biochemical characteristics of the recombinant protease from *F. islandicum* determined by casein hydrolysis. (a) Effect of temperature on islandisin activity (pH 8.0); (b) effect of pH on islandisin activity (80°C); (c) thermostability, determined with six replicates.

rpm (Heraeus Sepatech, Osterode, Germany) for 10 min. The absorbance of the supernatant at 280 nm was determined. Sample blanks were used to correct the nonenzymatic release of aromatic amino acids. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of aromatic amino acids (with tyrosine as a standard) per minute under the defined assay conditions. The protein concentration was measured by the method of Bradford (5) with bovine serum albumin as the standard.

Enzyme purification. Cells were suspended in 20 mM potassium phosphate buffer, pH 7.2, disrupted by sonication, and centrifuged at 15,000 \times g for 20 min. The resultant supernatant was heat treated at 80°C for 20 min and centrifuged (15,000 \times g; 20 min) to remove the denatured *E. coli* proteins. The heat-treated protein fraction was dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and loaded on a hydroxyapatite column (35 ml; Roche, Mannheim, Germany) equilibrated with the same buffer. The column was washed with 150 ml of equilibration buffer (10 mM sodium phosphate buffer, pH 7.2). The proteins were eluted by linearly increasing the ionic strength of the buffer from 10 mM sodium phosphate buffer (pH 7.2) to a final concentration of 500 mM sodium phosphate buffer (pH 6.8) at a flow rate of 1 ml/min. Active fractions were analyzed for their purity by SDS-PAGE on a 12% (wt/vol) polyacrylamide gel,

TABLE 2. Effects of metal ions on recombinant islandisin activity

Metal ion	Relative activity (%) at concn of:	
	1 mM	5 mM
Control	100	100
Mo ²⁺	0	0
Cu ⁺	0	0
W ⁺	0	0
Ni ²⁺	0	0
Zn ²⁺	16.7	0
Vo ²⁺	30.4	0
Mg ²⁺	45.8	55.4
Ca ²⁺	51.2	34.6
Mn ²⁺	71.4	47.1
Se ²⁺	188.1	266.7

followed by staining with Coomassie brilliant blue. The purified enzyme fractions were pooled and used for further characterization studies.

Effects of temperature and pH on islandisin activity. The temperature and pH range of islandisin were determined in universal buffer at pH 8 and 80°C, respectively (6). The thermostability was studied by incubating the enzyme in the universal buffer pH 8.0, at various temperatures (80, 90, and 100°C) in tightly closed screw caps. The residual proteolytic activity was measured at appropriate time intervals in six replicates at optimal temperature and pH.

Effects of inhibitors, metal ions, detergents, solvents, and denaturing and reducing agents on islandisin activity. The effect of the metal ions MoCl₂, CuCl, WCl, NiCl₂, ZnCl₂, VoCl₂, MgCl₂, CaCl₂, MnCl₂, and SeCl₄ and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), EDT, iodacetate, and 4-(2-aminoethyl)-benzylsulfonyl fluoride (Pefabloc SC) was investigated in final concentrations of 1 to 10 mM. The influence of other reagents, such as SDS, Triton X-100, ethanol, isopropanol, dimethylformamide, dimethyl sulfoxide, acetonitrile, urea, dithiothreitol (DTT), and β-mercaptoethanol, was also tested. Islandisin was preincubated with the respective reagents dissolved in universal buffer, pH 8, at room temperature for 1 h (6). The reaction was started by the addition of 0.25% (wt/vol) casein, giving a final volume of 500 μl. The enzyme solution was then incubated at 80°C for 40 min, and the residual enzyme activity was measured as described before. The activity without metal ions, inhibitors, detergents, solvents, or denaturing agents was considered as 100%.

Sequence similarities and construction of a phylogenetic tree. Computer-assisted DNA and protein sequence analyses were performed using CLUSTAL X 1.8 and DNASTRIDER 1.1. Protein sequence similarity searches were performed using the BLAST algorithm at the National Center for Biotechnology Information server (1). Signal peptides were predicted by the program SignalP, version 3.0 (4). An extensive search in databases (BLAST) was performed to collect amino acid sequence of subtilisins from bacteria, archaea, and eucarya. Amino acid sequences of 10 chosen enzymes, including islandisin, were aligned using CLUSTAL X with a BLOSUM62/PAM substitution matrix and default gap penalty parameters. PROTDIST was employed to analyze protein sequence similarities, and NEIGHBOR (Kimura two-parameter correction) was used to create a phylogenetic tree based on amino acid sequences.

Structural homology modeling. A structural model of islandisin was constructed by homology modeling using the 1.7-Å crystal structure of *Feravidobac-*

terium pennivorans feravidolysin as a template (17). A pairwise protein sequence alignment of feravidolysin and islandisin was generated by Toffee using default parameters (25), and aligned sequences were modeled in alignment mode by SwissModel (29), an automated comparative protein modeling web server. The validity of the model was checked by WhatCheck (11). The final model consisted of three chains, connecting amino acid residues 35 to 484, 490 to 518, and 524 to 658.

Nucleotide sequence accession number. The nucleotide sequence of the islandisin-encoding gene of *F. islandicum* has been deposited in the GenBank database (National Center for Biotechnology Information) under accession no. AY190029.

RESULTS

Identification and sequence analysis of the *fis* gene from *F. islandicum*. The 549-bp amplicon that was synthesized by PCR using degenerated primers showed high similarities to other known subtilisin-like enzymes in the database. The whole gene, comprising an open reading frame of 2,106 bp, was completed by inverse PCR. A putative Shine-Dalgarno sequence was detected 11 bp upstream of the start codon (AGGAGA). In addition, a putative hairpin structure for the mRNA terminating the translation was found in the sequence downstream of the *fis* gene (AGTGGT-TTT-ACCACT) (Fig. 1). It was amplified by PCR and ligated into the pET-15b vector (Novagen) (Fig. 2).

Amino acid sequence and sequence comparison of islandisin to other subtilases. The deduced amino acid sequence comprises 701 amino acids (aa) with a calculated molecular mass of 76.1 kDa. Islandisin contained four Cys residues, one of which was found in the putative signal sequence of the enzyme. A search for homology in protein databases showed that islandisin is 41% and 39% identical to feravidolysin from *Feravidobacterium pennivorans* and subtilisin C from *Bacillus licheniformis*, respectively. Multiple alignments allowed the construction of a phylogenetic tree (Fig. 2). Islandisin is grouped in the cluster of thermitase, together with feravidolysin and aerolysin from the hyperthermophilic archaeon *Pyrobaculum aerophilum* underlining the intermediate position *Thermotogales* have in the tree of life, located between the archaea and the bacteria. The three amino acid residues that form the catalytic triad and which have been found in all subtilases (32) were fully conserved in the islandisin sequence (Asp177, His215, and Ser391) (Fig. 3a). The latter was also involved in formation of the oxyanion hole, together with Thr390 (Fig. 3b).

Signal peptide analysis. A putative signal sequence, composed of 33 aa, was predicted by the program SignalP, version 3.0 (4). The molecular mass of the enzyme could be calculated with 76.1 kDa and differed from the molecular mass of 72 kDa that was estimated by SDS-PAGE analysis (data not shown). This fact supports the hypothesis that the enzyme possesses a signal sequence of 33 aa in length (= 3.7 kDa) revealed by computer analysis, that is cleaved off by a signal peptidase of *E. coli*, reducing the molecular mass to 72.4 kDa.

Recombinant enzyme production and purification. To obtain islandisin in an amount sufficient for biochemical characterizations, plasmid pET-15bFiS was constructed (Fig. 2), in which transcription of the islandisin-encoding gene is initiated by the T7 promoter. Plasmid pET-15b FiS was used to transform *E. coli* BL21(DE3)pLysS. Recombinant islandisin was expressed in *E. coli* BL21(DE3)pLysS after induction with IPTG. After cell lysis by sonication and centrifugation, island-

TABLE 3. Effects of protease inhibitors on recombinant islandisin activity

Protease inhibitor	Proteases inhibited	Final concn (mM)	Remaining protease activity (%)
PMSF	Serine proteases	5	0
		10	0
Pefabloc S ^a	Serine proteases	1	0
		5	0
Iodacetate	Cysteine proteases	1	93.2
		5	113.3
EDTA	Metalloproteases	1	77.2
		5	78.3

^a 4-(2-Aminoethyl)-benzylsulfonyl fluoride; Roche, Mannheim, Germany.

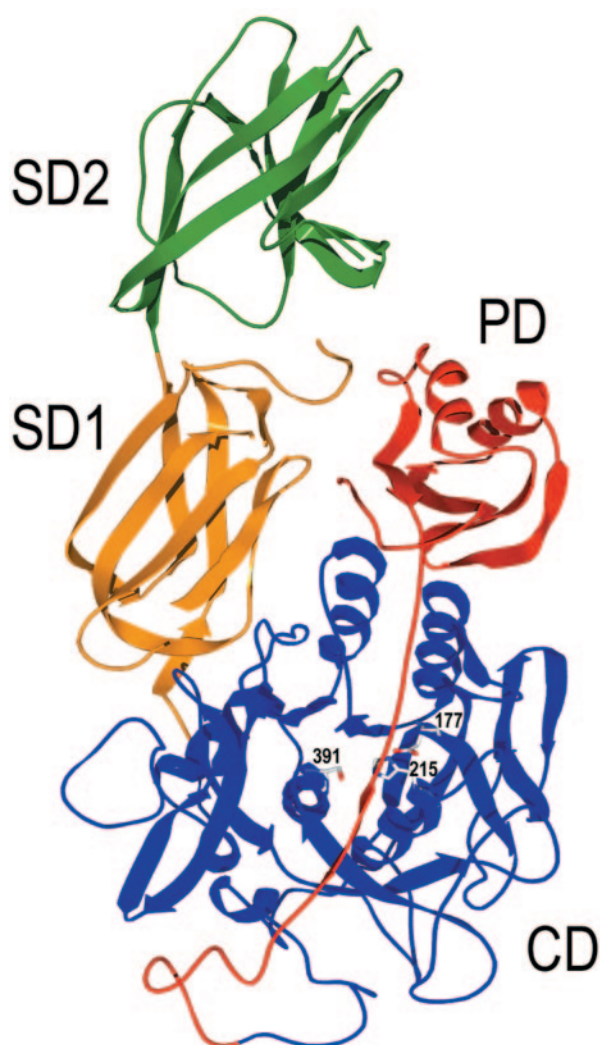


FIG. 6. Ribbon representation of islandisin model showing domain organization of the proenzyme and catalytic residues. Color coding: red, PD; blue, CD; orange, SD1; green, SD2. Putative catalytic residues: Asp177, His215, and Ser391. The figure was prepared by Swiss-PDBviewer 3.7 and rendered with POV-Ray, version 3.1g.

disin was detected in the soluble protein fraction. The recombinant islandisin was purified by two purification steps. In the first step, the crude extract was incubated for 20 min at 80°C to denature the nonthermostable *E. coli* proteins. These proteins were separated by centrifugation. In addition, an increase in enzymatic activity was observed after heat treatment (Table 1). In a second purification step, the recombinant enzyme was purified by hydroxyapatite column chromatography. The proteins were eluted by increasing the ionic strength of the elution buffer. Islandisin was purified 23.9 fold at a yield of 28.6%. The purified enzyme has a specific activity of 2.3 U/mg (Table 1). The protein fractions of the purification steps were analyzed by SDS-PAGE (Fig. 4).

Biochemical properties of islandisin. The enzyme activity of the recombinant protein was investigated by activity staining, showing a bright clearing zone after incubation of the gel at 80°C for 45 min (Fig. 4). Islandisin was active at a broad range

of temperatures (60 to 80°C) and pH values (pH 6 to 8.5). Maximal activity was detected at 80°C and pH 8.0 (Fig. 5 a and b). The temperature stability of the protein was examined by measuring residual activities after incubation of the pure enzyme at high temperatures (80, 90, and 100°C) (Fig. 5c). At 80°C, there was no loss of activity detectable over a period of 32 h, indicating the enzyme to be highly thermostable. The half-life was about 4 h at 90°C and 1.5 h at 100°C. Enzyme activity increased to 140% after incubation of the enzyme at 80°C for 30 min, showing that the recombinant protein is activated by heat treatment.

Influence of metal ions, protease inhibitors, and detergents on the activity of the recombinant islandisin. Of all metal ions tested, only selenium caused an increase in activity to 266%. All other metal ions tested lowered the enzymatic activity (Table 2). In the presence of EDTA, an inhibitor of metalloproteases, a 20% loss of enzyme activity was observed (Table 3). As expected from the amino acid sequence, the addition of PMSF and Pefabloc SC, two inhibitors of serine proteases, resulted in a complete loss of activity. No influence on enzyme activity was observed in the presence of iodacetate, an inhibitor of cysteine proteases. Several detergents, reducing agents, and solvents did not have a significant influence on the stability of recombinant islandisin (Table 4). Almost 100% of activity was detected in the presence of Triton X-100, ethanol, isopropanol, or β -mercaptoethanol; only a slight decrease of 20 to 30% of total activity was measured in the presence of dimethylformamide and dimethyl sulfoxide. The enzyme was activated by 0.5% (wt/vol) SDS, causing an increase of activity of up to 340%. Acetonitrile, urea, and DTT in final concentrations of 20% (vol/vol), 6 M, and 0.1% (wt/vol), respectively, caused a loss of 50% at 80°C.

Structural analysis of the islandisin model. A structural model for islandisin was constructed based on the 1.7-Å crystal structure of fervidolysin (17). The islandisin model shows a four-domain organization: a propeptide (PD) and a catalytic domain (CD), followed by two β -sandwich domains (SD1 and SD2) (Fig. 6). The propeptide, amino acid residues 1 to 135, forms an $\alpha\beta$ mixed globular structure with a C-terminal extension which runs through the active site of the CD (residues 136 to 450). At this point, it starts to form a short β -strand, possibly preventing autoprocessing of the PD at this site. Like other subtilases, the main CD has a rather conserved architecture. Asp177, His215, and Ser391 form a fully conserved catalytic triad, while backbone nitrogens of Thr390 and active-site nucleophile Ser391 make up an oxyanion hole required in stabilizing negative charges of the substrate during catalytic events. A conserved calcium binding site is present, consisting of residues Glu144, Asp186, Lys226, Asp228, and Lys230, at the bottom of the CD. Both C-terminal β -sandwich domains, comprising residues 451 to 556 and 557 to 668 for SD1 and SD2, respectively, have only been observed so far in subtilases of the *Fervidobacterium* lineage. Only islandisin SD1 could not be modeled in one chain, since it is different from fervidolysin in loops towards the CD, PD, and SD2.

DISCUSSION

A gene encoding a thermostable subtilisin-like serine protease, designated islandisin, from *Fervidobacterium islandicum*

TABLE 4. Effects of detergents, solvents, and denaturing agents on recombinant islandisin activity

Reagent ^a	Reagent group	Final concn	Relative protease activity (%)
Control			100
SDS	Detergent	0.5% (wt/vol)	342.9
Triton X-100	Detergent	0.5% (vol/vol)	92.1
Ethanol	Solvent	2% (vol/vol)	94.7
Isopropanol	Solvent	2% (vol/vol)	100.3
DMF	Solvent	2% (vol/vol)	75.3
DMSO	Solvent	1% (vol/vol)	86.4
		5% (vol/vol)	64.7
Acetonitrile	Solvent	10% (vol/vol)	64.9
		20% (vol/vol)	51.5
Urea	Denaturing agent	2 M (12% [wt/vol])	50.9
		6 M (36% [wt/vol])	63.1
DTT	Reducing agent	0.05% (wt/vol)	88.1
		0.1% (wt/vol)	61.6
β -Mercaptoethanol	Reducing agent	0.1% (vol/vol)	123.2
		0.5% (vol/vol)	113.1

^a DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

was cloned and expressed as an active enzyme in *E. coli*. The gene sequence showed high homology to those of the subtilisin family, the largest class of serine-type proteases. In the sequenced region, there is only one open reading frame encoding a protein 701 amino acids in length with a molecular mass of 76.1 kDa. Downstream of the Shine-Dalgarno sequence, the sequence started with ATG as translational initiation codon.

Computer analysis with the algorithm SignalP, version 3.0, suggests that the recombinant islandisin possesses a signal peptide with a length of 33 aa and 3.7 kDa. This was supported by molecular mass analysis. Subtilisin-like enzymes such as the subtilisins from *Bacillus* species (15, 34), aqualysin I from *Thermus aquaticus* (33), and proteinase K from *Tritirachium album* (10) are known to possess prosequences that play an important role as intramolecular chaperones for activating the enzyme (31). The prosequence from these enzymes is removed from the catalytic domain by autoprocessing or by another protease. The removal of this prosequence from the catalytic domain is necessary to generate active subtilisin molecules, because the uncleaved prosequence interacts with the active site of the catalytic domain and thereby inhibits its activity, although the

inhibitory and chaperone functions of the prosequence are not necessary (32). Due to the absence of conserved regions in the prosequence in islandisin, which can be found within many other subtilisin-like enzymes, and due to the enzymatic activity found by zymogram analysis, which correlated with a protein with a molecular mass of 72 kDa, it is assumed that the recombinant islandisin is synthesized as a proenzyme that is secreted into the periplasmic space of *E. coli*.

However, there is one subtilisin-like enzyme from the hyperthermophilic archaeon *Thermococcus kodakaraensis*, actively expressed as a recombinant protein in *E. coli*, which seems to possess a prosequence that is not cleaved off. In this case, it is assumed that the prosequence does not function as an intramolecular chaperone (16).

The recombinant enzyme was purified by two purification steps. In the first step, the crude extract was incubated for 20 min at 80°C to denature nonthermostable *E. coli* proteins that could be separated by centrifugation. The enzyme activity of islandisin increased after heat treatment, possibly indicating that the propeptide is released from the active site. However, SDS-PAGE analysis revealed that the prosequence is not cleaved off by autoprocessing and remains attached to active islandisin. The recombinant fervidolysin, on the other hand, did show autoprocessing but failed to become active, perhaps due to tight attachment of the propeptide (18). In a second purification step, the recombinant enzyme was purified by hydroxyapatite column chromatography. The recombinant enzyme was purified 23 fold at a yield of 28% recovery. The purified enzyme showed specific activity of 2.3 U/mg and optimal proteolytic activity at 80°C and pH 8.0. The enzyme was highly thermostable, showing no loss of activity over 32 h at 80°C. A decrease of activity was observed after the enzyme was incubated at 100°C (half-life, 1.5 h). These properties make the enzyme an attractive candidate in industrial processes running at elevated temperatures and controlled by varying the temperature.

It is known that thermostability of proteins correlates with an increased number of residues involved in pairs and networks of charge-charge and aromatic-aromatic interactions (36). By comparing the amino acid composition of islandisin to other thermostable subtilisins, it is obvious that the amount of charged and especially acidic amino acids increases with the temperature optimum of enzyme activity. The enzymes subtilisin BPN' from *Bacillus amyloliquefaciens* and thermitase from *Thermoactinomyces vulgaris*, with an activity optimum at a temperature of 60°C, are composed of 5% acidic amino acids. The proportion of acidic amino acids is much higher in islandisin (10.1%) as well as in stetterlysin from *Thermococcus stetteri* (T_{opt} , 85°C) and pyrolysin from *Pyrococcus furiosus* (T_{opt} , 115°C) (up to 12%). As shown in Table 5 the proportion of aromatic amino acids also increases with increasing temperature optima of the enzymes. The finding of a calcium binding site indicates that the enzyme is stabilized by calcium ions. However, at millimolar concentrations, calcium ions displayed an inhibitory effect. Similar effects have been observed for other enzymes such as the α -amylase from *Pyrococcus woesei* (23).

The subtilisin-like serine proteases are grouped into the six families: proteinase K, thermitase, subtilisin, pyrolysin, kexin, and antibiotic peptidase (32). Sequence alignments to other

TABLE 5. Percent amino acid composition of islandisin in comparison with those of several subtilisin-like proteases^a

Amino acid ^b	Protease (T_{opt})				
	Subtilisin BPN' (60°C)	Thermitase (60°C)	Islandisin (80°C)	Stetterlysin (85°C)	Pyrolysin (115°C)
Charged	12.4	12.1	19.8	17.0	17.7
Acidic	5.5	5.4	10.1	11.9	11.8
Aromatic	5.8	8.7	9.9	12.5	13.8
Alkaliphilic	4.7	5.4	8.7	3.3	4.3
Polar	28.4	31.8	25.6	21.5	10.6
Hydrophobic	36.7	35.1	36.6	35.5	35.8

^a Islandisin, *Fervidobacterium islandicum*; subtilisin BPN', *Bacillus amyloliquefaciens*; stetterlysin, *Thermococcus stetteri*; pyrolysin, *Pyrococcus furiosus*; thermitase, *Thermoactinomyces vulgaris*. Data are taken from Voorhorst et al. (36).

^b Charged, RKHDE; acidic, DE; alkaliphilic, KR; polar, NCYST; hydrophobic, AILFWV; aromatic, FWY.

subtilases indicated that islandisin is a member of the subtilase family. Because of the high amino acid identities to fervidolysin (44%), to aerolysin (33%), and to thermitase (29%), the islandisin was clustered in the thermitase group. Within this group, only fervidolysin and islandisin have been found to contain β -sandwich domains. These domains are speculated to be involved in the degradation of β -keratin, the main component of native feathers (17). Both *Fervidobacterium pennivorans* and *F. islandicum* AW-1 have been shown to degrade feathers while producing keratinases (9, 24). The disassembly of highly regular layers of β -structures in β -keratin might be essential for the protease to act, providing the bacteria with a supply of peptide nutrients. Future protein engineering studies may clarify the exact role of these unique domains.

In conclusion, a novel thermostable protease from *F. islandicum* was discovered. As the first representative of *Thermotogales*, the enzyme was cloned and successfully expressed as an active enzyme in *E. coli*. Maximal proteolytic activity was determined at 80°C and pH 8.0, showing a high thermostability. Within the class of subtilisins (clan SB and family S8) the enzyme, designated islandisin, was clustered in the thermitase group.

ACKNOWLEDGMENT

This work was supported by the DBU (Deutsche Bundesstiftung Umwelt, German Federal Environmental Foundation).

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