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Biochemical Properties of a Putative Signal Peptide Peptidase from the Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1

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We have performed the first biochemical characterization of a putative archaeal signal peptide peptidase ($SppA_{Tk}$) from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. $SppA_{Tk}$, comprised of 334 residues, was much smaller than its counterpart from *Escherichia coli* (618 residues) and harbored a single predicted transmembrane domain near its N terminus. A truncated mutant protein without the N-terminal 54 amino acid residues ($\Delta N54SppA_{Tk}$) was found to be stable against autoproteolysis and was examined further. $\Delta N54SppA_{Tk}$ exhibited peptidase activity towards fluorogenic peptide substrates and was found to be highly thermostable. Moreover, the enzyme displayed a remarkable stability and preference for alkaline pH, with optimal activity detected at pH 10. $\Delta N54SppA_{Tk}$ displayed a K_m of 240 ± 18 μM and a V_{max} of 27.8 ± 0.7 μmol min^{-1} mg^{-1} towards Ala-Ala-Phe-4-methyl-coumaryl-7-amide at 80°C and pH 10. The substrate specificity of the enzyme was examined in detail with a FRETS peptide library. By analyzing the cleavage products with liquid chromatography-mass spectrometry, $\Delta N54SppA_{Tk}$ was found to efficiently cleave peptides with a relatively small side chain at the P-1 position and a hydrophobic or aromatic residue at the P-3 position. The positively charged Arg residue was preferred at the P-4 position, while substrates with negatively charged residues at the P-2, P-3, or P-4 position were not cleaved. When predicted signal sequences from the *T. kodakaraensis* genome sequence were examined, we found that the substrate specificity of $\Delta N54SppA_{Tk}$ was in good agreement with its presumed role as a signal peptide peptidase in this archaeon.

In the domain *Archaea*, research on protein secretion and the machinery involved are still at an early stage (32). Sequence comparison of genome data have shed light on the features of the archaeal signal peptide (5) and have also indicated the presence or absence of individual components corresponding to eukaryotic or bacterial factors participating in protein secretion (12). Factors that have been analyzed in detail include those involved in flagellum formation in methanogens (8), the signal recognition particle of *Archaeoglobus fulgidus* (9) and *Sulfolobus solfataricus* (33), the Sec complex of *Methanocaldococcus jannaschii* (38), and the secretion ATPase of *Sulfolobus* spp. (1).

Signal peptide peptidases are enzymes considered to cleave the signal peptide chains of secreted proteins after they are removed from the precursor proteins by signal peptidases (15, 28). Eukaryotic signal peptide peptidases are intramembrane enzymes, with activity dependent on two aspartate residues (21, 39). They have become a center of attention in mammalian cells due to their involvement in immune surveillance. After signal peptide peptidase cleaves signal peptides of the major histocompatibility complex I molecules, the peptide products are presented on the cell surface by a nonclassical major histocompatibility complex class I molecule, HLA-E, indicating to natural killer cells that major histocompatibility complex synthesis is proceeding normally (11, 20).

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The bacterial signal peptide peptidase was initially identified in *Escherichia coli* as a cytoplasmic membrane protein named protease IV (15, 16, 27). The enzyme, encoded by the *sppA* gene (17, 34), was found to cleave the signal peptide of outer membrane lipoprotein after its release from the precursor protein. Further studies have indicated that protease IV (SppA) carries out only the initial breakdown of the signal peptide into smaller peptide fragments, followed by complete digestion through the functions of cytoplasmic peptidases including oligopeptidase A (25, 26). The gram-positive counterpart of SppA in *Bacillus subtilis* has also been studied, and has been shown to be involved in signal peptide degradation (10). Furthermore, a cytosolic peptidase, TepA, structurally related to both SppA and ClpP has also been found to actively participate in the degradation of signal peptides in this organism (10).

In terms of signal peptidases and signal peptide peptidases from the *Archaea*, the type I signal peptidase gene from *Methanococcus voltae* has been cloned and its product characterized, confirming that the protein exhibits signal peptidase activity (24). Residues critical for the peptidase activity of the protein have been determined (7). FlaK, the signal peptidase for preflagellin signal cleavage, has also been characterized from this organism and has been demonstrated to be an aspartic protease essential for preflagellin cleavage (6). In the Crenarchaeota, the homologue of bacterial type IV prepilin peptidases from *S. solfataricus* (PibD) has been characterized, and residues on the substrate that are important for recognition by PibD have been examined (2). In contrast to the progress on signal peptidases, experimental examinations of archaeal signal peptide peptidases have not been reported.

Thermococcus kodakaraensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara on Kodakara Island, Ka-

goshima, Japan (4, 23). The strain is an obligate anaerobe and grows optimally at 85°C. Only heterotrophic growth has been observed, and the strain can efficiently utilize and/or degrade amino acids, pyruvate, tryptone, chitin, and starch. The complete genome sequence of *T. kodakaraensis* has recently been determined and annotated (13). As expected from the growth characteristics of this strain, the genome sequence revealed the presence of a large number of extracellular enzymes, including chitinase (36), α-amylase (35), and subtilisin-like protease (19). An orthologue search also revealed that *T. kodakaraensis* harbors a set of factors involved in protein secretion equivalent to those found in various hyperthermophilic archaea (see the Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/).

In this study, we have examined the enzymatic properties of a putative signal peptide peptidase from *T. kodakaraensis*, revealing that the substrate specificity of the enzyme is consistent with its presumed role as a signal peptide peptidase in this archaeon.

MATERIALS AND METHODS

Strains, media, and plasmids. *T. kodakaraensis* KOD1 was cultivated as described elsewhere (4) in order to isolate genomic DNA (29). *E. coli* DH5α and plasmid pUC18 were used for gene cloning, sequencing, and DNA manipulation. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) and pET21a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium (10 g liter^{-1} of tryptone, 5 g liter^{-1} of yeast extract, and 10 g liter^{-1} of NaCl) with 100 μg ml^{-1} ampicillin at 37°C.

DNA manipulation and sequence analysis. Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan). Plasmid DNA was isolated with the Plasmid mini-kit from QIAGEN (Hilden, Germany). KOD Plus (Toyobo) was used as a polymerase for PCR, and a GFX PCR DNA and gel band purification kit (Amersham Biosciences, Buckinghamshire, United Kingdom) was used to recover DNA fragments from agarose gels after electrophoresis. DNA sequencing was performed using BigDye terminator cycle sequencing kit v.3.0 and a model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA). Sequence alignments and construction of the phylogenetic tree with the neighbor-joining method were performed with the ClustalW program available at the DNA Data Bank of Japan. Bootstrap resampling was performed 1,000 times with the BSTRAP program.

Expression of the *sppA$_{Tk}$* gene in *E. coli*. The *sppA$_{Tk}$* gene initiating with a Met residue preceding Gln30, omitting the transmembrane domain, was amplified from the genomic DNA of *T. kodakaraensis* using the primer set sppN1 and sppC1 (sppN1, 5′-GTTCTCCATATGCAGGTCAATCCCCCCGCTGT-3′; sppC1, 5′-CAGAATTCAACCACCCCCAATGAGGG-3′). The gene for the truncated protein initiating with a Met residue preceding Cys55 was amplified with sppN2 and sppC1 (sppN2, 5′-ACTTTACGCATATGTGTGAAGGCAGTGTTAAC-3′). After confirming the sequences of the DNA fragments, they were inserted into pET21a(+) at the NdeI and EcoRI sites. After introduction into *E. coli* BL21-CodonPlus(DE3)-RIL cells, gene expression was induced with 0.1 mM isopropylthiogalactopyranoside (IPTG) at the mid-exponential growth phase with further incubation for 6 h at 37°C.

Purification of recombinant SppA$_{Tk}$. After inducing gene expression, cells were washed with 50 mM Tris-HCl (pH 8) and resuspended in the same buffer. Cells were sonicated on ice, and the supernatant after centrifugation (20,000 × *g*, 30 min at 4°C) was applied to heat treatment at 85°C for 15 min, immediately cooled on ice, and then centrifuged (20,000 × *g*, 30 min at 4°C). The soluble protein sample was brought to 35% saturation with ammonium sulfate and the precipitate which included SppA$_{Tk}$ was dissolved in 50 mM Tris-HCl (pH 8) at a concentration of 1 mg ml^{-1}. This was applied to anion exchange chromatography (ResourceQ, Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 8), 0.2 M NaCl, and proteins were eluted with a linear gradient (0.2 to 1.0 M, 42 ml) of NaCl. After desalting with a HiPrep26/10 column (Amersham Biosciences), the sample was applied to gel filtration chromatography (Superdex 200 HR 10/30, Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 8), 0.15 M NaCl, and the fractions obtained were used for enzyme analysis. Approximately 3 to 7 mg of purified octameric protein was obtained per liter of culture.

Protein analysis of purified recombinant SppA$_{Tk}$. The native molecular mass of the purified protein was examined by gel-filtration chromatography using Superdex 200 HR 10/30 in 50 mM Tris-HCl (pH 8), 0.15 M NaCl. The retention time was calibrated with those of the standard proteins thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa). Protein concentration was determined with the protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Determination of N-terminal amino acid sequences of proteins were performed with a protein sequencer (model 491 cLC, Applied Biosystems) after separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electroblotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA).

Enzyme activity measurements. Most activity measurements were performed with peptidyl-MCA substrates [peptidyl–α-(4-methylcoumaryl-7-amide) substrates] available from Peptide Institute (Osaka, Japan). Release of 7-amino-4-methylcoumarin was monitored consecutively with a fluorescence spectrophotometer capable of maintaining the cuvette at desired temperatures between 30 and 100°C. Excitation and emission wavelengths were 380 nm and 460 nm, respectively. Standard activity measurements were performed at 60°C in a final volume of 1 ml with 0.1 μg of purified protein and Ala-Ala-Phe-MCA (200 μM) in 50 mM CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid; pH 10.0). The final concentration of dimethyl sulfoxide used to dissolve the substrate was constant at 3% of the reaction mixture. Kinetic parameters were calculated with SigmaPlot (SPSS Science, Chicago, IL).

Effects of temperature and pH on enzyme activity. All buffers were prepared so that they would reflect accurate values at the applied temperatures. In examining the effect of temperature, the standard assay method was applied at each temperature. The effect of pH was examined in the presence of 50 mM of morpholineethanesulfonic acid (MES)-NaOH (pH 6.0 to 7.0), HEPES-NaOH (pH 7.0 to 8.0), Bicine-NaOH (pH 8.0 to 9.0), CHES-NaOH (pH 9.0 to 10.0), and CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)-NaOH (pH 10.0 to 12.0), respectively. Thermostability of the protein was analyzed by measuring the residual activity of the protein after incubation at various temperatures in 50 mM CHES-NaOH (pH 10.0). The initial activity of the enzyme incubated at 60°C was designated as 100%. Alkaline stability was analyzed by measuring the residual activity of the protein after incubation at various pH in 50 mM CAPS-NaOH at 60°C. The initial activity of the enzyme incubated in 50 mM CAPS-NaOH (pH 10.0) was designated as 100%. In measuring thermostability and alkaline stability, the protein concentration during incubation was 1 μg ml^{-1}. Residual activities were measured with the standard assay method described above.

Effect of protease inhibitors. The effects of various protease inhibitors at concentrations of 200 μM, 1 mM, or 10 mM were examined at 60°C and pH 10. The substrate Ala-Ala-Phe-MCA was present at a concentration of 200 μM. Activity in the absence of inhibitors was defined as 100%.

Determination of substrate specificity. Substrate specificity was examined with a FRETS peptide library (25Xaa series, Peptide Institute) (37). These peptide substrates harbor a highly fluorescent 2-(*N*-methylamino)benzoyl group linked to the side chain of the amino terminal D-2,3-diaminopropionic acid residue (D-A$_2$pr), along with a 2,4-dinitrophenyl group (quencher) linked to the ε-amino group of a Lys residue. In between the D-A$_2$pr and Lys residue lies the peptide Gly-Zaa-Yaa-Xaa-Ala-Phe-Pro, where Zaa is a mixture of Phe, Ala, Val, Glu, and Arg, Yaa is a mixture of Pro, Tyr, Lys, Ile, and Asp, and the Xaa residue is a defined single amino acid of choice (see below). Excitation and emission wavelengths were 340 nm and 440 nm, respectively.

In the initial assay to examine the preference for residues at the P-1 position, 1 μg of purified enzyme was added to the reaction mixture with a final volume of 1 ml containing 30 μM substrate in 50 mM CHES (pH 10). The final concentration of dimethyl sulfoxide used to dissolve the substrate was constant at 3% of the reaction mixture. A second assay to identify the cleavage sites and the preference towards residues at the P-2 to P-4 positions was performed on selected substrates. Aliquots (100 μl) from the cleavage reactions were taken at various time intervals that corresponded to 15 to 30% cleavage of the substrates, and subjected to liquid chromatography (LC)-mass spectrometry analysis. An ODS A-302 column (YMC, Kyoto, Japan) was used for separation with 0.05% trifluoroacetic acid in H_2O as eluant A and 0.05% trifluoroacetic acid in CH_3CN as eluant B. The gradient was 5–40% of eluant B in A at a flow rate of 1.0 ml min^{-1} over a time span of 55 min. Aliquots taken from the cleavage reactions were injected and the cleaved products were monitored with absorbance at 220 nm, as well as fluorescence intensity in order to identify the N-terminal segments. The structures of the cleaved products were deduced from the theoretical molecular weights.

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Tko    1  MDDRIWKYVSAVLTLLLALSIVSVAVLYYQVNPPAVPANQTGFVIETPSNFTLVCEGSVN   60
Mja    1  ------------------------------------MKKIYIILLILFVILISLIGASI   23
Tac       ------------------------------------------------------------
PaI    1  -----MAKALALAGLVLAIAALALGIALWWGLLNAERTITMKIDDVRSSITAEVKTLNSS   55
Neq       ------------------------------------------------------------
Hba    1  ---------------------------------------------MTNPIDKHAKTLAT   14
Bsp    1  --------------------MNAKRWIALVIALGIFGVSIIVSISMSFFESVKGAQTDLT   40
Eco  244  AANRQIPAEQVFPGAQGLLEGLTKTGGDTAKYALENKLVDALASSAEIEKALTKEFGWSK  303

Tko   61  TTSTEVAELRDQIAFLQRLVSSNQGGTT---IAVVPIFGIIDDYTALQVIPLLRNLAMNE  117
Mja   24  LLVMSLSGENVDLFGGEKIAKVYLCNEIYFDYNQGDGIFPQQKKDARYYINLLDDLEKDD   83
Tac    1  ----------------------------MYILRTRIEGTITQQLYRSYYPIFSFAENKR   31
PaI   56  LTERLASINKTLEELTVRVKRLEKRAAAEREIVIVPVDQPIFDYYVDFLIKYVKKLQFDN  115
Neq    1  MNDIKDIIKRNSWILFLVGIILFSHIYSILTKGNVAVIYIDKPITSDFAEKVIDFLKEAK   60
Hba   15  SWTFIIVVAAIVGGVGGVALQSGSDSGPENSVAVVNIESAITGGTGDAVAKELRSIRGND   74
Bsp   41  SLTDESQEKTLENGSPSSKIAVLEVSGTIQDNGDSSSLLGADGYNHRTFLKNLERAKDDK  100
Eco  304  TDKNYRAISYYDYALKTPADTGDSIGVVFANGAIMDGEETQGNVGGDTTAAQIRDARLDP  363

Tko  118  SVGGVLLWIESPGGVVGPVINIHSEIKKLSLV--KPVVAYSGDIIASGGYYIAVGAQKIV  175
Mja   84  SVKGVLLVVNSPGGEVIASEKLARKVEELAKK--KPVVVYVEGLDASGAYMVSAPADYIV  141
Tac   32  SVAGLVLVFNSGGGDAVASQLMFEMIRKIRKK--KPVYSFIQGICASGAYWISAGSSKIY   89
PaI  116  KTAGVILLINSPGGAVGATERLYSTIKGLN----KTVYAVVAGLAASGAYYTAVAAGRIY  171
Neq   61  DYKAIVLYIDSPGGAPEPTYRIIKYLDRIN----KTKISYIAQYGTSASYWIATHTNKIF  116
Hba   75  SIDAVVLRVSSPGGAVSGSEVQYRAVKRLAQE--KPVVTSVRGPAASGGYYTIAPTDKIY  132
Bsp  101  TVKGIVLKVNSPGGGVYESAEIHKKLEEIKKETKKPIYVSMGSMAASGGYYISTAADKIF  160
Eco  364  KVKAIVLRVNSPGGSVTASEVIRAELAAARAAG-KPVVVSMGGMAASGGYWISTPANYIV  422
                *   * ☆☆                  *        ☆  ☆       *

Tko  176  ASPLAEVGSIGVIYVHYDLEKNYEMNGIKVNVFKTGKHKDMGAEWRDLTPEEREKITEMV  235
Mja  142  AEKHSIVGSIGVRMDLMHYYGLMKKLGINVTTIKAGKYKDIGSPFRPMTKEEKEYLQKMI  201
Tac   90  SLDTSLIGSIGVISMIPYIKPLLDKIGVEMKIYKVGKYKDMLSSYREPSDEENEHYMRVL  149
PaI  172  ATPSSWVGSIGVVALLWPDEYLID---LPDYIYTTGPFKYYGMDLTEFYNDIEKTRANFV  228
Neq  117  ANELSFVGSVGVLIGKIDLSGLLSKLGVKYYSFSKGKYKEFSNPLLPLDNYTKQYYNELA  176
Hba  133  VTPSSLVGSVGVISSVSENNGVPSR-------WKSAPDKGTTGPADKARARAATFRQSFL  185
Bsp  161  ATPETLTGSLGVIMESVNYSKLADKLGISFETIKSGAHKDIMSPSREMTKEEKNIMQSMV  220
Eco  423  ANPSTLTGSIGIFGVITTVENSLDSIGVHTDGVSTSPLADVS-ITRALPPEAQLMMQLSI  481
               ☆* ☆*                           *

Tko  236  NTYFQAFISAVSEGRNMTIDEVKNFSTGETWFAENVTG-ALVDELGGMDTAIDVLEKLMN  294
Mja  202  NETYMDFVKWVAEHRHLSINYTLKIADGKIYSGEDAKKVGLVDEVGTEEDALKKLEQLAN  261
Tac  150  NDVYRKFRDSVMTERNIPEDKMEDIAQGQIFSPSMAMENRLIDGIGTMDSMMDDMYR-QL  208
PaI  229  AAVLKGRAGRLKADP-------EVFETAKIFNAETALRLGLIDKIGGLWDAVEDMARELG  281
Neq  177  DKLYNFFLTDVLEHRDIKKECLSKVKESTIFLGIEAKKCGLIDYIGTMDDVKAYLEKTLK  236
Hba  186  DVVMNERGDDLTVDR-------ETIGRAQIYAGNKAVEIGLADEIGGLDAAIADAADRAS  238
Bsp  221  DNSYEGFVDVISKGRGMPKAEVKKIADGRVYDGRQAKKLNLVDELGFYDDTITAMKKDHK  280
Eco  482  ENGYKRFITLVADARHSTPEQIDKIAQGHVWTGQDAKANGLVDSLGDFDDAVAKAAELAK  541
                     *                          * ☆  *

Tko  295  VSGAKVVVVYKDLETPEEFGVYGSTALYIDPRYLTPLIGGG--------------------  334
Mja  262  VSNPEIVEYGLEENKGLFGLTY-----YLGYGIGKGIGEVLYGMEKINGRVELLS-----  311
Tac  209  GRKYKTRDILPRRPWIMRFLGT--------------------------------------  230
PaI  282  LKNYTVVDIYEKYNATGFGIVVPLISGNKIPLQFLMNVSAPPVFYLWPGAIQIPHHPINV  341
Neq  237  IKVKFEPFREKALFYLSFPI----------------------------------------  256
Hba  239  VQNYGVVYRSPGGLGGLLSIIGGNSDTGAANAVSASEFCTNEYLAYAPQAGPDLEVIQNA  298
Bsp  281  DLKNASVISYEESFGLGSLFSMGANKMFKSEIDFLNMREILSQSGSPRMMYLYAK-----  335
Eco  542  VKQWHLEYYVDEPTFFDKVMDNMSGSVRAMLPDAFQAMLPAPLASVASTVKSESDKLAAF  601
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FIG. 1. Sequence alignment of putative signal peptide peptidase proteins from *E. coli*, *B. subtilis*, and representative archaeal strains. The 19 archaeal SppA sequences described in Fig. 2 were aligned with the sequences of SppA from *E. coli* and *B. subtilis*. After alignment, representative sequences were selected. Asterisks indicate highly conserved residues that were present in at least 18 of the 21 sequences aligned. Among these, residues that were conserved in all sequences examined are indicated by stars. The bar above the alignment indicates the putative transmembrane domain of signal peptide peptidase from *T. kodakaraensis*. Arrowheads indicate the residues that immediately follow the artificial Met residue incorporated in ΔN29SppA$_{Tk}$ and ΔN54SppA$_{Tk}$. Tko, *T. kodakaraensis*; Mja, *Methanocaldococcus jannaschii*; Tac, *Thermoplasma acidophilum*; PaI, *Pyrobaculum aerophilum* I; Neq, *Nanoarchaeum equitans*; Hba, *Halobacterium* sp. strain NRC-1; Bsp, *B. subtilis*; Eco, *E. coli*. Due to their lengths, not all residues are shown for *Pyrobaculum aerophilum* I (608 residues), *Halobacterium* sp. strain NRC-1 (300 residues), and *E. coli* (618 residues).

RESULTS

Putative signal peptide peptidase gene on the genome of *T. kodakaraensis*. Using the primary structure of the signal peptide peptidase from *E. coli* (SppA$_{Ec}$), we performed a BLAST search against the protein sequences of *T. kodakaraensis*. Although significantly smaller in size than SppA$_{Ec}$ (618 amino acid residues), one open reading frame, encoding TK1164 (334 residues), displayed significant similarity (27% identical) to SppA$_{Ec}$, and was therefore designated *sppA$_{Tk}$* (Fig. 1). The deduced molecular mass of the protein was 36,211 Da. A BLAST search against the complete genome sequences of various archaeal strains was performed with the SppA$_{Ec}$ and SppA$_{Tk}$ sequences. Similar orthologues were found in many genera of the *Euryarchaeota*, including *Picrophilus*, *Pyrococcus*,

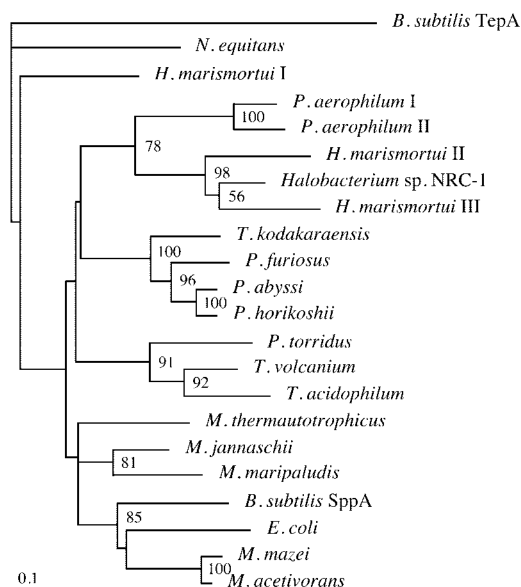

FIG. 2. Phylogenetic tree of putative archaeal SppA sequences. SppA sequences from archaea that displayed high similarity to $SppA_{Ec}$ and $SppA_{Tk}$ were analyzed along with the sequences of $SppA_{Ec}$ and the SppA and TepA from *B. subtilis*. The proteins used (accession numbers) were *B. subtilis* SppA (CAB14931) and TepA (CAB13552), *E. coli* (BAA15557), *Haloarcula marismortui* I (AAV45638), II (AAV46904), and III (AAV47811), *Halobacterium* sp. strain NRC-1 (AAG19125), *Methanocaldococcus jannaschii* (AAB98642), *Methanococcus maripaludis* (CAF30625), *Methanosarcina acetivorans* (AAM07395), *Methanosarcina mazei* (AAM30562), *Methanothermobacter thermautotrophicus* (AAB85306), *Nanoarchaeum equitans* (AAR39164), *Picrophilus torridus* (AAT42796), *Pyrobaculum aerophilum* I (AAL65089) and II (AAL64441), *Pyrococcus abyssi* (CAB49512), *Pyrococcus furiosus* (AAL81707), *Pyrococcus horikoshii* (BAA30681), *Thermococcus kodakaraensis* (BAD85353), *Thermoplasma acidophilum* (CAC11222), and *Thermoplasma volcanium* (BAB59171). Only bootstrap values above 50 are indicated.

FIG. 3. SDS-PAGE analysis of purified $\Delta N29SppA_{Tk}$ (lane 1) and $\Delta N54SppA_{Tk}$ (lane 2). Solid dots to the right of lane 1 indicate the purified $\Delta N29SppA_{Tk}$ and the major degradation products.

and *Thermoplasma*, as well as the methanogens and the haloarchaea. We also found orthologues in *Nanoarchaeum* and the crenarchaeon *Pyrobaculum*.

A phylogenetic analysis of these sequences along with several selected bacterial sequences is shown in Fig. 2. Although the catalytic residues have not been experimentally verified, effects of various inhibitors have suggested that $SppA_{Ec}$ is a serine protease (16). We indeed observed multiple serine residues that were highly conserved among the archaeal and bacterial SppA sequences (Fig. 1). As in the case of $SppA_{Ec}$, $SppA_{Tk}$ is structurally categorized in the S49 family of the SK clan of serine proteases (MEROPS, the peptidase database, http://merops.sanger.ac.uk/) (31). Another common feature was that both proteins harbored a putative transmembrane region(s) near their N termini. Based on these similar features, we set out to express the $sppA_{Tk}$ gene and examine the enzymatic properties of the recombinant protein.

Expression of the $sppA_{Tk}$ gene in *E. coli* and purification of the recombinant protein. The putative transmembrane domain of $SppA_{Tk}$ corresponds to residues Lys7 to Tyr29 (Fig. 1). In order to characterize the catalytic domain of the protein, we omitted this region when constructing the expression vector. An artificial Met residue was incorporated in the place of Tyr29, and this gene was expressed in *E. coli*. As expected, we obtained a soluble protein ($\Delta N29SppA_{Tk}$) which was resistant to heat treatment at 85°C for 15 min. We found that the thermostable protein exhibited peptidase activity towards peptide substrates such as Ala-Ala-Phe-MCA. The recombinant protein was purified with ammonium sulfate fractionation, anion exchange chromatography, and gel filtration chromatography. During these procedures, we observed a gradual decrease in the molecular weight of the protein as judged by SDS-PAGE, leading to three major molecular species (Fig. 3).

We determined the N-terminal amino acid sequences of each species and found that in the smaller proteins, degradation at the N-terminal region had occurred, probably due to autoproteolysis. As the smallest species harbored the Cys55 at its extreme N terminus, we reconstructed an expression plasmid so that translation initiated at a Met residue adjacent to Cys55. The protein produced ($\Delta N54SppA_{Tk}$) was purified with the methods mentioned above (Fig. 3), and was found to be relatively stable in terms of proteolytic degradation. This protein, $\Delta N54SppA_{Tk}$, was therefore used for further biochemical examination. Although not shown in the results described below, $\Delta N29SppA_{Tk}$ and $\Delta N54SppA_{Tk}$ displayed similar tendencies in terms of specific activity, pH dependency, and substrate preference using the FRETS peptide library.

Oligomeric form of $\Delta N54SppA_{Tk}$. The molecular mass of the purified $\Delta N54SppA_{Tk}$ using gel filtration chromatography was estimated to be slightly larger than that of catalase (232 kDa). The molecular mass of a single subunit of $\Delta N54SppA_{Tk}$

is 30,421 Da, suggesting that the protein formed an octamer. Besides this major peak, we observed a second peak at 460 to 500 kDa. The octameric form of the protein was used for further examination. It should be noted that the results obtained here are those of a truncated protein, and may not accurately reflect the oligomeric state of the native protein, which can be assumed to be associated with the membrane. It has previously been reported that the oligomeric form of the native $SppA_{Ec}$ was suggested to be tetrameric through cross-linking experiments, while the results of native PAGE raised the possibility of an even higher oligomeric form (17).

Optimal pH and temperature. The effects of pH and temperature on the activity of $\Delta N54SppA_{Tk}$ were examined with the substrate Ala-Ala-Phe-MCA. As shown in Fig. 4A, the protein exhibited maximal activity at an unexpectedly high pH range of 10 to 10.5. High levels of activity were maintained at even higher pH values of 11.5 (58%) and 12 (50%). The effects of temperature on activity were analyzed with the same substrate at pH 10. Maximum activity under the conditions examined was observed at approximately 80°C (Fig. 4B). The Arrhenius plot gave a constant slope from 30°C to 60°C, and the activation energy of the reaction was calculated to be 54 kJ mol^{-1} (Fig. 4C). The thermostability of the enzyme was examined for 72 h at pH 10 (Fig. 4D), and alkaline stability was measured at 60°C for 48 h (Fig. 4E). In terms of temperature, we observed extremely high stability at 60 and 70°C, with over 60% of the initial activity remaining after 72 h. At 80°C, we detected a relatively greater decrease in activity at the initial phases of incubation, which was consistently reproduced in multiple experiments. However after 30 min, the enzyme seemed to stabilize and follow the usual deactivation kinetics, where the deactivation rate ($-dN_E/dt$) is proportional to the amount of enzyme (N_E), or $-dN_E/dt = kN_E$. We still observed over 30% residual activity after 48 h at 80°C and pH 10. $\Delta N54SppA_{Tk}$ also exhibited high alkaline stability. Nearly 50% residual activity was observed after 24 h at pH 11.5 and 60°C, and the calculated half-life at pH 12 was approximately 10 h.

Effects of various inhibitors. The effects of various protease inhibitors were examined at 60°C and pH 10 (Fig. 5). Leupeptin, chymostatin, and antipain exhibited relatively strong inhibition, leading to an 80% or higher decrease in activity at 200 μM. Diisopropyl fluorophosphate, which specifically reacts with serine residues, also strongly inhibited the activity of $SppA_{Tk}$ at 1 mM. In contrast, addition of EDTA, a typical inhibitor of metalloproteases, and pepstatin, an inhibitor of aspartate proteases, did not result in significant decreases in activity. Although the effects of phenylmethylsulfonyl fluoride were lower than expected, the results as a whole agree with the assumption that $SppA_{Tk}$ is a serine protease.

Preference of $\Delta N54SppA_{Tk}$ for various peptidyl-MCA substrates. We examined the activity of $\Delta N54SppA_{Tk}$ on various peptidyl-MCA substrates shown in Table 1. At pH 10, we found that Ala-Ala-Phe-MCA was by far the preferred substrate, followed by moderate activities towards *N*-glutaryl (Glt)-Ala-Ala-Phe-MCA and *N*-benzyloxycarbonyl (Z)-Val-Lys-Met-MCA. At pH 8, the preference became stricter, with Ala-Ala-Phe-MCA and Glt-Ala-Ala-Phe-MCA the only substrates leading to significant cleavage.

Kinetic analysis. A kinetic analysis of the reaction catalyzed by $\Delta N54SppA_{Tk}$ was carried out at 80°C and pH 10 with the substrate Ala-Ala-Phe-MCA. The reaction followed Michaelis-Menten kinetics with a K_m of 240 ± 18 μM and a V_{max} of 27.8 ± 0.7 μmol min^{-1} mg $protein^{-1}$. The k_{cat} of the enzyme was calculated to be 14.1 s^{-1}.

Examining the substrate preference of $\Delta N54SppA_{Tk}$ with a FRETS peptide library. We next evaluated the peptidase activity of $\Delta N54SppA_{Tk}$ against a FRETS peptide library described in Materials and Methods. This analysis provides detailed information on the preference of a peptidase towards substrate residues at the P-1, P-2, P-3, and, in some cases, the P-4 position. An initial analysis was carried out with 19 substrates corresponding to all amino acids at the Xaa site with the exception of cysteine (Fig. 6A). We detected a preference of $\Delta N54SppA_{Tk}$ for substrates with rather small residues (Gly, Ser, Ala, and Thr) at the Xaa position (Fig. 6B). Cleavage rates of substrates with charged or aromatic residues were low.

We next selected four substrates with high cleavage rates (Xaa = Gly, Ser, Ala, and Val), and subjected the cleaved products to LC-mass spectrometry analysis. In order to identify products generated from the initial cleavage reaction of the substrate, reactions were stopped at various intervals corresponding to 15 to 30% substrate cleavage. The most abundant cleavage products for each of the four substrates are shown in Fig. 6C. In the case of Xaa = Gly, we found that cleavage predominantly occurred at the C-terminal amide bond of Ala, and not the expected Gly residue. In this case, we can obtain insight on the preferred residues at the P-3 and P-4 sites. At the P-3 site, Ile led to the highest levels of cleavage, followed by Tyr and Pro. Products with Asp at the P-3 position could not be found. Concerning the P-4 position, we found a high preference for Arg. We also detected products with Phe or Val at the P-4 site. As in the case of the P-3 residue, we could not find products with the negatively charged Glu residue at the P-4 site. Among the products that were actually cleaved at the Gly residue, Tyr and Lys were found at the P-2 site, and Phe and Val at the P-3 site.

With Xaa = Ala, Ser, or Val, we found a common tendency with the results described above for the Xaa = Gly substrate. In all three cases, the most preferred P-3 residue was Ile or Phe, followed by Val, while cleavage products with negatively charged residues at this site were not found. A preference for the positively charged Arg at the P-4 site was also observed for all three substrates. The results also indicated a broad substrate specificity of $\Delta N54SppA_{Tk}$ towards the P-2 residue.

DISCUSSION

We have described the biochemical properties of $\Delta N54SppA_{Tk}$, a truncated form of the putative signal peptide peptidase from *T. kodakaraensis*. One remarkable feature of the enzyme was its high activity at high alkaline pH. Alkaliphilic proteases have attracted much attention due to their high demand in application, particularly in the detergent industry (3, 14, 18, 22). A number of enzymes are commercially available, including Savinase, subtilisin Carlsberg, and subtilisin BPN′. Various strategies in the protein engineering field have been applied to alkaliphilic proteases with the aim to improve their (thermo)stability, and this has led to significant improvements in terms of catalytic efficiency and stability (14). However, as $\Delta N54SppA_{Tk}$ was obtained from a hyperthermophile,

FIG. 4. (A) Effect of pH on the activity of $\Delta N54SppA_{Tk}$. Measurements were performed at 60°C in the following buffers at a concentration of 50 mM: MES-NaOH (open triangles), HEPES-NaOH (solid squares), Bicine-NaOH (open squares), CHES-NaOH (solid circles), and CAPS-NaOH (open circles). (B) Effect of temperature on the activity of $\Delta N54SppA_{Tk}$. Reactions were carried out in 50 mM CHES-NaOH (pH 10.0). (C) Arrhenius plot of B, indicating the activation energy of substrate hydrolysis catalyzed by $\Delta N54SppA_{Tk}$. (D) Thermostability of $\Delta N54SppA_{Tk}$ at various temperatures. Incubation of the enzyme was carried out in 50 mM CHES-NaOH (pH 10.0). Symbols: 60°C, open circles; 70°C, solid circles; 80°C, open squares; 90°C, solid squares. (E) Stability of $\Delta N54SppA_{Tk}$ at various pHs. Enzyme incubation was carried out at 60°C in 50 mM CAPS-NaOH (pH 10.0, open circles; pH 10.5, solid circles; pH 11.0, open squares; pH 11.5, solid squares; pH 12.0, open triangles). All activity measurements (A to E) were carried out with 200 μM Ala-Ala-Phe-MCA.

the stability of the enzyme can be regarded as exceptionally high in comparison with previously known alkaline proteases/peptidases. We have detected over 50% residual activity after incubation at 70°C and pH 10 for 3 days.

As the enzyme exhibits intrinsic thermophilic and alkaliphilic properties, $\Delta N54SppA_{Tk}$ should be an attractive target for future protein engineering focusing on the modification of its substrate specificity. We have examined whether $\Delta N54SppA_{Tk}$

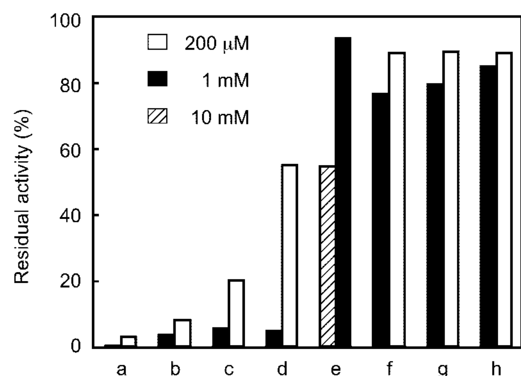

FIG. 5. Effect of various protease inhibitors on the activity of ΔN54SppA$_{Tk}$. The inhibitors examined were leupeptin (a), antipain (b), chymostatin (c), diisopropyl fluorophosphate (d), phenylmethylsulfonyl fluoride (e), pepstatin (f), elastatinal (g), and EDTA (h). Activity measurements were performed at 60°C with the indicated inhibitor concentrations.

exhibits hydrolase activity towards proteins at 60°C and pH 10. Using bovine serum albumin, ovalbumin, α-casein, hemoglobin, and lysozyme, we found that protease activity of ΔN54SppA$_{Tk}$ was dependent on the particular protein substrate. Degradation of bovine serum albumin and ovalbumin was not observed, while α-casein, hemoglobin, and lysozyme were degraded to various extents (data not shown).

SppA$_{Tk}$ is structurally categorized in the S49 family of serine proteases, a prokaryotic family of proteases of which still little is understood. There are no three-dimensional structures available, and moreover, the catalytic mechanism of signal peptide peptidases and the residues involved have not yet been experimentally determined. Information on the enzymatic properties of previously identified signal peptide peptidases is also limited. The specific activity of SppA$_{Ec}$ has been measured in several studies and ranges between 0.733 μmol mg^{-1} min^{-1} at 25°C against Z-valine *p*-nitrophenyl ester (27) and 13.6 μmol mg^{-1} min^{-1} at 37°C against Z-valine β-naphthyl ester (17).

TABLE 1. Activity of ΔN54SppA$_{Tk}$ towards various peptidyl-MCA substrates[a]

Substrate[b]	Sp act (μmol mg protein^{-1} min^{-1})	
	pH 10.0 (%)	pH 8.0
Ala-Ala-Phe-MCA	9.80 (100)	4.70
Glt-Ala-Ala-Phe-MCA	2.85 (29)	0.98
Z-Val-Lys-Met-MCA	1.05 (11)	0.05
Phe-MCA	0.15 (1.5)	0.04
Suc-Ala-Ala-Ala-MCA	0.06 (0.6)	0.04
Suc-Ile-Ile-Trp-MCA	0.03 (0.3)	n.d.
Suc-Leu-Leu-Val-Tyr-MCA	0.02 (0.2)	0.03
Ala-MCA	n.d.	0.04
Suc(OMe)-Ala-Ala-Pro-Val-MCA	n.d.	0.01
Suc-Ala-Ala-Pro-Phe-MCA	n.d.	0.01
Z-Leu-Leu-Glu-MCA	n.d.	n.d.
Z-Ala-Ala-Asn-MCA	n.d.	n.d.
Z-Leu-Leu-Leu-MCA	n.d.	n.d.
Z-Leu-Arg-Gly-Gly-MCA	n.d.	n.d.

[a] Activities were examined with a substrate concentration of 300 μM in 50 mM CHES (pH 10.0) or 50 mM HEPES (pH 8.0) at 60°C. n.d., activity not detected.
[b] Suc, *N*-succinyl; Suc(OMe), *N*-methoxysuccinyl.

Taking into account the V_{max} at 80°C and the effects of temperature, the specific activity of the archaeal SppA$_{Tk}$ was calculated to be 6.6 μmol mg^{-1} min^{-1} at 40°C against Ala-Ala-Phe-MCA.

Residues preferred at the cleavage site have also been examined for SppA$_{Ec}$ with both synthetic substrates and signal peptides. The former revealed a preference of cleavage at the C-terminal side of Ala, Leu, Val, Gly, and Phe (27), while the latter indicated a preference for Val, Leu, Ile, Gly, Thr, and Ala (25). As in the case of the specific activity described above, the substrates used and the conditions applied in these experiments vary greatly, making it difficult to accurately compare the two enzymes. One point that is worthy of note is that many residues (Ala, Val, Thr, and Gly) are commonly preferred at the P-1 site by both SppA$_{Ec}$ and SppA$_{Tk}$.

By performing a BLAST search against the genome sequences of archaea, we were able to identify SppA orthologues in most strains of Euryarchaeota (Fig. 2). As exceptions, we could not identify highly similar orthologues on the *A. fulgidus* and *Methanopyrus kandleri* genomes. Interestingly, most members of the Crenarchaeota do not seem to utilize structurally related signal peptide peptidases. The two protein sequences from *Pyrobaculum aerophilum*, the only SppA orthologues from *Crenarchaeota*, contained exceptionally long extensions in their C-terminal domains. Residues that are highly conserved among bacterial and archaeal SppA sequences are indicated in Fig. 1 and may be involved in catalysis and/or substrate specificity. In particular, Ser162 in SppA$_{Tk}$ is conserved in all SppA sequences and is also conserved in the functionally and structurally related *B. subtilis* TepA protease (10). Although this serine residue of TepA has not been experimentally analyzed, it is included in a region which displays similarity to the region containing the active site serine of *E. coli* ClpP, raising the possibilities that this residue is the nucleophile in all of these proteins. However, the His and Asp/Glu residues that constitute the conventional catalytic triad found in ClpP are not conserved among the archaeal proteins and are not even present in the SppA proteins from *E. coli* and *B. subtilis*. In order to accurately determine the active-site residues of SppA proteins, including the nucleophilic serine, site-directed mutagenesis studies will be necessary.

Using the FRET peptide library, we have been able to clarify some of the preferences of ΔN54SppA$_{Tk}$ towards residues at the P-1, P-2, P-3, and P-4 sites. A relatively small side chain seems to be preferred at the P-1 position. The specificity at the P-2 position can be regarded as broad. Hydrophobic and/or aromatic residues are recognized most at the P-3 site, while the positively charged Arg enhances the activity of ΔN54SppA$_{Tk}$ when present at the P-4 position. Our results also indicate that the presence of acidic residues at any one of the sites from P-2 to P-4 has a negative effect on the substrate recognition of ΔN54SppA$_{Tk}$. With the MCA substrates, a direct comparison among substrates to determine the residue preference of ΔN54SppA$_{Tk}$ was difficult, as multiple factors differ even between two given substrates. One point that can be noted is that a negative charge at the P-4 position has a large negative effect on substrate recognition (Ala-Ala-Phe-MCA > Glt-Ala-Ala-Phe-MCA).

Taking into account the specificity of ΔN54SppA$_{Tk}$, we examined a vast number of putative signal sequences that were

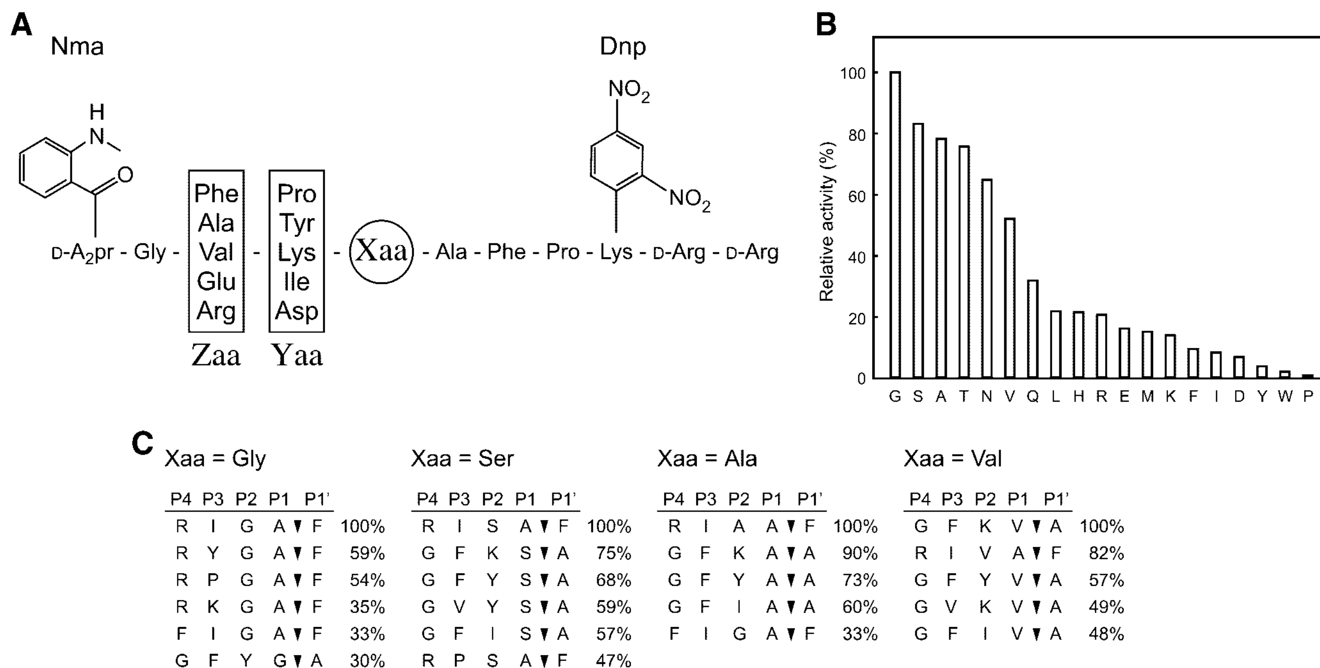

FIG. 6. (A) Structure of the peptide substrates from a FRETS peptide library utilized in B and C. (B) Peptidase activity towards various substrates from the FRETS peptide library. Amino acid residues at the Xaa position are indicated. Each substrate was examined at a concentration of 30 μM. (C) The major cleaved products of four substrates (Xaa = Gly, Ser, Ala, and Val) were detected by LC-mass spectrometry. Relative quantities are indicated to the right of the sequences.

identified on the *T. kodakaraensis* genome using the SOSUI program. Some representative signal sequences, those from four proteins that have been experimentally proven to be secreted from *T. kodakaraensis* (19, 30, 35, 36; unpublished data), are shown in Fig. 7. As in the case of most putative signal sequences on the *T. kodakaraensis* genome, acidic residues are not found, consistent with the fact that ΔN54SppA$_{Tk}$ does not cleave peptides with acidic residues in the P-2 to P-4 sites. Further, we found a number of candidate sequences in each signal sequence that can be presumed to be efficiently recognized and cleaved by ΔN54SppA$_{Tk}$.

Although future gene disruption studies will be necessary to confirm the physiological role of SppA$_{Tk}$, the similarity in primary structure to SppA$_{Ec}$ as well as the enzymatic properties of the enzyme are in good agreement with the assumption that SppA$_{Tk}$ functions as a signal peptide peptidase in *T. kodakaraensis*.

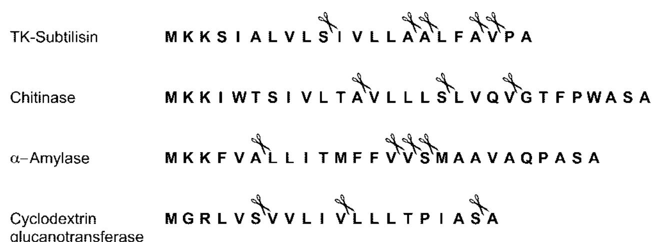

FIG. 7. Predicted signal sequences of four *T. kodakaraensis* proteins that have been biochemically characterized. Prediction was carried out with the SOSUI program. The signal sequence of α-amylase has been confirmed experimentally. Scissors indicate sites that can be presumed to be cleaved by SppA$_{Tk}$ when its substrate specificity is taken into account.

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