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Hydrolysis of the Amyloid Prion Protein and Nonpathogenic Meat and Bone Meal by Anaerobic Thermophilic Prokaryotes and *Streptomyces* Subspecies

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Transmissible spongiform encephalopathies are caused by accumulation of highly resistant misfolded amyloid prion protein PrPres and can be initiated by penetration of such pathogen molecules from infected tissue to intact organism. Decontamination of animal meal containing amyloid prion protein is proposed thanks to the use of proteolytic enzymes secreted by thermophilic bacteria *Thermoanaerobacter*, *Thermosipho*, and *Thermococcus* subsp. and mesophilic soil bacteria *Streptomyces* subsp. Keratins α and β, which resemble amyloid structures, were used as the substrates for the screening for microorganisms able to grow on keratins and producing efficient proteases specific for hydrolysis of β-sheeted proteic structures, hence amyloids. Secretion of keratin-degrading proteases was evidenced by a zymogram method. Enzymes from thermophilic strains VC13, VC15, and S290 and *Streptomyces* subsp. S6 were strongly active against amyloid recombinant ovine prion protein and animal meal proteins. The studied proteases displayed broad primary specificities hydrolyzing low molecular mass peptide model substrates. Strong amyloidolytic activity of detected proteases was confirmed by experiments of hydrolysis of PrPres in SAFs produced from brain homogenates of mice infected with the 6PB1 BSE strain. The proteases from *Thermoanaerobacter* subsp. S290 and *Streptomyces* subsp. S6 are the best candidates for neutralization/elimination of amyloids in meat and bone meal and other protein-containing substances and materials.

KEYWORDS: Prion; meat and bone meal; thermophilic; *Streptomyces*; hydrolysis

INTRODUCTION

The use of bacteria secreting proteases into their medium during their life cycle in the search for peptides and amino acids necessary for their growth finds increased application for the hydrolysis of industrial or other protein substrates, because this method is energy saving and is most ecologically sound. Some proteins, and keratins in particular, are resistant to proteases, because of their structural features (insolubility and the presence of large quantities of disulfide intra- and intermolecular bridges, high content of β structures in the case of β-keratins and extended forms of α-keratins, and the high degree of aggregation) (*1*, *2*). Such proteins can be destroyed only by proteases with strong activity of broad specificity, such as subtilisin or subtilisin-like enzymes, secreted by the bacteria of *Bacillus* species (*3*−*7*), or by proteases active under highly denaturing conditions destabilizing the molecules of recalcitrant protein substrates (*8*). The use of thermophilic bacteria such as

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Fervidobacterium pennivorans, *Thermoanaerobacter keratinophilus*, *Thermoactinomyces candidus*, and *Fervidobacterium islandicum* for keratinolytic purposes has been reported (*9*−*12*).

Transmissible spongiform encephalopathies (TSE) or "prion diseases" form a group of fatal infectious neurodegenerative disorders represented by bovine spongiform encephalopathy (BSE), scrapie in sheep and goats, and Kuru and Creutzfeldt−Jakob disease (CJD) in humans. Because TSE are transmissible, they can be distinguished from amyloidoses. CJD is the most important TSE and was first described in 1920. In 1996, a variant form of CJD (vCJD) was identified in young people in the United Kingdom and a connection between BSE and this new entity was strongly suspected. There is now strong evidence that vCJD is caused by the agent responsible for BSE. The causative agent of these diseases is yet to be fully characterized, but it is clear that the host-encoded "prion protein" (PrP), and its highly aggregated amyloid isoform called PrPres (for proteinase-resistant), has a central role in the TSE pathology.

Prions are characterized by an extraordinary resistance to most physical and chemical methods classically used for inactivation of conventional pathogens. Many studies have investigated the contribution of various processes to either reduce the infectiousness or to eliminate TSE agents altogether (*13*). Steam-autoclaving (134 °C for 18 min) and treatments with sodium hypochlorite or hydroxide are recommended as the most efficient procedures. Unfortunately, these treatments are incompatible with many industrial processes and with all instruments and materials used in health care. Additionally, the TSE crisis generated by improper treatment and use of meat and bone meal (MBM) in animal nutrition stopped recycling of animal wastes in food. Storage and disposal of these unrecyclable wastes create important ecologic and sanitary problems all over Europe. Thus, new environmentally friendly, economically sound, and safe methods of decontamination and disposal are badly needed. The methods should transform ordinary organic matter and destroy the amyloids present. In this sense screening diverse groups of microorganisms for proteases and other enzymes, such as lipases and reductases, for the fragmentation of amyloids and elimination of infectiousness is an important task. The effects of many proteases, such as trypsins, under nondenaturing conditions are negligible. However, some conventional proteases, such as pronases, demonstrated significant effects in titer reduction (*13*). Consequently, the identification of efficient bacterial strains and enzymes could greatly improve the efficiencies of composting and decontamination of slaughterhouse and rendering effluents. They could also provide new methods for disinfection of surgical equipment and other material and devices used in health care. The use of thermophile microorganisms for the biodegradation of highly resistant amyloid molecules may provide a unique opportunity due to the activity of thermophilic enzymes on the PrPres structure under denaturing conditions.

The production of required enzymes can be induced by the cultivation of microorganisms on suitable protein substrates. The collections of anaerobic thermophilic prokaryotes from deep-sea and terrestrial sources, as well as representatives of the genus *Streptomyces*, were screened for their ability to grow on the insoluble protein keratin, which forms fibrillar structures similar to amyloids. The activities of the proteases toward amyloid structures were studied using keratins and recombinant prion ovine protein as substrates.

MATERIALS AND METHODS

Materials. The components of media, sea salts, oxidized B-chain of bovine insulin, bovine gelatin, 3-(*N*-morpholino)propanesulfonic acid (MOPS), and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Purified preparations of recombinant ovine prion were prepared according to methods described by Rezaei et al. (*14*). Keratin prepared from chicken feathers was provided by Dr. Yu. Zuev, Kazan Centre of Science, Russian Academy of Sciences, Kazan, Russia. Porcine hair was obtained from SIFDDA Co., Plouvara, France.

Bacterial Strains, Media, and Growth Conditions. Deep-sea thermophilic anaerobic strains were isolated from samples collected at Rainbow site (36° 14′ N, 33° 54′ W, 2300 m depth) on the Mid-Atlantic Ridge. Strains VC13 and VC15 were isolated from a black smoker sample (AT2E01-08), and strain VC34 was isolated from a shrimp sample (AT2E09-10). Strains VC15 and VC34 were enriched at 60 °C in SP medium (30 g/L sea salts, 2 g/L porcine hair). Strain VC13 was enriched at 80 °C in SPS medium (SP supplemented with 5 g/L sulfur). Subcultures for protease assays were done under the same conditions. SSU rDNA PCR amplifications and sequencing (data not shown) indicated that strains VC15 and VC34 are thermophilic bacteria of genus *Thermosipho* subsp. and that strain VC13 is an archaea that likely belongs to the hyperthermophilic *Thermococcus* genus.

Strain S290 was isolated from the hot spring of caldera Uzon, Kamtchatka, and was identified as a representative of the genus *Thermoanaerobacter* by the reaction with species-specific 16S-rRNA-targeted oligonucleotide probe (*15*). Strain S290 was cultivated at 60 °C for 5 days on the mineral medium Th (0.33 g/L KH_2PO_4, 0.33 g/L NH_4Cl, 0.33 g/L $MgCl_2$, 0.33 g/L $CaCl_2$, 0.33 g/L KCl, 0.5 g/L $NaHCO_3$, 0.5 g/L Na_2S), supplemented with 2 g/L porcine hair or feathers.

The strains of *Streptomyces* subsp. BC-1 (S1), 444-K-P (S2), MA-6 (S3), 14-8 (S4), 741-6/71 (S5), and 695-206 (S6) were from the collection of the Department of Microbiology, Sofia University, Bulgaria. BC-1 is a *Streptomyces* subsp. isolated from meadow soil of Bulgarian mountain Strandja, 444-K-P is a morphological variant of *Streptomyces albogriseolus* 444 prepared after regeneration of protoplasts, MA-6 is *Streptomyces* subsp. MA-6 isolated from Bulgarian forest soil, 14-8 is *Streptomyces* subsp. 14-8 isolated from Guinean soil, 741-6/71 is a variant of *Streptomyces* subsp. 741 producing the antifungal antibiotic bifamicin, and 695-206 is a variant of *Streptomyces galbus* var. *achromogenes* 695 selected after exposure to UV and nitrosoguanidine. Strains S1−S6 were cultivated at 30 °C for 7−10 days on the mineral medium St (1 g/L KNO_3, 0.5 g/L $MgSO_4$, 0.5 g/L KH_2PO_4, 0.5 g/L NaCl, 0.02 g/L $FeSO_4$), supplemented with 20 g/L starch. For the growth of *Streptomyces* subsp. on keratin, the starch was replaced by 2 g/L of fine (∼1 mm diameter) particles of porcine hair.

For cultivation on animal MBM, keratin in all media was replaced by 2 g/L meals of wool, meat, blood, or mixed MBM from these three components. The MBM meals were heterogeneous, polydisperse industrial samples provided by SARIA Industries Bretagne, Issé, France, a rendering company (particle size varying between 0.1 and 2 mm diameter).

Protease Assays. After cultivation, the bacteria and the insoluble medium components were precipitated by centrifugation during 15 min at ∼20000*g*. Proteolytic and peptidase activities were measured in the resulting supernatants.

The presence of proteases in the supernatants was determined according to the method of zymogram. Proteases were separated by SDS-PAGE containing hydrolyzed substrate−0.01% bovine gelatin polymerized in the gel. Twenty microliters of the lysis buffer was added to 40 μL of supernatants, and 10, 12, or 15% SDS-PAGE was made (*16*). After electrophoresis, the gels were washed from SDS in 2.5% Triton X-100 and three times in water and then were incubated in 20 mM MOPS, pH 7.2, 100 mM NaCl, and 2 mM $CaCl_2$ at growth temperatures for 1−10 h. Gels were colored with silver nitrate (*17*).

Keratinolytic activity of supernatants was measured according to an *o*-phthaldialdehyde (OPA) method determining the quantity of liberated amino groups (*18*). One hundred microliters of supernatants was added to 100 μL of 2 mg/mL of β-keratin (feathers) or 4 mg/mL α-keratin (porcine hair) in 20 mM MOPS, pH 7.2, and was incubated at growth temperatures for 72 h. Control experiments were carried out without bacterial supernatants. The OPA reagent was freshly prepared by mixing and diluting to a final volume of 50 mL with distilled water

the following reagents: 25 mL of 100 mM sodium tetraborate, pH 9.2, 2.5 mL of 10% SDS, 40 mg of OPA (dissolved in 1 mL of methanol), and 100 μL of β-mercaptoethanol. Digestion mixtures were centrifuged during 10 min at 10000*g*, and 50 μL of supernatant was added to 1 mL of OPA reagent. The assay solution was incubated for 2 min at room temperature. The absorbance at 340 nm was then measured. Glycine was used for the measurement of the standard curve.

The activity of proteases from the supernatants of bacteria in the hydrolysis of recombinant prion and nonpathogenic animal MBM was determined by electrophoresis. Fifty microliters of supernatants was added to 50 μL of 2 mg/mL MBMs or 1 mg/mL of recombinant prion in 20 mM MOPS, pH 7.2, and incubated at different temperatures for 8 or 24 h. The lysis buffer was added to the aliquots from the reactions, aliquots were boiled during 10 min, and then 15% SDS-PAGE was performed. Proteins on gels were colored with Coomassie Brilliant Blue R-450.

Peptidase activity and specificity of proteases from the bacterial supernatants were detected by measuring the fragmentation of the oxidized B-chain of bovine insulin. One hundred microliters of supernatants was incubated with 100 μL of 2 mg/mL insulin B-chain in 20 mM MOPS, pH 7.2, at different temperatures for 10 h. The products of hydrolysis were separated by reversed-phase HPLC on Nucleosil 300-5 C_{18} equilibrated by solvent A, with a 30-min gradient of solvent B (A, 0.1% formic acid; B, 60% CH_3CN and 0.1% formic acid). Collected peptide fractions were diluted 5 times with solvent C (50% CH_3CN and 0.5% formic acid) and subjected to mass spectrometry (MS) analysis.

Mass Spectrometry. Peptide analyses were performed on a Finnigan LCQ ion trap spectrometer (Finnigan MAT, San Jose, CA). The heated desolvation capillary was held at 200 °C, and the electron multiplier was set to 1.0 kV. Spectra were acquired in automated MS mode. The scan range for MS mode was set between the masses of 200 and 2000.

Sites of oxidized insulin B-chain cleavages were identified using the program FindPept, ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics.

Purification of the PrPres from the Mouse-Adapted 6PB1 BSE Strain. PrPres was prepared from 20% brain homogenates obtained from C57/BL6 mice infected with the 6PB1 BSE strain; the purification of PrPres was performed using the BSE Purification Kit (Bio-Rad) and a treatment with 10 μg/mL of proteinase K solution for 10 min at 37 °C. The 6PB1 BSE strain has been adapted and stabilized in C57/BL6 mice (*19*). The disease incubation period in these animals is 150 days on average, and average mice die 180 days after intracerebral inoculation of 105 times the 50% lethal dose. For the negative-PrP controls, the PrPres purification was repeated using brain homogenate from healthy mice instead of brain homogenates from 6PB1 BSE-infected mice. At the end of the PrPres purification, PrPres and negative-PrP controls were resuspended in Milli-Q water.

PrPres Treatment with Bacterial Extracts and Detection of PrPres by Western Blot. The 6PB1 BSE PrPres SAFs were treated for 24 h with different bacterial supernatants (v/v) at various temperatures. At the end of the incubations, the fate of PrPres was detected by Western blot. Samples were run on 6−3% polyacrylamide gels and transferred onto a nitrocellulose membrane. Immunoblotting was performed with the monoclonal peroxidase-conjugated anti-mouse PrPres SAF83 antibody (*20*). Immunoreactivity was revealed with a chemiluminescence enhancement kit using ECL hyperfilm (Amersham Bioscience) and the Super Signal R-West Extended Duration Substrate (Pierce) and visualized by autoradiography. The specific bands were quantified by densitometry (NIH 1.2, W. Rasband, National Institutes of Health, Bethesda, MD). Results were considered as positive when one signal corresponding to one of the PrPres glycoforms was evidenced. Two complementary controls, that is, cross-reaction (CR) control and extemporaneous (E) control, were performed in parallel to demonstrate the absence of cross-reactions between SAF83 and bacterial supernatants (CR control) and the absence of deleterious effects of bacterial supernatants on PrPres detection by Western blot, respectively. The CR control was realized by running on nonspiked bacterial supernatants and immunoblotting with SAF83. The E control was performed by extemporaneously treating PrPres with various bacterial supernatants.

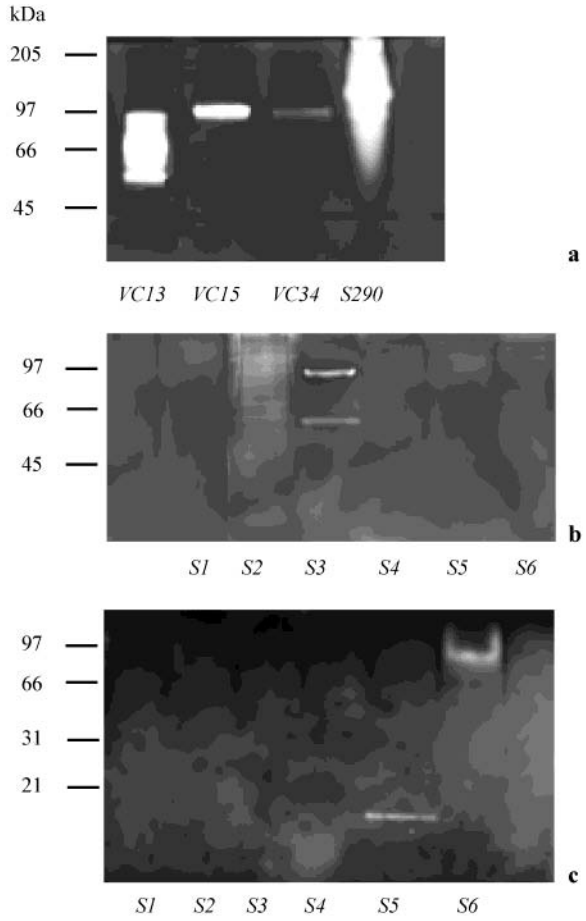


Figure 1. Zymograms of the supernatants of (**a**) thermophilic bacteria (VC13, VC15, VC34, and S290) and (**b**) *Streptomyces* subsp. strains (S1–S6) grown on starch-containing media; (**c**) zymogram of the supernatants of *Streptomyces* subsp. strains (S1–S6) grown on keratin-containing media.

RESULTS

Growth of Microorganisms under Study on the Keratin-Containing Media. Thermophilic bacteria strains S290, VC13, VC15, and VC34 and *Streptomyces* subsp. strains S1−S6 were tested for the ability to grow on keratin-containing media.

All of the thermophilic bacteria were able to use porcine hair keratin (α-keratin) as the main nutrient source. Time of cultivation was 3 days. Strain S290 grew relatively well on the feather keratin (β-keratin). In the latter case the bacteria culture reached the same density as when growing on hair keratin for 7 days.

Streptomyces subsp. strains S1−S6 grew relatively rapidly on porcine hair keratin. The appearance of the first colonies was marked in 3 days. At the same time, strain S6 grew only on feather keratin. In the latter case the growth was much slower—the first colonies appeared in 15 days.

Zymograms of Proteases Produced by Thermophilic Bacteria and *Streptomyces* Subspecies. To identify the proteases secreted by these bacterial strains, zymograms of the bacterial culture supernatants were examined. The main feature of enzymes from thermophilic bacteria was their stability to denaturation (*21*). Therefore, it would be expected that thermophilic proteases would maintain their activity after SDS-PAGE. Data presented in **Figure 1a** summarize the proteolytic activity of enzymes from the thermophilic strains VC13, VC15, and VC34, grown on α-keratin. Strain VC13 formed a group of proteolytic bands with molecular masses in the range of 45−

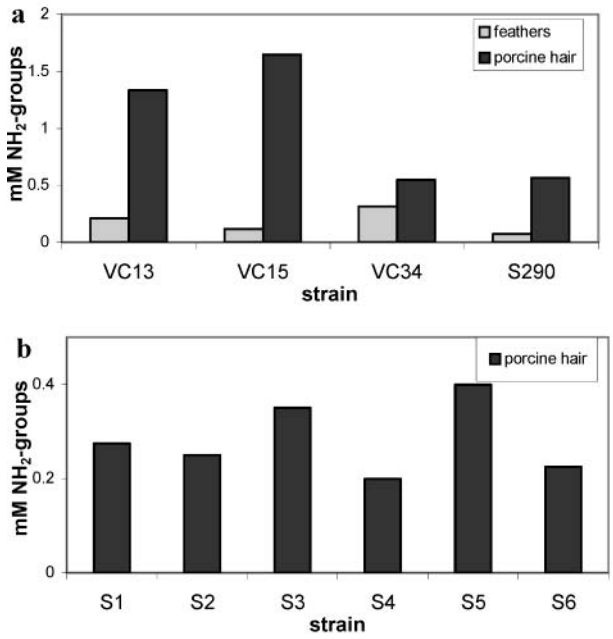


Figure 2. Accumulation of amino groups in soluble fractions after 3 days of incubation of (**a**) thermophilic and (**b**) *Streptomyces* subsp. bacterial supernatants with insoluble feathers keratin (1 mg/mL) and porcine hair keratin (2 mg/mL).

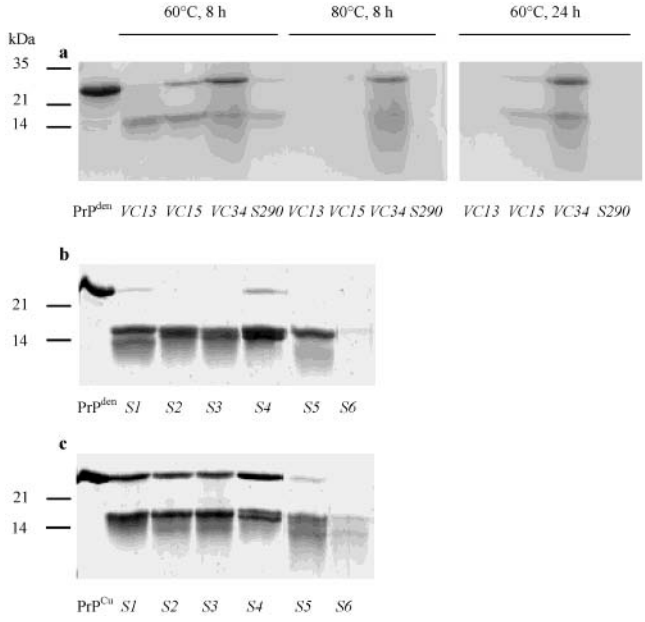


Figure 3. Hydrolysis of thermally denatured amyloid recombinant prion (PrP^{den}) by the supernatants of (**a**) thermophilic bacteria VC13, VC15, VC34, and S290 at 60 and 80 °C and (**b**) *Streptomyces* subsp. S1–S6 strains at 30 °C; (**c**) hydrolysis of PrP–Cu^{2+} (PrP^{Cu}) complex by *Streptomyces* subsp. strains.

100 kDa. These activities may belong to different forms of one protease (*22*). Zymograms of supernatants of strains VC15 and VC34 displayed only a single band of proteolytic activity, corresponding to a molecular mass of ~100 kDa.

The thermophilic anaerobe *Thermoanaerobacter* strain S290 produced a range of bands of proteolytic activities with molecular masses of ~60−200 kDa and above (**Figure 1a**), which were active at 60 °C.

Zymograms of proteases secreted by different strains of *Streptomyces* subsp., grown on a mineral medium with starch, revealed the presence of two bands of proteolytic activity in strain S4, corresponding to molecular masses of approximately 60 and 90 kDa. A broad band of activity was observed in the case of strain S3 (**Figure 1b**). The content of secreted proteases changed when strains S1−S6 were cultivated on porcine hair keratin. The bands observed previously in starch media in the case of strains S3 and S4 disappeared when these strains were cultivated on keratin. Under these conditions strains S5 and S6 produced new proteases with molecular masses of approximately <20 and 80 kDa, respectively (**Figure 1c**). The absence of clear bands of proteolytic activity in the supernatants of other strains can be explained by the weak activity or irreversible inactivation of proteases after SDS-PAGE, because all strains were able to grow on keratin; that is, they should produce keratinases degrading this substrate. Consequently, the results unambiguously show the induction of proteolytic enzyme synthesis by substrates and, hence, the need to cultivate microorganisms on a suitable substrate such as keratin.

Hydrolysis of Keratins by Bacteria Supernatants. The activity of proteolytic enzymes from the supernatants of thermophilic bacteria and *Streptomyces* toward α-keratin from porcine hair and *β*-keratin from feathers was studied by measuring the accumulation of NH_2 groups in soluble fractions.

Figure 2a demonstrates the ability of studied supernatants of thermophilic bacteria to hydrolyze the keratins. *β*-Keratin of feathers was hydrolyzed efficiently by the proteases produced by strain VC34 and to a smaller extent by proteases from VC13, VC15, and S290. α-Keratin of porcine hair was digested best by supernatants of VC15 and VC13.

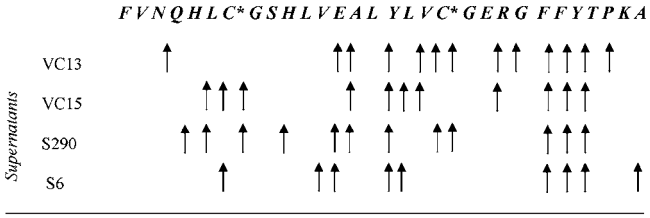
No protease activity against feather keratin was detected in the supernatants of *Streptomyces* subsp. during 72 h of incubation. The absence of growth of five studied strains of *Streptomyces* subsp. on feather keratin confirmed that they are unable to use this protein as substrate. Only strain S6 was able to digest feathers. However, its growth was very slow (it was 15 days before the appearance of the first colonies), and keratinolytic activity was not detected during 3 days of supernatant incubation with feathers. It should be noted that the keratin obtained from feathers is the most resistant to proteolysis.

All six strains of *Streptomyces* subsp. were able to grow rapidly on the porcine hair, and they all produced enzymes, which degraded keratin. **Figure 2b** demonstrates similar activities of proteases from the supernatants of *Streptomyces* subsp. toward porcine α-keratin.

Activity of Supernatants of Thermophilic Bacteria and *Streptomyces* Subspecies against Recombinant Amyloid Prion. It has been shown that the recombinant ovine prion molecule denatured at 80 °C forms amyloid structures and therefore can represent the model of PrPres structures (*23*). The ability of thermophilic bacteria and *Streptomyces* subsp. supernatants to digest thermally denatured ovine prion was studied.

Results presented in **Figure 3a** show that supernatants of strains VC13, VC15, and S290 hydrolyzed amyloid prion in 8 h until the complete disappearance of the electrophoretic band at 80 °C, which corresponds to strain VC13 optimal growth temperature. Strain VC34 displayed weaker activity toward this substrate. However, at 60 °C (growth temperature of VC15, VC34, and S290) amyloid prion was not hydrolyzed completely by these strains. In this case, as observed previously with proteinase K (*24*), a large hydrolytic fragment accumulated. The resistance of this fragment to hydrolysis can be caused by its aggregation. At 80 °C, this aggregate could be destabilized/denatured and the activity of the thermophilic proteases would increase considerably. The large hydrolytic fragment is cleaved

Table 1. Peptide Bonds Cleaved in the Molecule of Oxidized Insulin B-Chain by Proteases from Supernatants of Thermophilic VC13, VC15, and S290 Strains and *Streptomyces* Subsp. Strain S6



much more rapidly at 80 °C. Nevertheless, proteases from VC13, S290, and, to a lesser extent, VC15 were able to digest amyloid recombinant prion completely. The stable fragment at 60 °C was also hydrolyzed after the incubation time had been extended to 24 h (**Figure 3a**).

Supernatants of *Streptomyces* subsp. strains S1−S5 degraded thermally denatured ovine prion during 24 h at 30 °C with the accumulation of its large fragment (**Figure 3b**). The enzymes secreted by strain S6 were the most efficient because they cleaved prion completely (**Figure 3b**). These results are in agreement with data on the growth of this strain on keratins. Only strain S6 was able to grow on both α- and *β*-keratins.

It was shown that the introduction of copper ions can facilitate the transformation of prion molecules into their amyloid form and increase the resistance of PrP to proteolysis (*25*, *26*). Thus, native recombinant ovine prion in the presence of copper ions represents the other model of PrPres structure. It was demonstrated that only strain S6 hydrolyzed prion and its stable fragment completely after 24 h of incubation in the presence of 1 mM Cu^{2+}. Strain S5 showed less hydrolytic activity. The proteases secreted by strains S1−S4 of *Streptomyces* subsp. only converted prion to its large hydrolytic fragment (**Figure 3c**). The study of thermophilic protease activity is not possible under these conditions because of the rapid degradation of prion at high temperature in the presence of copper ions (data not shown).

Thus, the results demonstrate that thermophilic strains VC13, VC15, and S290 and *Streptomyces* subsp. strain S6 are the best candidates for decontaminating animal MBM of amyloid aggregates, and these strains were selected for more extended study.

Specificity of Proteolytic Activity of Studied Bacterial Supernatants. The hydrolysis of oxidized B-chain of insulin, a standard substrate of proteolytic specificity tests by selected bacterial strains, was studied in order to get more precise information about active proteases. The B-chain of insulin is composed of large numbers of hydrophobic amino acid residues, and it contains also several charged side-chain residues (**Table 1**). Products of oxidized insulin B-chain cleavage by supernatants of studied strains were separated by HPLC and subjected to MS analysis. Results of B-chain hydrolysis are presented in **Table 1**.

Proteases from thermophilic strains VC13 and VC15 hydrolyze peptide bonds after hydrophobic and after negatively charged amino acid residues (**Table 1**). Residues of Cys are oxidized, and this creates two additional negative charges. Besides cleavages at oxidized Cys, the additional hydrolytic sites of the insulin molecule by enzymes from VC13 were identified as Asn3-Gln4, Arg22-Gly23, and Pro28-Lys29. If the hydrolysis of the peptide bond after the Asn residue is due to the deamidation, which may be possible at 80 °C, creating a negative charge at this site, then the other cleavage sites are of particular interest. Enzymes from strain VC15 hydrolyzed the peptide bond after the His5 residue (not observed in the case of VC13). Strain S290 displayed specificity somewhat similar to that of VC15 with additional histidine sites Gln4-His5 and Ser9-His10. Observed broad specificities were confirmed by the ability of the studied supernatants to hydrolyze *p*-nitroanilides of succinyl-Ala-Ala-Pro-Phe and succinyl-Ala-Ala-Pro-Asp (data not shown).

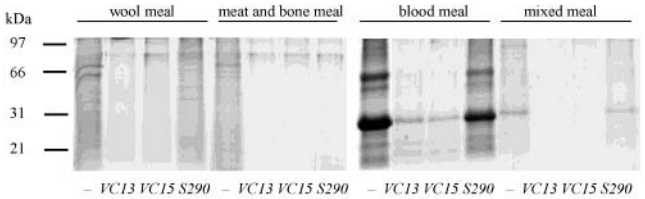


Figure 4. MBMs hydrolysis by the supernatants of thermophilic strains VC13 (at 80 °C), VC15 (at 80 °C), and S290 (at 60 °C) after 24 h of incubation. Lines marked by "–" are negative controls, MBM without hydrolysis.

Protease from *Streptomyces* subsp. strain S6 cleaves peptide bonds at the P1 position, at which a residue of hydrophobic amino acid or Lys is situated (**Table 1**). Such dual chymotryptic and tryptic activity of the enzyme was confirmed by using *p*-nitroanilides of succinyl-Ala-Ala-Pro-Phe, succinyl-Ala-Ala-Pro-Lys, and succinyl-Ala-Ala-Pro-Arg as substrates. However, it should be remembered that the studied extracellular proteases may be a complex mixture of more than one enzyme.

Thus, both thermophilic strains VC13, VC15, and S290 and mesophilic *Streptomyces* subsp. strain S6 display proteolytic activities with broad specificities. Broad specificities should achieve the fragmentation of protein substrates to small peptides, and this explains well the efficacy of the selected strains in the hydrolysis of otherwise highly resistant amyloid prions.

Digestion of Nonpathogenic MBM by Thermophilic Bacteria and *Streptomyces* Subspecies. The ability of thermophilic bacteria strains VC13, VC15, and S290 and *Streptomyces* subsp. strains S1−S6 to grow on the nonpathogenic meals of wool, meat-and-bone, and blood and on meal made of a mixture of these three components was screened.

All strains of thermophilic bacteria proved to be able to grow well on MBM. Moreover, the studied bacteria grew on the meals more quickly than on keratins. This is not surprising because animal meals are rich sources of all necessary substances for growth: proteins, lipids, carbohydrates, vitamins, and microelements. The lowest growth yield of all microorganisms was obtained when the meal made of blood was used as the substrate, maybe because of the toxicity of porphyric components and heme adducts.

Hydrolytic enzymes present in the supernatants of strains VC13, VC15, and S290 allowed partial dissolution of the treated substrates after 24 h of incubation of the meals of wool, meat, blood, and mixed meal. **Figure 4** demonstrates that supernatants of all studied bacterial strains hydrolyzed efficiently the proteins of meal of meat, whereas the meals of wool, blood, and mixed meal were more efficiently degraded by the enzymes present in the supernatants of strains VC13 and VC15 at 80 °C. The enzymes of strain S290 (moderate thermophile) showed lower activities at 60 °C, which resulted probably from the lower temperature of incubation. It should be highlighted that all studied thermophilic strains hydrolyzed the meals to small peptides without accumulation of large products. It should be noted also that remaining inorganic particles did not contain any proteins detectable by SDS-PAGE.

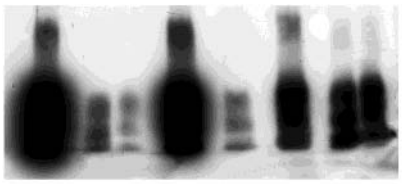


Figure 5. Western blot of BSE 6PB1 PrPres after incubation without (PC) or with supernatants of *Streptomyces* subsp. strain S6 and its 5× concentrate, thermophilic strains S290, VC13, and VC15, during 24 h.

The incubation of the meals with the supernatants of *Streptomyces* subsp. during 72 h did not lead to a significant dissolution of the substrates. Electrophoresis did not reveal any noticeable hydrolysis of meals (data not shown). However, the ability of all strains of *Streptomyces* subsp. to grow rapidly on all studied meals suggests the production of indispensable enzymes by these bacteria and their potential application for the utilization of meals.

Hydrolysis of PrPrec Deposits in Mouse Brain by Selected Bacteria Supernatants. The digestion of purified PrPres obtained from brain homogenates of mice infected with the 6PB1 BSE strain by supernatants from cultures VC13, VC15, and S290 and *Streptomyces* subsp. S6 was performed to verify the ability of proteolytic enzymes from the selected bacterial strains to hydrolyze effectively the pathogenic amyloid prion SAF in multicomponent systems.

The electrophoretic profile of the PrPres obtained from 6PB1 mouse-adapted BSE strain is a type 4 profile, exclusively associated with vCJD, and was present in all BSE-infected animals (**Figure 5**, positive control). PrPres was detected only in PrPres-contaminated samples. No signal corresponding to PrPres was evident in the negative control and in various CR controls (data not shown). On the other hand, PrPres was detected in untreated positive controls (**Figure 5**). Nevertheless, after 24 h of incubation at 80 °C, the amount of PrPres had decreased by 46% in positive control (**Figure 5**, PC80 vs PC60 and PC30). No significant difference between positive controls and E (extemporaneous) controls was detected at all temperatures tested and for different bacterial supernatants tested (data not shown). These bacteria supernatants have no effects on the PrPres detection by Western blot, at least with the SAF83 antibody.

All bacterial supernatants were efficient against BSE 6PB1 PrPres SAFs with a significant decrease in the PrP signal. At 30 °C, the *Streptomyces* subsp. S6 supernatant decreased by 88% the intensity of the PrPres signal (**Figure 5**). The 5-times-concentrated S6 supernatant decreased by 95% the intensity of the PrPres signal (**Figure 5**). At 60 °C, the PrPres signal was decreased by 85% after 24 h of treatment with the S290 supernatant (**Figure 5**). At 80 °C, the VC13 and VC15 supernatants decreased by 45% the intensity of the PrPres signal (**Figure 5**). Surprisingly, the proteolytic activities of enzymes from thermophilic strains VC13 and VC15 when incubated at 80 °C were relatively weak if compared with activities of these supernatants toward amyloid recombinant ovine prion. This phenomenon can be explained by inhibition or inactivation of proteases by remaining, despite purification, brain components or can be due to the inhibition by detergents used for the purification of PrPres.

DISCUSSION

Transmissible spongiform encephalopathies are diseases caused by the accumulation of amyloid forms of prion proteins in brains of mammals. The danger created by these pathologies consists of the fact that they can be transmitted also by the ingestion of tissues from sick animals. Ingested pathogenic prions would catalyze the transformation of normal, native forms of prion protein of the host into amyloid pathogenic forms. Therefore, the use of meals produced of fallen animals presents serious risks. The disposal of these materials is also very risky for wild fauna and the environment. The present work attempts to propose a solution for these problems. First, it demonstrates efficient hydrolysis of amyloid (including that of pathogenic BSE amyloids). Second, it demonstrates that it is possible to eliminate and sterilize excessive MBM, using microorganisms capable of breaking down these proteins and using them as growth substrates.

The high stability of amyloid prion proteins to hydrolysis by proteolytic enzymes from mesophilic organisms (*13*, *24*) has stimulated the study of the amyloidolytic capacities of proteases from heterotrophic thermophilic bacteria because they require high temperature for growth. In these conditions, proteins and prion amyloids, in particular, are destabilized. Keratins can represent the model substrates for screening because the structure of β-keratins, in particular, is so similar to that of amyloids. Keratins, which are highly insoluble, are also characterized by significant resistance to proteolysis due to their fibrillar highly aggregated structures. Therefore, screening for microorganisms able to grow on keratins was a first step of the present work. Thermophilic keratinolytic anaerobic bacteria from marine and terrestrial Euro-Asiatic geothermal springs strains VC13, VC15, VC34, and S290 and some strains of the soil bacteria *Streptomyces* subsp. secreting the necessary proteolytic enzymes for keratin digestion were selected for this study.

It was found that the thermophilic bacterial strains VC13, VC15, VC34, and S290 secrete a wealth of proteolytic high molecular mass (50−200 kDa) enzymes (aggregates or the isoforms of single proteases), which display high lytic activities against gelatin after SDS-PAGE. Production of these enzymes enables bacterial growth on keratins. It was demonstrated that the cultivation of six studied *Streptomyces* subsp. strains on different substrates induced the production of different proteases. When grown on keratins (model of amyloid structure), bands of proteolytic activity with molecular masses of <20 and 80 kDa were obtained on the zymograms of the proteases from strains S5 and S6, respectively.

Proteases produced by strains VC13, VC15, VC34, and S290 were able to hydrolyze keratins of feathers and porcine hairs. Supernatants of *Streptomyces* subsp. strains display proteolytic activity toward α-keratins composing porcine hairs but not toward β-keratin from feathers, which is confirmed by the inability of selected strains to grow on this substrate. Nevertheless, all studied *Streptomyces* subsp. are able to grow on MBM, likely because of the presence of the hydrolases on their cell surface.

The study of the activity of thermophilic proteases toward the model amyloid form of recombinant ovine prion resulting from the protein thermal denaturation demonstrated that enzymes of the strains VC13, VC15, and S290 hydrolyzed the amyloid prion molecule completely at 60 and 80 °C. Strain VC34 had a weak activity toward amyloid prion at both temperatures.

It was found that strain S6 is the most active of all selected *Streptomyces* subsp. strains toward both thermally denatured prion and prion-Cu^{2+}, which represent the second model of

PrPres structure at moderate temperatures in vitro. This is a remarkable result because the proteases of *Streptomyces* subsp. are active at relatively low growth temperatures (30 °C), when the amyloid structure is still not unfolded. It was shown that at physiological temperature proteinase K degraded amyloid prion only to a large fragment (*24*). Thus, proteases from *Streptomyces* subsp. strain S6 present an interesting class of proteases displaying strong hydrolytic activities toward aggregated structures, which are otherwise highly resistant to the action of majority of proteolytic enzymes.

The study of the hydrolysis of oxidized B-chain of insulin by enzymes from selected thermophilic strains VC13, VC15, and S290 and *Streptomyces* subsp. strain S6 demonstrated the broadness of their specificities. Proteases from strains VC13, VC15, and S290 hydrolyzed peptide bonds after hydrophobic and after negatively charged amino acids. Besides this, the cleavages after His (for VC15), Pro (for VC13), and Ser residues (for S290) were detected. Proteases from the *Streptomyces* subsp. strain S6 cleaved peptide bonds in position P1 with hydrophobic and positively charged amino acid residues, that is, they were displaying chymotrypsin and trypsin activities. Thus, all of the selected strains produce proteases with broad specificities, which explains well their powerful hydrolytic activities, making them potentially applicable for the degradation of amyloid proteins and other resistant substrates.

Selected thermophilic and *Streptomyces* subsp. bacterial strains were able to grow on and to dissolve nonpathogenic solid MBM. Maximal activity toward MBM proteins was displayed by thermophilic strains VC13 and VC15 at 80 °C. *Streptomyces* subsp. strains grew better on this substrate than on keratins; however, no proteolytic activities of supernatants toward MBM proteins were detected during all applied incubation times. Thus, to resolve the problem of MBM dissolution, the time of digestion by *Streptomyces* subsp. was increased considerably. It can be also supposed that the majority of proteases active in the growth of *Streptomyces* subsp. on the MBM is associated with their cellular walls and is absent from the liquid phase of the growth medium.

Surprisingly, the supernatants of thermophilic strain S290 and *Streptomyces* subsp. strain S6 hydrolyzed effectively and rapidly the PrPres obtained from a mouse-adapted BSE strain. This result is particularly surprising in the case of mesophilic *Streptomyces* bacterial strain S6 because no detectable proteolytic activity of this strain toward MBM proteins was observed. In contrast, thermophilic strains VC13 and VC15 were unable to hydrolyze the BSE PrPrec structures from murine-infected brains. This is caused possibly by the inhibition of proteases or by inactivation by small brain molecules or by detergents persisting after the purification of PrPres. The obvious hydrolytic activities of supernatants of VC13 and VC15 against keratins and amyloid recombinant prion protein indicate that these strains can be applied for the hydrolysis of safe and infectious MBM (containing infectious amyloid prion proteins).

Hence, the problem of efficient proteolytic degradation of amyloid deposits can be solved using hyperthermophilic archaea *Thermococcus* VC13, thermophilic eubacteria *Thermosipho* VC15, and *Thermoanaerobacter* S290 or mesophilic *Streptomyces* subsp. S6 (695−206) strains able to grow on keratins and MBM and secreting proteases with broad specificities. Moreover, the application of soil *Streptomyces* bacteria for the neutralization of meal containing amyloid structures is the most attractive from technological and practical points of view, because the cultivation of these microorganisms does not require high-temperature or anaerobic conditions.

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LITERATURE CITED

(1) Arai, K.; Naito, S.; Dang, V. B.; Nagasawa, N.; Hirano, M. Cross-linking structure of keratin. VI. Number, type, and location of disulfide cross linkages in low-sulfur protein of wool fiber and their relation to permanent set. *J. Appl. Polym. Sci.* **1996**, *60*, 169−179.

(2) Goddard, D. R.; Michaelis, L. A study on keratin. *J. Biol. Chem.* **1934**, *106*, 604−614.

(3) Atalo, K.; Gashe, B. A. Protease production by a thermophilic *Bacillus* species (P-001A), which degrades various kinds of fibrous proteins. *Biotechnol. Lett.* **1993**, *15*, 1151−1156.

(4) Cheng, S. W.; Hu, H. M.; Shen, S. W.; Takagi, H.; Asano, A.; Tsai, Y. C. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 2239−2243.

(5) Lin, X.; Lee, C. G.; Gasale, E. S.; Shih, J. C. H. Purification and characterisation of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl. Environ. Microbiol.* **1992**, *58*, 3271−3275.

(6) Williams, C. M.; Richter, C. S.; MacKenzie, J. M., Jr.; Shih, J. C. H. Isolation, identification and characterization of a feather-degrading bacterium. *Appl. Environ. Microbiol.* **1990**, *56*, 1509−1515.

(7) Kristie, L.; Evans, J. C.; Crowder, J.; Miller, E. S. Subtilisins of *Bacillus* subsp. hydrolyze keratin and allow growth on feathers. *Can. J. Microbiol.* **2000**, *46*, 1004−1011.

(8) Han, X. Q.; Damodaran, S. Stability of protease Q against autolysis and in sodium dodecyl sulphate and urea solutions. *Biochem. Biophys. Res. Commun.* **1997**, *240*, 839−843.

(9) Friedrich, A. B. Degradation of keratin by a novel thermophilic anaerobic bacterium, *Fervidobacterium pennivorans*. Ph.D. Thesis, Technical University of Hamburg, Hamburg, Germany, 1994.

(10) Riessen, S.; Antranikian, G. Isolation of *Thermoanaerobacter keratinophilus* subsp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. *Extremophiles* **2001**, *5*, 399−408.

(11) Ignatova, Z.; Gousterova, A.; Spassov, G.; Nedkov, P. Isolation and partial characterisation of extra cellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. *Can. J. Microbiol.* **1999**, *45*, 217−222.

(12) Nam, G. W.; Lee, D. W.; Lee, H. S.; Kim, B. C.; Choe, E. A.; Hwang, J. K.; Suhartono, M. T.; Pyun, Y. R. Native-feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase-producing thermophilic anaerobe. *Arch. Microbiol.* **2002**, *178*, 538−547.

(13) Taylor, D. M. Inactivation of transmissible degenerative encephalopathy agents: A review. *Vet. J.* **2000**, *159*, 10−17.

(14) Rezaei, H.; Marc, D.; Choiset, Y.; Takahashi, M.; Hui Bon Hoa, G.; Haertlé, T.; Grosclaude, J.; Debey, P. High yield purification and physico-chemical properties of full-length recombinant allelic variants of sheep prion protein linked to scrapie susceptibility. *Eur. J. Biochem.* **2000**, *267*, 2833−2839.

(15) Subbotina, I. V.; Chernykh, N. A.; Sokolova, T. G.; Kublanov, I. V.; Bonch-Osmolovskaya, E. A.; Lebedinskii, A. V. Oligonucleotide probes for the detection of Thermoanaerobacter. *Mikrobiologiia* **2003**, *72*, 374−382.

(16) Laemmli, U. K. Cleavage of structural protein during the assemblage of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(17) Nesterenko, M. V.; Tilley, M.; Upton, S. J. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J. Biochem. Biophys. Methods* **1994**, *28*, 239−242.

(18) Church, F. C.; Swaisgood, H. E.; Porter, D. H.; Catignani, G. L. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J. Dairy Sci.* **1983**, *66*, 1219−1227.
(19) Lasmézas, C. I.; Deslys, J.-P.; Demaimay, R.; Adjou, K. T.; Hauw, J.-J.; Dormont, D. Strain specific and common pathogenic events in murine models of scrapie and bovine spongiform encephalopathy. *J. Gen. Virol*. **1996**, *77*, 1601−1609.
(20) Bach, S.; Talarek, N.; Andrieu, T.; Vierfond, J. M.; Mettey, Y.; Galons, H.; Dormont, D.; Meijer, L.; Cullin, C.; Blondel, M. Isolation of drugs active against mammalian prions using a yeast-based screening assay. *Nat. Biotechnol.* **2003**, *21*, 1075−1081.
(21) Niehaus, F.; Bertoldo, C.; Kahler, M.; Antranikian, G. Extremophiles as a source of novel enzymes for industrial application. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 711−729.
(22) Halio, S. B.; Bauer, M. W.; Mukund, S.; Adams, M. W. W.; Kelly, R. M. Purification and characterization of two functional forms of intracellular protease PfpI from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **1997**, *63*, 289−295.
(23) Rezaei, H.; Choiset, Y.; Eghiaian, F.; Treguer, E.; Mentre, P.; Debey, P.; Grosclaude, J.; Haertlé, T. Amyloidogenic unfolding intermediates differentiate sheep prion protein variants. *J. Mol. Biol.* **2002**, *322*, 799−814.
(24) Prusiner, S. B. Prions. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13363−13383.
(25) McKenzie, D.; Bartz, J.; Mirwald, J.; Olander, D.; Marsh, R.; Aiken, J. Reversibility of scrapie inactivation is enhanced by copper. *J. Biol. Chem.* **1998**, *273*, 25545−25547.
(26) Sigurdsson, E. M.; Brown, D. R.; Alim, M. A.; Scholtzova, H.; Carp, R.; Meeker, H. C.; Prelli, F.; Frangione, B.; Wisniewski, T. Copper chelation delays the onset of prion disease. *J. Biol. Chem.* **2003**, *278*, 46199−46202.

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