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Extracellular serine proteases from *Stenotrophomonas maltophilia*: Screening, isolation and heterologous expression in *E. coli*

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ABSTRACT

A large strain collection comprising antagonistic bacteria was screened for novel detergent proteases. Several strains displayed protease activity on agar plates containing skim milk but were inactive in liquid media. Encapsulation of cells in alginate beads induced protease production. *Stenotrophomonas maltophilia* emerged as best performer under washing conditions. For identification of wash-active proteases, four extracellular serine proteases called StmPr1, StmPr2, StmPr3 and StmPr4 were cloned. StmPr2 and StmPr4 were sufficiently overexpressed in *E. coli*. Expression of StmPr1 and StmPr3 resulted in unprocessed, insoluble protein. Truncation of most of the C-terminal domain which has been identified by enzyme modeling succeeded in expression of soluble, active StmPr1 but failed in case of StmPr3.

From laundry application tests StmPr2 turned out to be a highly wash-active protease at 45 °C. Specific activity of StmPr2 determined with *suc*-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as the substrate was 17 ± 2 U/mg. In addition we determined the kinetic parameters and cleavage preferences of protease StmPr2.

1. Introduction

The genus *Stenotrophomonas* comprises eight species which are found throughout the environment (Ryan et al., 2009). Although *Stenotrophomonas* is ubiquitous, the type species *Stenotrophomonas maltophilia* was preferentially recovered from the rhizosphere of plants like wheat, oat, cucumber, maize and cabbage (Berg et al., 1996). Due to the beneficial interactions with plants which promote plant growth, the gram-negative bacterium has become important for biotechnological applications in agriculture. For instance, *S. maltophilia* is applied for biological control of fungal plant diseases (Berg et al., 1996; Dunne et al., 2000) and supports plant development on marginal soil (Taghavi et al., 2009). Furthermore, the property of metabolizing a broad range of organic compounds in conjunction with a high metal tolerance makes it attractive for bioremediation purposes (Alonso et al., 2000).

In the last years, *S. maltophilia* has also emerged as a human pathogen in immunosuppressed patients (Looney et al., 2009). Although not highly virulent, the bacterium can cause various bacteraemic infection diseases or pneumonia and has been isolated from cystic fibrosis patients (Gross et al., 2004).

S. maltophilia produces numerous hydrolytic enzymes like chitinases, glucanases, lipases, laccases and proteases (Ryan et al., 2009). The latter are known to contribute to the biocontrol activity. For instance, an extracellular protease from strain G-2 was shown to be an important factor in virulence against a plant-parasitic nematode (Huang et al., 2009). Overproduction of extracellular proteolytic activity by mutagenesis of strain W81 resulted in significantly enhanced suppression of the phytopathogenic fungi

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Phytium ultimum (Dunne et al., 1997). Besides, also from clinical sources extracellular serine proteases were isolated and characterized (Windhorst et al., 2002; Nicoletti et al., 2010).

In this study a screening of a culture collection comprising different antagonistic bacteria isolated from plants and clinical sources (Berg, 2009) was performed for identification of new detergent proteases for application as detergent enzymes. Despite the large efforts in the past regarding screening for novel detergent proteases, the potential of antagonistic bacteria has not been assessed systematically. New enzymes are required to adjust the currently used detergents to the changing washing habits. Besides a high activity at low temperatures and alkaline pH, detergent proteases have to be compatible with detergent components like surfactants, bleaches or perfumes and have to tolerate the high ionic strength of the detergent solution (Gupta et al., 2002). This work reports on screening of antagonistic bacteria for identification of new proteolytic detergent enzymes. Screening of 534 strains, comprising a broad variety of gram-negative and gram-positive bacteria, resulted in identification of *S. maltophilia* as producer of high potential proteases. The modified and heterologously expressed proteases were evaluated for addition to detergent formulations.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were of analytical grade and purchased from Sigma (Austria). Components for culture media were purchased from Roth (Germany). Tetrapeptides were derived from Bachem (Germany).

2.2. Bacterial strains and plasmids

Altogether 534 bacterial strains from SCAM (Strain Collection for Antagonistic Microorganisms, Graz University of Technology, Environmental Biotechnology), which are known for their antagonistic properties towards fungal pathogens, were used. *S. maltophilia* 19580 (=c1) was obtained from clinical origin (Berg et al., 1999; Minkwitz and Berg, 2001).

Vector pMS470Δ8 (Balzer et al., 1992) was used for expression in *E. coli* BL21-Gold (Stratagene), *E. coli* ORIGAMI B (Merck) and *E. coli* Rosetta2 (Merck).

2.3. General recombinant DNA techniques

All DNA manipulations described in this work were performed by standard methods (Sambrook et al., 1989). *Phusion* DNA Polymerase (Finnzymes), HotStar*Taq* DNA Polymerase (Qiagen) and dNTP's from MBI Fermentas (Germany) were used for all PCRs. The PCRs were performed in a Gene Amp® PCR 2200 thermocycler (Applied Biosystems, USA). Digestion of DNA with restriction endonucleases (New England Biolabs, USA), dephosphorylation with alkaline phosphatase (Roche, Germany) and ligation with T4 DNA-ligase (Fermentas, Germany) were performed in accordance to the manufacturer's instructions. Plasmid Mini Kit from Qiagen (Germany) was used to prepare plasmid DNA. Plasmids and DNA fragments were purified by Qiagen DNA purification kits (Qiagen, Germany).

2.4. Standard protease assays

For determination of protease activity with azocasein, 75 μl of protease preparation were mixed with 125 μl of a solution of 2% azocasein in 0.1 M Tris–HCl pH 8.6 and incubated at 37 °C for 30 min. The reaction was stopped by adding 600 μl of 10% trichloroacetic acid. After incubation at room temperature for 15 min, the reaction mixture was centrifuged (5 min, 13,000 rpm, 20 °C). Then 600 μl supernatant was neutralized by addition of 700 μl 1 M NaOH and the absorbance was measured at 440 nm. One unit was defined as the amount of enzyme that increases the absorbance by 1.0/h.

Quantitative determination of protease activity was also performed by measurement of *p*-nitroaniline which is released during hydrolysis of suc-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as described in Ribitsch et al. (2010). The protocol was also used for other tetrapeptides: suc-AAPE-pNA, suc-AAPM-pNA, suc-AAP-Nva-pNA, suc-AAPV-pNA, suc-AA-pNA, suc-AAA-pNA, suc-AAF-pNA, suc-AAV-pNA, suc-ALPF-pNA (each with 100 mM concentration); suc-F-pNA (70 mM); suc-FAAF-pNA (71.14 mM) because of a lower solubility of these tetrapeptides in buffer.

2.5. Determination of protein concentration and SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970); protein-containing samples were denatured with 20% trichloroacetic acid, protein bands were stained with Coomassie Brillant Blue R-250. Protein concentration was determined using the Uptima BC Assay protein quantification kit (Interchim, France) and bovine albumin as protein standard.

2.6. Screening for bacteria with high proteolytic activity

In a primary screening 534 bacterial strains were cultivated on NB agar plates at 30 °C until formation of single colonies (approx. 48 h for most isolates) which were subsequently transferred to agar plates containing 1% skim milk and incubated at 30 °C for 3 days. Strains showing a cleared halo around the colonies were selected, cultivated again on NB agar plates and subsequently used for inoculation of liquid cultures (LB medium) supplemented with 1% skim milk. After 3 days of cultivation at 30 °C the cultures were harvested (4 °C, 4000 rpm, 20 min). Culture supernatants showing proteolytic activity with azocasein were subjected to a secondary screening for identification of proteases with thermal stability and EDTA tolerance. Therefore enzyme solutions were incubated at 50 °C for 30 min and in the presence of 0.01 M EDTA (room temperature, 30 min) before performing the azocasein assay.

2.7. Cultivation in alginate beads for induction of protease activity

A 3% alginate suspension (alginic acid sodium salt from brown algae, Roth, Germany) was filtered two times through a sterile syringe filter (0.45 μm cellulose acetate, Millipore). 15 ml of alginate suspension were mixed with 15 ml 2-fold LB medium and 30 μl cell culture. Alginate beads were prepared by extruding the alginate-cell-suspension through a 300 nm needle fitted (JANOME Desktop Robot JR2200N mini, Pulsar Robotics & Automation Systems) with a syringe into a stirred, ice cold $CaCl_2$ solution (100 mM). After 1 h incubation on ice the beads were washed with sterile water, transferred into 100 ml LB-medium supplemented with 1% skim milk and stirred at 30 °C for 48 h.

2.8. Two-dimensional electrophoresis

In the first dimension extracellular proteins from *S. maltophilia* 19580 were separated by preparative isoelectric focusing (IEF). Therefore 60 ml of protein solution (10 ml culture supernatant diluted with 50 ml water) were mixed with 3 ml Bio-Lyte 3/10 Ampholyte (40%) and loaded onto a 50 ml Rotofor Cell (BioRad). Focusing was carried out at 340 V for 3 h and 4 °C using 0.1 M H_3PO_4 as the anode solution and 0.1 M NaOH as the cathode solution. Twenty fractions of 3 ml were collected and pH values were measured by pH strips.

In the second dimension IEF fractions were separated on 12% SDS-gels supplemented with 0.12% skim milk for detection of proteases. After electrophoresis, gels were washed with buffer (30 min, 50 mM Tris–HCl pH 7.4 supplemented with 2.5% Triton X-100) and incubated over night with 50 mM Tris–HCl pH 7.4 and 5 mM $CaCl_2$. Gels were stained with Coomassie Blue R-250. Active proteases acting on skim milk were identified by cleared bands on a blue background.

2.9. Cloning of extracellular proteases from S. maltophilia 19580

Protease genes from *S. maltophilia* were amplified by touchdown PCR from chromosomal DNA of strain 19580. The reaction was done in a volume of 50 μl with 25 pMol forward primers and 25 pMol reverse primers shown in supplementary file 1, 0.2 pM dNTP-mix, 0.5U HotStar*Taq* DNA Polymerase and 1× reaction buffer provided by the supplier. The primers were designed on the basis of genome sequences from *S. maltophilia* R551-3 including restriction sites *Nde*I and *Hind*III. For PCR amplification the DNA was heated to 95 °C for 15 min and amplified in a Gene Amp® PCR 2200 thermocycler (Applied Biosystems, USA) using the following temperature program: 18 cycles with 30 s denaturation at 95 °C, 30 s annealing starting at 65 °C and reducing the temperature every successive cycle by 1 °C, 90 s extension at 72 °C followed by 35 cycles with 30 s denaturation at 95 °C, 30 s annealing at 56 °C and 90 s extension at 72 °C. The PCR products were subcloned by the use of the TOPO TA Cloning *Kit* (Invitrogen) and subsequently cloned into pMS470Δ8 for expression in *E. coli* BL21-Gold. The genes were sequenced using the primers listed in supplementary file 1. StmPr4_R551-3 was synthesized by Mr. Gene (Germany).

2.10. C-terminal truncation of proteases

Truncated proteases were amplified from plasmids constructed by cloning genes (*stmpr*1, *stmpr*2, *stmpr*3 and *stmpr*4) in pMS470Δ8 (see Section 2.9). The PCR was carried out in a volume of 50 μl with 25 pMol forward primers and 25 pMol reverse primers shown in supplementary file 1, 0.2 pM dNTP-mix, 3% DMSO, 0.5U *Phusion* DNA Polymerase (Finnzymes) and 1× reaction buffer provided by the supplier. The DNA amplification was performed in 30 cycles according to the instructions for the DNA polymerase. The PCR products, named *stmpr*1-*C*520, *stmpr*2-*C*477 and *stmpr*3_*C*455, were gel-purified, digested with *Nde*I and *Hind*III and ligated to pMS470Δ8. The plasmids were transformed into *E. coli* BL21-Gold.

2.11. Expression of proteases in E. coli

Expression of proteases was performed as described for HP70 in Ribitsch et al. (2010).

2.12. Purification of protease from culture supernatant

Recombinant protease was purified with FPLC (Äkta purifier, GE Healthcare) by anion exchange chromatography. First the culture supernatant containing protease was desalted using PD-10 desalting columns (GE Healthcare) according to the manufacturer's protocol and buffered with 20 mM Tris–HCl pH 8.6. Then the enzyme solution was loaded on a Q-Sepharose Fast Flow column (GE Healthcare, 20 ml bed volume) and eluted by a gradient of 20 mM Tris–HCl pH 8.6 (buffer A) and 1 M NaCl in 20 mM Tris–HCl pH 8.6 (buffer B) at a flow rate of 5 ml/min using the following gradient program: 1 CV (column volume) 0% B, 5 CV 10% B, 5 CV 10% B, 5CV 100% B and 5 CV 0% B. Protease eluted with 10% B. Fractions showing protease activity were collected, analysed by SDS-PAGE and stored at −20 °C.

2.13. Characterization of StmPr2

Biochemical characterization, determination of kinetic parameters, protein sequencing and determination of specific proteolytic activities towards insulin B were performed as described for HP70 in Ribitsch et al. (2010).

2.14. DNA sequencing, database searches and alignments

DNA was sequenced as custom service (Agowa, Germany). Analysis and handling of DNA sequences was performed with Vector NTi Suite 8 (Invitrogen, USA). BLAST searches were performed using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics and sequences of related proteins were aligned using the Clustal W program (Swiss EMBnet node server).

2.15. Deposition of sequence data

The nucleotide sequences of proteases were deposited in the Genbank under accession numbers JF317275 (*stmpr*1, *strain* 19580), JF317276 (*stmpr*2, *strain* 19580), JF317277 (*stmpr*3, *strain* 19580) and JF317278 (*stmpr*2, *strain PS*5).

3. Results and discussion

3.1. Screening of antagonistic bacteria for protease activity

In order to isolate new proteases for application as detergent enzymes, a strain collection containing antagonistic bacteria isolated from clinical and environmental sources was screened. The primary screening of 534 strains was performed on agar plates supplemented with skim milk for induction and detection of protease activities. 249 strains produced clear halos around the colonies were consequently cultivated in skim milk liquid medium. Protease activity with azocasein was found in culture supernatants of 119 strains. Interestingly, 50 strains produced clear halos on skim milk – agar plates but did not show proteolytic activity in liquid cultures. Apparently, a high cell density is needed for induction of protease expression. For this reason a novel procedure involving entrapment of the organisms in alginate beads followed by incubation in liquid medium was developed (Fig. 1). Immobilization of cells at a high density may provide many benefits. The hydrogel matrix protects cells from harmful environmental conditions like pH, temperature or organic solvents (Park and Chang, 2000) and enables a controlled delivery of macromolecules. In addition, alteration of microbial physiology was observed (Galazzo and Bailey, 1990). In this work, indeed encapsulation of *S. maltophila* as well as other gram-negative and gram-positive bacteria enabled induction of protease expression in case of 20 strains.

Altogether culture supernatants of 139 strains showed high activity with azocasein. These strains were subjected to a secondary screening for identification of strains producing alkaline proteases that work at 50 °C in the presence of chelating agents like EDTA as it is required for detergent enzymes. About 60 strains out of this screening cycle, mainly consisting of different species of *Pseudomonas*, *Stenotrophomonas*, *Serratia* and *Bacillus*, were evaluated in laundry application tests and compared to the alkaline protease from *Bacillus lentus* as a benchmark. Out from these, all strains belonging to *S. maltophilia* (20 strains) displayed higher activities than the other strains.

3.2. Identification of S. maltophilia as best protease producer

Under washing conditions culture supernatants of *S. maltophilia* performed best. *Stenotrophomonas* occurs in a wide range of

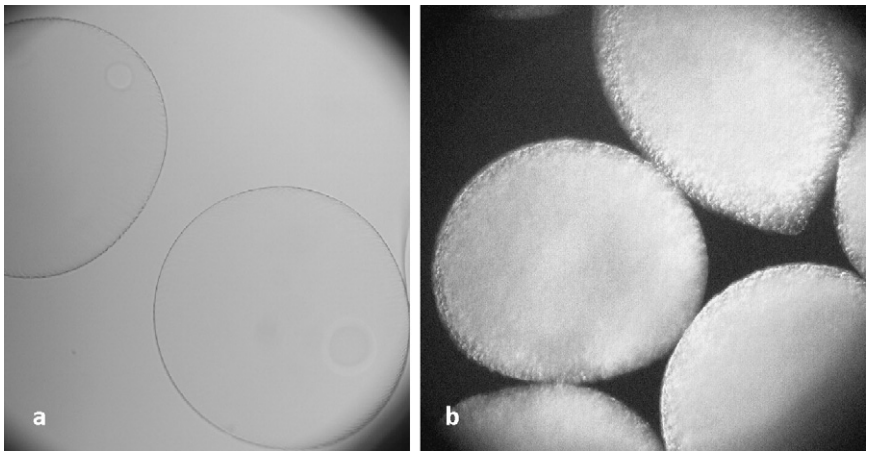

Fig. 1. Cultivation of antagonistic bacteria in alginate beads. (a) Beads without cells. (b) Beads filled with *Stenotrophomonas maltophilia* 19580 after cultivated for 48 h at 37 °C.

habitats but is commonly found in the rhizosphere of many plants. In recent years *S. maltophilia* has emerged as resistant, opportunistic bacterial pathogen in the increasing population of patients who are immunocompromised by diseases or medical treatments (Hayward et al., 2010). From the strains tested for production of new proteases fulfilling the criteria as washing-active enzymes, *S. maltophilia* strains PS5 and 19580 were selected. Strain PS5 has been isolated from the rhizosphere of oilseed rape (Berg et al., 1996) whereas strain 19580 was obtained from a clinical isolate (Berg et al., 1999).

3.3. Detection of extracellular proteases activities from S. maltophilia 19580 by 2D-electrophoresis

To get first insights into extracellular protease activities of *S. maltophilia*, the culture supernatant from strain 19580 was analysed by two-dimensional electrophoresis. In a first dimension the enzyme solution was separated by preparative IEF using a gradient of pH 3–10. Twenty fractions were obtained which were analysed regarding activity with azocasein (supplementary file 2). Low protease activities were detected in fractions 2–6 (pH 3–5) whereas in fractions 18–20 (pH 9–10) very high activities were measured. In a second dimension active fractions were loaded on SDS-gels containing 0.12% skim milk (Fig. 2). After electrophoresis, proteins were allowed to refold upon removal of the denaturant and incubated over night at 37 °C. Staining with Coomassie Blue revealed uncoloured bands derived from active protease on a dark background. Interestingly, several active protease bands appeared at low pH in each fraction which may result from post-translational processing. At high pH, only two bands were observed in each fraction. Unfortunately, incorporation of skim milk into the gel hindered the use of a protein marker and MS-analysis of active bands.

3.4. Cloning and sequence analysis of extracellular serine proteases from S. maltophilia 19580

Strain 19580 was selected to identify extracellular protease(s) exhibiting washing-activity. A search for extracellular serine proteases in the genome of *S. maltophilia* R551-3 (GenBank CP001111) revealed five genes coding for subtilases, named in this work StmPr1 (EAX21823), StmPr2 (EAX22204), StmPr3 (EAX23930) and StmPr4 (EAX23141) as well as an ATP-dependent metalloprotease FtsH (EAX21005). Since detergent enzymes have to be compatible with complexing agents, the metalloprotease was not investigated further. On the basis of the four extracellular subtilase-sequences primers were designed and used for amplification of protease genes from chromosomal DNA of *S. maltophilia* 19580 by PCR. Three genes were isolated, *stmpr*1 (1893 bp), *stmpr*2 (1743 bp) and *stmpr*3 (1767 bp) coding for StmPr1 (63.6 kDa), StmPr2 (58.4 kDa) and StmPr3 (61.5 kDa). In order to screen all potentially washing-active enzymes, *stmpr*4 (1506 bp) from strain R551-3 was synthesized by a commercial supplier for expression of StmPr4 (51.9 kDa) which was included in further work.

Sequence alignment revealed, that the proteases isolated from strain 19580 differ considerably from the corresponding enzymes of strain R551-3 (79% similarity to StmPr1, 97% to StmPr2 and 91% to StmPr3) which reflects the high genomic diversity of the genus *Stenotrophomonas*. As an example, *S. maltophilia* R551-3 was isolated from *Populus trichocarpa* and represents the second most commonly found endophytic species in poplar (Rocco et al., 2009) whereas strain 19580, as mentioned above, was obtained from clinical sources.

Proteases StmPr1–StmPr3 from strain 19580 and StmPr4 from strain R551-3 differ strongly among each other as it is shown in Table 1. Highest similarity was observed between StmPr1 and StmPr2 (55%), lowest between StmPr3 and StmPr4 (26%). All of them are members of the S8A subfamily as classified by the MEROPS peptidase database (www. merops.sanger.ac.uk). Amino acids of the catalytic triad appeared in the order Asp, His, and Ser in the

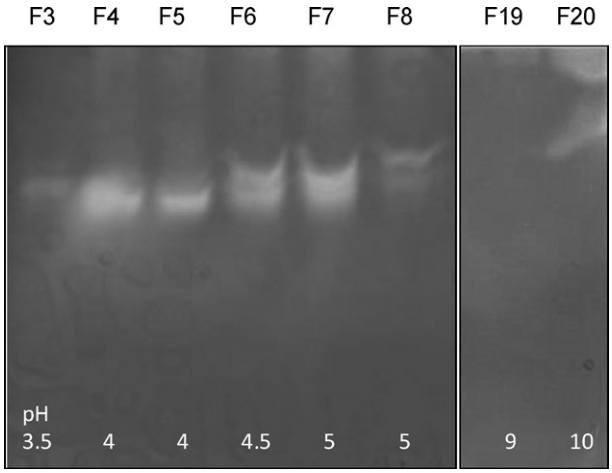

Fig. 2. Activity stain of IEF fractions separated on 12% SDS-gels containing 0.12% skim milk. After electrophoresis gels were washed and proteases were allowed to refold by incubation the gels in 50 mM Tris–HCl pH 7.4 and 5 mM $CaCl_2$ at 37 °C over night. Staining with Coomassie Blue revealed uncoloured protein bands resulting from protease activities.

Table 1
Similarities between StmPr1-3 from strain 19580 and StmPr4 from strain R551-3.

	StmPr1 (%)	StmPr2 (%)	StmPr3 (%)	StmPr4 (%)
StmPr1	100	55	28	39
StmPr2		100	27	47
StmPr3			100	26
StmPr4				100

primary sequence (supplementary file 3). StmPr1, StmPr2 and StmPr4 aligned very well in the catalytic regions whereas StmPr3 was out of this alignment. Like other subtilases, proteases StmPr1–StmPr3 from strain 19580 have a multi-domain structure including a signal peptide for translocation (supplementary file 3, underlined), a pro-peptide for maturation, the mature peptide and a C-terminal domain. Interestingly, the annotated sequence of StmPr4_R551-3 carries no C-terminal extension.

Although extracellular serine proteases are considered to be important pathogenic factors, the extracellular proteases from *S. maltophilia* are poorly characterized in literature. Solely the major extracellular protease StmPr1, derived from a specimen of an immunocompromised patient (GenBank CAC42085, 79% similarity to StmPr1 from strain 19580), is well characterized (Windhorst et al., 2002). Proteolytic activity towards insulin B revealed that the protease is an endopeptidase with broad substrate specificity working best at pH 9.0. StmPr1 was reported to be a relevant virulence factor of *S. maltophilia* (Nicoletti et al., 2010) which is also considered for StmPr2. The sequence of the minor protease StmPr2 was determined (GenBank AAP13815, Windhorst, 2002, Ph.D. thesis). Recently, protease HP70 (GenBank CBM43244) was identified in the course of a metagenome screening showing 92% similarity to StmPr2 (Ribitsch et al., 2010). Furthermore, a protease from *S. maltophilia* S-1 was isolated and basically characterized (Miyaji et al., 2005). According to the results of N-terminal amino acid sequencing the protease has highest similarity to StmPr3 and a native molecular mass of approximately 36 kDa as determined by gel filtration. Interestingly, the protease featured an extremely high pH optimum of 12.0 measured with casein as the substrate. No information is available for StmPr4.

3.5. Prediction of structural domains by enzyme modeling

As mentioned above, subtilases have a multi-domain structure. In this work, the boundaries between the different domains of the three proteases from *S. maltophilia* (signal peptide, propeptide I, mature protease, C-terminal domain) were predicted based on a sequence comparison with HP70, for which a reasonable homology model is available (Ribitsch et al., 2010), as well as with other proteases for which the experimental structure was deposited in the Protein Data Base (PDB).

The protease StmPr1 is slightly similar to HP70 exhibiting 37% identity and 44% similarity. StmPr1 features a signal peptide (27 residues, 2.8 kDa) and a prosequence (123 residues, 12.8 kDa, Windhorst et al., 2002). A multiple sequence alignment with StmPr2 and HP70 as well as a BLAST search against sequences in the PDB predicts the linker region between the protease domain (about 370 residues, 37.4 kDa) and the C-terminal domain (about 110 residues, 11.0 kDa) to be in the range of residues 520–528.

Protease StmPr2 is very similar to HP70 and shows about 69% sequence identity (75% similarity). The signal peptide is cleaved after Ala32 (3.4 kDa). A prosequence is predicted from Gly33 to Thr136 (104 residues, 11.0 kDa). The protease domain of HP70 starts at Leu138. The corresponding residue in StmPr2 (i.e. the approximate boundary between the propeptide and the protease domain) is Leu137 (341 residues, 33.4 kDa). From a multiple sequence alignment the boundary between the protease domain and the C-terminal domain and thus a suitable site for gene truncation is predicted at Pro477 (103 residues, 10.6 kDa). The sequence contains a Pro475-Asn476-Pro477 motif in the linker between the two domains. Truncation of HP70 at Pro477 produced active protease in high amounts (Ribitsch et al., 2010).

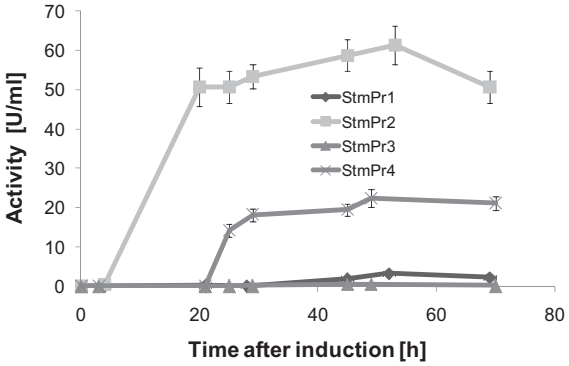

Fig. 3. Expression of StmPr1–StmPr4 in *E. coli* BL21-Gold. Protease activity of the cultivation broth during induction at 20 °C, measured with azocasein at 37 °C and pH 8.6. StmPr1, StmPr2 and StmPr4 were expressed in *E. coli* BL21-Gold, StmPr3 in *E. coli* Origami B.

StmPr3 is the most remote sequence in this set and shows only 13% identity and 27% similarity with HP70. A BLAST search against the PDB as well as fold recognition using GenThreader (Jones, 1999), however, still indicates the same fold as HP70. The most similar protein with known structure is the kexin-like serine protease from *Aeromonas sobria* (Kobayashi et al., 2009) with 37% identity and 53% similarity. In this structure (PDB-entry: 3hjr) the linker between the protease domain and the C-terminal domain is the range from Gln430 to Leu435. This corresponds to the stretch between Gln450 and Leu455 in StmPr3. According to this sequence structure alignment, the protease domain (about 419 residues, 43.5 kDa) is predicted to start close to the N-terminus with only a short (or even absent) propeptide. The signal sequence is cleaved most likely between Ala31 and Gln32 (31 residues, 3.2 kDa).

As mentioned above, StmPr4 reveals no C-terminal domain. The signal peptide is most likely cleaved after Ala24 (2.4 kDa), the catalytic domain starts in the range of residues 140–150 (~36 kDa).

3.6. Expression of extracellular proteases in E. coli BL21-Gold

In analogy to HP70, proteases from *S. maltophilia* were primarily expressed at 20 °C in *E. coli* BL21-Gold. Samples were withdrawn from cultures at different times after induction and analysed regarding activity towards suc-AAPF-pNA (Fig. 3) and expression levels by SDS-PAGE (supplementary file 4). Generally, the proteases differed strongly in terms of expression behavior. Activity was mainly found in culture supernatants. In raw lysates only marginal activities were detected directly after induction.

Expression of StmPr1 resulted in 3 U/ml culture supernatant. On SDS-gel, no characteristic band was identified in the medium which could be clearly traced back to the active protease. StmPr1 was mainly found in the insoluble cell fraction as precursor protein (64 kDa; supplementary 4, lane 3). Obviously, processing of the signal peptide and the prosequence could not be sufficiently achieved in the expression host.

In case of StmPr2, 60 U/ml culture supernatant were reached. Two major protein bands were identified by SDS-gel analysis (supplementary 4, lane 7), one below the 50 kDa protein standard matching best with the mature protease carrying the C-terminal domain (44 kDa predicted) and a second one at 40 kDa derived from the host strain itself.

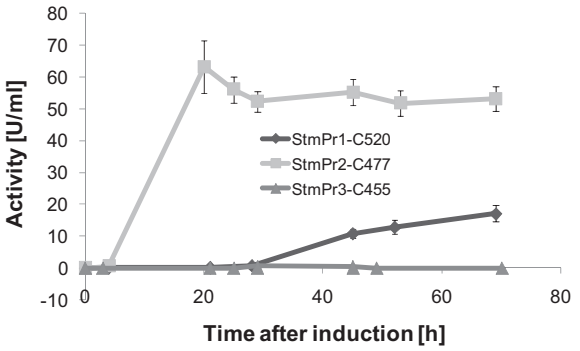

Fig. 4. Expression of truncated proteases in *E. coli* BL21-Gold. Protease activity of the cultivation broth during induction at 20 °C, measured with azocasein at 37 °C and pH 8.6. StmPr1-C520, StmPr2-C477 and StmPr4 were expressed in *E. coli* BL21-Gold, StmPr3-C455 in *E. coli* Origami B.

Unlike StmPr2, no activity was detected after expression of StmPr3. On SDS-gel, no protein bands were observed referring to the protease (data not shown). In a second step, different *E. coli* strains, including ROSETTA 2 and ORIGAMI B, were tested as expression hosts. Again, no activity was found in the soluble cell fractions of the mentioned strains but similar to StmPr1, unprocessed protease (61.5 kDa; supplementary 4, lane 10) was detected in the insoluble fraction by expression analysis.

Expression of the synthetic gene coding for StmPr4 resulted in soluble protein with an activity of 20 U/ml culture supernatant. As shown in supplementary file 4 (lane 14), the protease was fully processed (about 40 kDa) in the expression host. No insoluble protease was detected.

3.7. C-terminal truncation of proteases for improved expression rates

C-terminal truncation of HP70 strongly enhanced expression of active protease (Ribitsch et al., 2010). Analogously to HP70, proteases from *S. maltophilia* 19580 were shortened around the length of the predicted C-terminal domain to achieve higher expression levels of soluble protein. The truncated variants were expressed in *E. coli* BL21-Gold and analysed regarding activity with azocasein (Fig. 4) and expression levels (supplementary file 5). As for untruncated proteases, activities were found mainly in the culture supernatants.

StmPr1 was truncated at Glu520 yielding StmPr1-C520. A 6-times higher activity (18 U/ml culture supernatant) was measured compared to StmPr1. SDS-gel analysis revealed that, in contrast to StmPr1, full N-terminally processed StmPr1-C520 (37.4 kDa) was present in the culture supernatant (supplementary file 5, lanes 4–6). The insoluble cell fractions still contained unprocessed enzyme (53 kDa, data not shown). However, truncation of the C-terminal domain of StmPr1 strongly enhanced processing of N-terminal prosequences and hence production of active enzyme.

StmPr2 was shortened like HP70 at Pro477. Although StmPr2 has high similarity to HP70, truncation of the C-terminal domain did not result in production of higher amounts of soluble protein as it was observed for HP70-C477. Expression of StmPr2-C477 resulted in 55–60 U/ml culture supernatant which is in the same range of the untruncated enzyme. But different to StmPr1, no unprocessed protease was detected by electrophoresis (supplementary file 5, lane 10).

C-terminal truncation of StmPr3 did not result in expression of soluble protein. Like StmPr3, StmPr3-C455 was found in high amounts of N-terminally unprocessed protein (46.7 kDa; supplementary file 5, lanes 12 and 13) in the insoluble cell fractions.

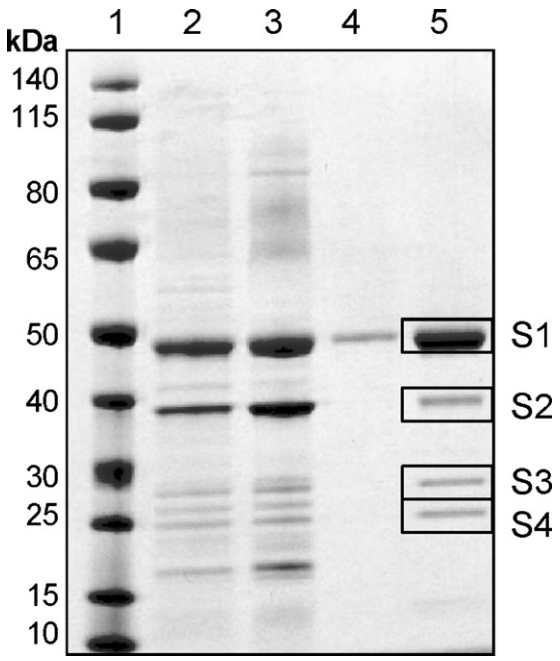

Fig. 5. Purification of StmPr2_PS5 from culture supernatant after expression in *E. coli* BL21-Gold. *Lane* 1: molecular weight standard; *lane* 2: culture supernatant before dialysis; *lane* 3: culture supernatant after dialysis; *lane* 4: fraction 4 of purification run; *lane* 5: fraction 5 of purification run. Marked protein bands were excised for analysis by MS.

3.8. Characterization of StmPr2

StmPr2 performed best under washing conditions. Apart from strain 19580, *S. maltophilia* PS5, which has been isolated from the rhizosphere of oilseed rape, emerged from the screening as highly active strain. Since strain 19580 has been derived from a clinical source, StmPr2 from strain PS5 was expressed in *E. coli* and purified (98% similarity to StmPr2 from strain 19580) from the culture supernatant by anion exchange chromatography for further characterization. SDS-gel analysis (Fig. 5, lane 5) revealed a major band below 50 kDa (protein band S1) which matches to the mature protein carrying the C-terminal domain and additionally three minor bands at lower molecular weight (protein bands S2–S4). All protein bands were excised from the gel, digested and analysed by MS. StmPr2 was unambiguously identified in all gel bands with high sequence coverage (S1: Mascot score 17524, 75% sequence coverage; S2: score 12095, 74% sequence coverage; S3: score 1282, 33% sequence coverage; S4: score 630, 21% sequence coverage), thus the purified protease (S1) undergoes autoproteolysis during purification leading to protein fragments S2–S4.

Isoelectric focusing resulted in p*I* = 8.5 as expected for a subtilase (Gupta et al., 2002). The protease worked best at 45 °C (pH = 8.6) and showed a specific activity of 17 ± 2 U/mg at pH = 9.5 and 25 °C towards suc-AAPF-pNA. These results are in accordance with data derived from the homologous protease HP70. Calculation of kinetic parameters (40 °C, pH = 9.5) resulted in $K_M = 0.24 \pm 0.01$ mM and $k_{cat} = 38.2 \pm 2.1\ s^{-1}$ for suc-AAPF-pNA. Compared to HP70, K_M is very similar (0.23 ± 0.01 mM) whereas k_{cat} ($167.2 \pm 3.6\ s^{-1}$) is much lower.

To determine preferential sites for proteolytic cleavage, purified StmPr2 was incubated with oxidized insulin B chain (30 amino acids, calculated mass 3493.6435; $[MH]^+ = 3494.6$) for 10 min and 4 h. Like for HP70, MALDI–TOF–TOF analysis (Fig. 6 and supplementary file 6) revealed the amide bond between Leu15 and Tyr16 as the preferential cleavage site leading to protonated fragments 1798.8 and 1714.8. Additionally, bonds between

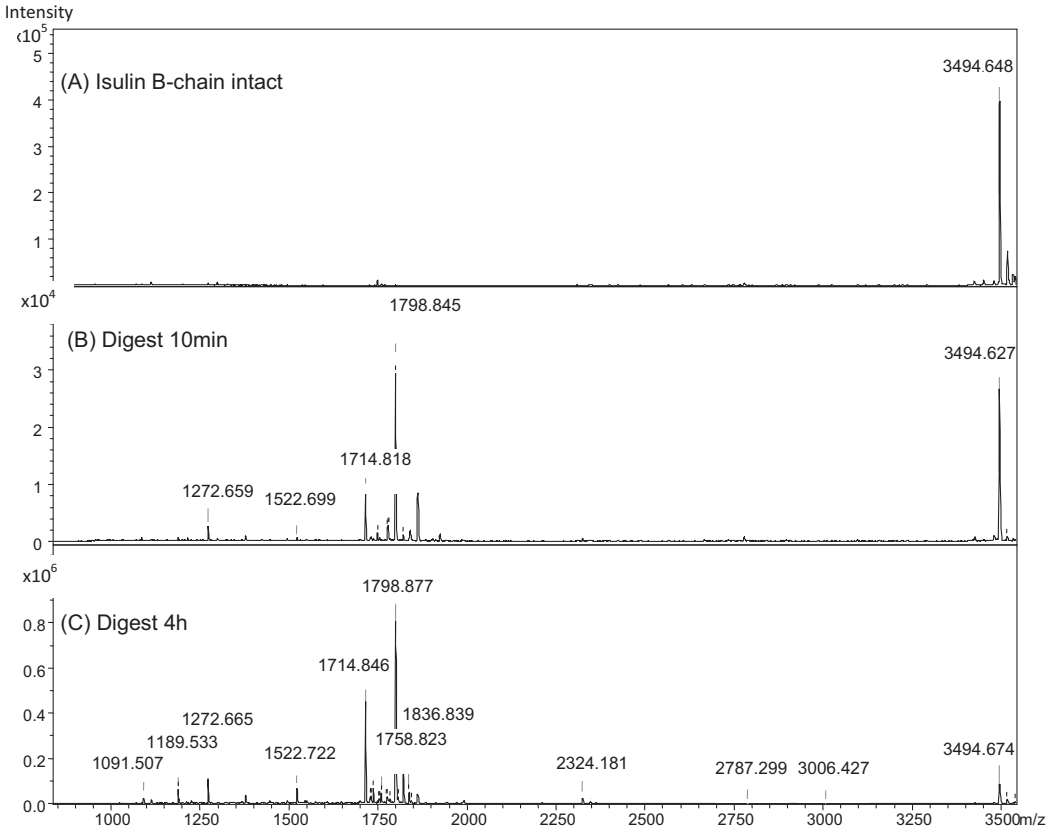

Fig. 6. MALDI–TOF spectra of oxidized insulin B chain before (A) and after cleavage by StmPr2 for 10 min (B) or 4 h (C).

Leu17-Val18 and Cys19-Gly20 were cut by StmPr2 generating fragments 1522.7 and 1272.7. After 4 h of digest, also bonds between Phe24-Phe25 and Gln4-His5 were cleaved. In summary, StmPr2 appeared to have the same cleavage preference like HP70 but a higher specificity.

4. Conclusions

In an attempt to identify novel types of microbial proteases for application in laundry detergents a culture collection of antagonistic bacteria was screened for protease activity on agar plates supplemented with skim milk. Cultivation of best protease producers in liquid media led to the interesting observation that some of the strains secreted proteases only at very high cell density which could be achieved by encapsulation of living cells in alginate beads. Further screening of potent producers of protease activity that performed best under washing conditions led to the selection of *S. maltophilia*. Three proteases produced by strain 19580 (StmPr1, StmPr2 and StmPr3) were cloned for expression in *E. coli*. An additional protease was expressed from a synthetic gene designed according the genome sequence of *S. maltophilia* R551-3. Although the four proteases differed significantly in primary structure they nevertheless shared common features, i.e. the presence of a signal sequence, a prosequence and with three of them (StmPr1, StmPr2 and StmPr3) a C-terminal domain that is common to some of secreted bacterial peptidases. The modification at the C-terminus by removing most of this domain improved intracellular maturation and efficiency of secretion with one of the proteases (StmPr1-C520). In summary, based on optimized production conditions, enzymatic properties and good performance under washing conditions StmPr2 features as a promising candidate for potential practical application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.09.025.

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