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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.