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A novel metalloprotease from *Bacillus cereus* for protein fibre processing

Fernanda Sousa [a], Susana Jus [b], Anita Erbel [c], Vanja Kokol [b], Artur Cavaco-Paulo [d], G.M. Gubitz [a,*]

[a] *Graz University of Technology, Environmental Biotechnology, Petersgasse 12, 8010 Graz, Austria*
[b] *Department of Textile Engeneering, Maribor University, Smetanova 17, SI-2000 Maribor, Slovenia*
[c] *Reserach Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria*
[d] *Department of Textile Engineering, Minho University, Campus Azruém, 4800 Guimaraes, Portugal*

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Abstract

A novel protease produced by *Bacillus cereus* grown on wool as carbon and nitrogen source was purified. *B. cereus* protease is a neutral metalloprotease with a molecular mass of 45.6 kDa. The optimum activity was at 45 °C and pH 7.0. The substrate specificity was assessed using oxidized insulin B-chain and synthetic peptide substrates. The cleavage of the insulin B-chain was determined to be Asn^3, Leu^6, His^{10}-Leu^{11}, Ala^{14}, Glu^{21}, after 12 h incubation. Among the peptide substrates, the enzyme did not exhibit activity towards ester substrates; with *p*-nitroanilide, the kinetic data indicate that aliphatic and aromatic amino acids were the preferred residues at the P_1 position. For furylacryloyl peptides substrates, which are typical substrates for thermolysin, the enzyme exhibited high hydrolytic activity with a K_m values of 0.858 and 2.363 mM for *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide and *N*-(3-[2-Furyl]acryloyl)-Gly-Leu amide, respectively. The purified protease hydrolysed proteins substrates such as azocasein, azocoll, keratin azure and wool.

Keywords: Metalloprotease; Specificity; Kinetics; Wool fibre

1. Introduction

The wool surface morphology requires a special wool textile finishing process. The cuticle cells (or scales) of wool fibres overlap with each other forming the surface of the wool. They are largely responsible for important properties of wool such as wettability, felting behaviour, dyeability and printability. The high concentration of cystine crosslinkages on wool surface is a special feature of keratin fibres. Reaction of cystine crosslinkages with oxidants and reductants are of major importance, being an integral part of the wool chemistry involved in isolating wool proteins [1]. This component of the keratin structure contributes largely to the physical and mechanical properties of the wool fibre. The hydrophobic character and the exposed edges of the cuticle cells are responsible for a differential-friction effect (DFE) resulting in all fibres to move to their root end when mechanical action is applied in the wet state [2,3]. It is well known that the DFE results in the felting shrinking of wool. Therefore, the most successful and commercial shrink-proofing processes are based on modification of fibre surface either by oxidative or reductive methods and/or by the application of a polymer resin onto the surface. The most frequently used commercial process (the chlorine/Hercosett process) consists of a chlorination step followed by a dechlorination step and polymer application [4]. Although this process is highly efficient in reducing shrinking, chlorination produces adsorbable organohalogens (AOX) which appear in the effluent and generate toxicity. With the increasing of ecological and economical restrictions imposed to the textile industry, industries are required to find environmentally favourable alternatives in wool treatment processes [5]. In addition, coating with synthetic polymers largely compromises the natural feeling of wool. The applications of biopolymers has been investigated as possible substitution [6], but until now were not successful in conferring degrees of resistance.

As an alternative, wool can be treated by enzymes, especially proteolytic enzymes, in order to develop environmentally friendly alternative processes. Enzyme technology, especially protease technology, has been explored intensively [7], alone

* Corresponding author.
E-mail address: guebitz@tugraz.at (G.M. Gubitz).

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or in combined processes [8]. To improve the proteolytic anti-shrinkage process, different types of proteases have been studied. A thermo- and alkaliphilic protease has been applied on an industrial scale in the pre-washing step of a wool dyeing process [9] and a protease from *Aspergillus flavus*, isolated from wool, has been examined [10]. Enzymatic processes published in the recent years also comprise combined processes. One example is the enzyme incorporated in the alkaline peroxide treatment, after which chitosan is applied to the wool [11]. However, the major problem in the application of proteases to wool is due to the enzyme penetration between cuticle scales into the interior of the fibre and break down of the cell membrane complex resulting in an irreversible damage of the fibre [9].

Only very little is known about the specificity of proteases toward wool due to its heterogeneous nature and to the amount of amino acids varying depending upon the source of the wool fibre.

The present study describes the purification and characterization of new wool-degrading enzyme from *Bacillus cereus* with potential application for wool treatment with further incorporation in detergent formulations. *B. cereus* is an aerobic, endospore-forming, mobile Gram-positive rod, but grows well anaerobically, being commonly found in soil, air and water [12].

2. Materials and methods

2.1. Isolation and selection of the strains

Several different bacteria growing on wool samples [Drummond Parkland of England, Huddersfield, UK] were cultivated in minimal medium agar plates overlaid with 0.1 g of wool. The growth was monitored under the microscope, after staining with DAPI (0.1 $\mu g\,ml^{-1}$ in phosphate-buffered saline 50 mM buffer). The separation of the individual strains was made by platting on Standard I nutrient agar. After separation of colonies the different bacteria were selected based on proteolytic activity by plating on the surface of skim milk agar plates consisting of ($g\,l^{-1}$): 13.6 agar, 4.5 pancreatic digest casein, 2.3 yeast extract, 0.9 glucose and 90 ml skim milk solution (0.1%, w/v). Plates were incubated at 30 °C for 24 h and examined for clear zones around the colony, which indicates the presence of proteases. For a more specific selection, the strains with proteolytic activity on skim milk agar plates were further cultivated in a minimal medium with wool as source of carbon and nitrogen (described below).

2.2. Growth conditions

For enzyme production, cultivation was performed in 2000 ml baffled flasks containing 500 ml of minimal medium with wool as source of carbon and nitrogen at pH 7.0, consisting of ($g\,l^{-1}$): 2.5 KH_2PO_4, 3.0 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 5 ml trace elements Stelkes–Ritter, and 5 g of wool (the wool was washed and sterilized separately) at 30 °C on a rotary shaker at 120 rpm for 24 h. The growth was monitored by RNA content and protease activity on azocasein was monitored.

2.3. Determination of the cellular RNA content

The RNA content was measured in order to monitor the bacterial growth in the presence of insoluble components of the cultivation medium. This assay was performed according to Benthin et al. [13]. Fermentation broth samples of 10 ml were collected during fermentation, centrifuged in chilled test tubes, the supernatant was discarded and the cells were frozen for subsequent analysis. The cells were washed three times with 3.0 ml cold 0.7 M $HClO_4$ to destroy the cell walls of the bacteria. Then, the samples were digested with 3.0 ml 0.3 M KOH for 60 min at 37 °C with occasional mixing in order to hydrolyse the RNA. The extracts were cooled and neutralized with 1.0 ml 3.0 M $HClO_4$. The post-hydrolysis supernatant was collected and the precipitate was washed twice with 4.0 ml cold 0.5 M $HClO_4$. Finally, the extracts were made up to 15.0 ml with 0.5 M $HClO_4$ and the solutions were centrifuged to remove any non-visible precipitate of $KClO_4$ that might be in the extracts. The RNA concentration was determined by measuring the absorbance at 260 nm using average nucleotide data for calibration [14].

2.4. Azocasein assay

Proteolytic activity with azocasein (Sigma–Aldrich) was determined according the method of Tomarelli et al. [15]. Azocasein 2% (w/v) was prepared by dissolving in 50 mM buffer pH 7.5. The solubility of azocasein becomes limiting at pH values below 6.0. After the azocasein had dissolved, it was clarified by centrifugation at 12,000 × *g* for 10 min. The reaction mixture containing 250 μl of azocasein and 150 μl of enzyme was incubated at 37 °C for exactly 30 min. The reaction was stopped by addition of 1.2 ml of 10% (w/v) trichloroacetic acid (TCA). After centrifugation at 8000 × *g* for 5 min, 1.2 ml of the supernatant was mixed with 1.4 ml of NaOH and the absorbance was determined at 440 nm. Controls were prepared by the same procedure except that TCA was added before the enzyme. One unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 h^{-1}, under the conditions of the assay.

2.5. Activity detection by zymogram activity detection

Pre-made, commercially available 12.0% acrylamide casein zymography gels were used. Gels were inserted into a Mini-Protean 3 Cell apparatus (Bio-Rad Laboratories, Inc., Hercules, USA). The chamber was filled with 25 mM Tris, 192 mM glycine and 0.1% SDS buffer. Samples to test were diluted 1 part sample with 2 parts zymography sample buffer (62.5 mM Tris–HCl, pH 6.8, 4.0% SDS, 25.0% glycerol and 0.01% Bromophenol Blue) and loaded into the gel. The gel run at 100 V constant, 25 mA, 80–100 min and ended 100 V constant, 12 mA. The gels were removed from the apparatus and incubated for 30 min at room temperature in 100 ml of renaturation buffer (50 mM Tris–HCl buffer (pH 7.4) containing 2.5% Triton X-100) on an orbital shaker. The renaturation buffer was removed and 100 ml of 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35 development buffer was added. Gels were incubated on the orbital shaker at room temperature overnight. The development buffer was poured off and the gels were washed with water. Coomassie Brilliant Blue R-250 was added and the gels were incubated on an orbital shaker for 30 min at room temperature. The gels were distained with 10% methanol, 5% acetic acid until clear bands appeared against the blue background.

2.6. Purification of the protease

The culture medium supernatant (4000 ml) was pre-filtrated through a metallic sieve (20 μm) to remove residual wool, than clarified by centrifugation to remove bacterial cells, and lyophilized. The lyophilized material was dissolved in 200 ml of 50 mM Tris–HCl buffer pH 7.5 and frozen. After defrosting, the sample was centrifuged in order to eliminate some polysaccharides that might have precipitated. All chromatographic purification steps were carried out using an Aekta Purifier FPLC system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protease activities in the fractions were determined with azocasein substrate.

Samples of 10 ml were supplemented with 0.3 M $(NH_4)_2SO_4$ and then loaded onto a Phenyl Sepharose CL-4B (GE Healthcare Bio-Sciences AB; 1.0 cm × 2.5 cm) column with a flow rate of 1.0 ml min^{-1}, previously equilibrated with 50 mM Tris–HCl buffer containing 1 M $(NH_4)_2SO_4$ at pH 7.5. The protein was eluted from the column using 50 mM Tris–HCl buffer pH 7.5 containing 15% isopropanol. The peak fractions (1 ml) with proteolytic activity were pooled together (10 ml) and then desalted with a Desalting Hitrap™ column (GE Healthcare Bio-Sciences AB; 1.6 cm × 2.5 cm), pre-equilibrated with 50 mM Tris–HCl buffer pH 7.5 containing 15% of isopropanol. The run was performed with a flow rate of 0.5 ml min^{-1} and 0.5 ml fractions were collected. Positive fractions with protease activity were collected (2 ml) and then 0.2 ml were loaded through Superdex 75 HR column (GE Healthcare Bio-Sciences AB; 1.5 cm × 12 cm) previously equilibrated with 50 mM Tris–HCl buffer pH

7.5 containing 15% isopropanol and 200 mM NaCl, with a flow of 0.5 ml min^{-1}. Fractions (1 ml) were collected and tested for protease activity and subjected to SDS-PAGE to confirm purity.

2.7. SDS-PAGE and protein content

SDS-PAGE was carried out by the method described by Laemmli [16] using 12% polyacrylamide resolving gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 Broad range pre-stained molecular weight markers (200.0–14.4 kDa) (Bio-Rad Laboratories, Inc.) were used to estimate the molecular weight of the protease.

Protein was determined by the Lowry method [17].

2.8. Effect of inhibitors and metal ions on protease activity

To determine the class of protease that yielded the proteolytic activity, enzymatic extract was pre-treated with following inhibitors: PMSF (serine protease inhibitor), E-64 (cysteine protease inhibitor), Pestatin A (aspartic protease inhibitor), Aprotinin (serine protease inhibitor) and EDTA (metalloprotease inhibitor). After the incubation at 37 °C for 30 min, the remaining activity was measured with azocasein assay.

The following metal ions were tested for their ability to stimulate or inhibit protease activity: Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}. The assay was carried out by pre-incubation of the enzyme with the divalent cations to a final concentration of 3 mM for 1 h at 37 °C and thereafter the remaining activity was measured with azocasein assay.

Enzyme activity measured in the absence of any inhibitor or any ion was taken as 100%.

2.9. Effect of pH and temperature on enzyme activity and stability

The optimum pH was determined at 37 °C for 30 min. The substrate (2%, w/v, azocasein) was prepared over a range of pH values between 6 and 12 in Britton–Robison universal buffer [18]. Protease activity was measured at different pH values under standard assay conditions. For pH stability, the enzyme was pre-incubated in the same buffer as above in a range of pH between 4 and 12 for 1 h at 37 °C. Then, the residual activity was determined with azocasein.

Using a standard reaction mixture for azocasein assay, the temperature optimum was a determined different temperature raging from 25 to 90 °C for 30 min.

The thermal stability was determined by pre-incubation of the enzyme at 37 and 50 °C for various periods and then quickly cooled. Standard enzyme assay was used to determine the residual activity.

2.10. Effect of various reagents on protease activity

The stability of *B. cereus* protease was determined with the following reagents: detergents, SDS and Triton X-100 in a concentration of 0.1, 0.5 and 2.0%; organic solvents: DMSO, isopropanol and acetonitrile in a concentration of 1.0, 5.0 and 20.0%; reducing agents: DTT, thioglycolate and β-mercaptoethanol in a concentration of 10.5, 2.0 and 8.0%. After pre-incubation at 37 °C for 30 min with the solutions above, the residual proteolytic activity was measured under standard conditions with azocasein. A control in absence of a reagent was taken as 100%.

2.11. Azocoll assay

Proteolytic activity with azocoll (Sigma–Aldrich) was determined according the method of Chavira et al. [19]. To 25 mg of azocoll, 5 ml of 100 mM potassium phosphate buffer, pH 7.5 was added, and equilibrated to 37 °C for 5 min. To this mixture, 100 μl of protease was added and was incubated exactly 30 min at 37 °C in a shaking water bath. Controls were prepared by the same procedure in absence of enzyme. After incubation, the suspension was immediately filtered through 0.45 μm syringe filters, and the absorbance of the filtered solution was determined at 520 nm. One unit is defined as the amount of enzyme which catalyses the release of azo dye causing an absorbance change of 1.0 min^{-1}.

2.12. Keratin azure assay

Hydrolysis of keratin azure (Sigma–Aldrich) by *B. cereus* protease was determined according to the method of Wainwright [20]. The reaction mixture comprising 200 μl of enzyme solution and 20 mg of keratin azure in 50 mM sodium phosphate buffer pH 7.5, in a total volume of 5 ml was incubated at 37 °C with constant shaking over 26 h period. The unreacted soluble substrate was removed by centrifugation at 8000 × *g* for 5 min and the liberation of the azo-dye in the supernatant was measured at 595 nm. One unit of keratinolytic activity was defined as the amount of enzyme that led to an increase in $A_{595\,nm}$ of 0.01 after 30 min under the standard assay conditions.

2.13. Protease assays with synthetic substrates

Activity with pNA substrates was tested at 30 °C in 50 mM Tris–HCl buffer, pH 8.0. The substrate was prepared just before the experiment since nitroanilide substrates show varying degrees of autolysis during storage.

The pNA substrates used were: L-Leucine-pNA (1.0 mM), *N*-Succinyl-Ala-Ala-Pro-Phe-pNa (1.0 mM), *N*-Succinyl-Gly-Gly-Phe-pNA (3.0 mM), *N*-Succinyl-Ala-Ala-Ala-pNA (3.0 mM), *N*-Succinyl-Ala-Ala-Pro-Leu-pNA (1.0 mM), *N*-Succinyl-Gly-Gly-Gly-pNA (2.5 mM), Sar-Pro-Arg-pNA dihydrochloride (3.0 mM) and BAPNA (3.0 mM). The increase in absorbance due to the hydrolysis and release of *p*-nitroanilide was measured at 400 nm. Extinction coefficient (ε) was taken to be 10500 M^{-1} cm^{-1} at pH 8.0. One unit of activity is defined as the amount of the enzyme that catalysis the transformation of 1 μmol substrate per minute.

Hydrolytic activity of *B. cereus* protease with esters substrates was performed at 30 °C in 50 mM Tris–HCl buffer, pH 7.5. The substrates with the respective concentration used for the activity were: BAEE, 4.0 mM (ε_{253} = 1150 M^{-1} cm^{-1}); BTEE, 2.0 mM (ε_{256} = 964 M^{-1} cm^{-1}); ATEE, 3.0 mM (ε_{237} = −0.0075 ml μmol^{-1} cm^{-1}); 4-nitrophenyl acetate and 4-nitrophenyl butyrate, 2.0 mM (ε_{405} = 9.36 ml μmol^{-1} cm^{-1}). One unit of activity is defined as the amount of the enzyme that splits of 1 μmol substrate per minute.

Protease activity using furylacryloylpeptidyl amides was determined according to the method described by Feder et al. [21]. The assay was carried out using 100 μl of enzyme solution and 1 mM of substrate at 30 °C. The stock solution (10 mM) of the substrates was prepared by adding DMSO in a sufficient amount for dissolving the substrates and then the final volume was done with 50 mM Tris–HCl buffer, pH 7.2, containing 50 mM Mes, 2 mM $CaCl_2$ and 0.01% Triton-X-100. The absorbance was recorded by continuously monitoring the decrease at 340 nm for 5 min. The extinction coefficients were ε_{340} = −0.600 ml μmol^{-1} for *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide and ε_{340} = −0.359 m μmol^{-1}.

2.14. Determination of the kinetic parameters

The kinetic parameters were determined for the following substrates: L-Leucine-pNA (0.05–1.0 mM), *N*-Succinyl-Ala-Ala-Pro-Phe-pNa (0.1–1.0 mM), *N*-Succinyl-Ala-Ala-Pro-Leu-pNA (0.4–2.5 mM), *N*-Succinyl-Gly-Gly-Gly-pNA (0.4–2.5 mM), *N*-Succinyl-Gly-Gly-Phe-pNA (0.8–4.0 mM), *N*-(3-[2-Furyl]acryloyl)-Gly-Leu amide (0.05-1.0 mM) and *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide (0.05–1.0 mM).

The kinetic studies were performed in triplicate and the data obtained fitted to the Michaelis–Menten equation. The K_m and V_{max} values were determined through non-linear regression analysis using the program Origin 5.0.

2.15. Determination inhibition kinetics

The inhibition kinetics were carried out with *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide substrate at different inhibitor (EDTA) concentrations. The enzyme-catalysed hydrolysis of the substrate (1 mM) was followed in the absence of EDTA to establish the inhibited rate of substrate hydrolysis (V_0). The inhibitor, EDTA was added in no more than 5% of the total assay volume. The reaction was allowed to procedure until the rate of hydrolysis has relaxed to a new steady state, thus establishing the inhibition rate (V_i). The substrate concentration was kept constant, by not allowing more than 5% hydrolysis. To have more accurate results, the experiment was repeated with several different concentrations of EDTA (0.001–0.3 mM).

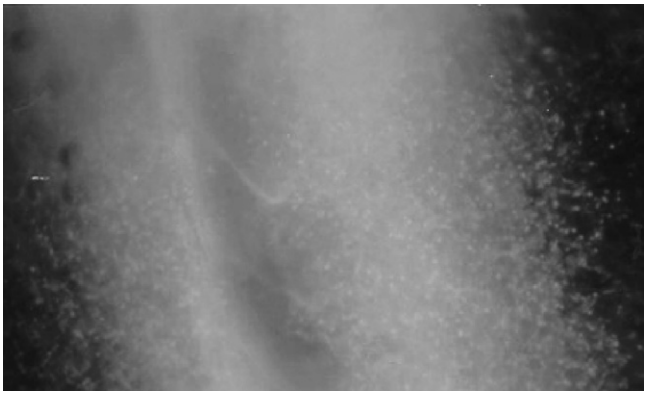

Fig. 1. Fluorescence microscopy images of the mixed population plated on overlaid wool agar plates.

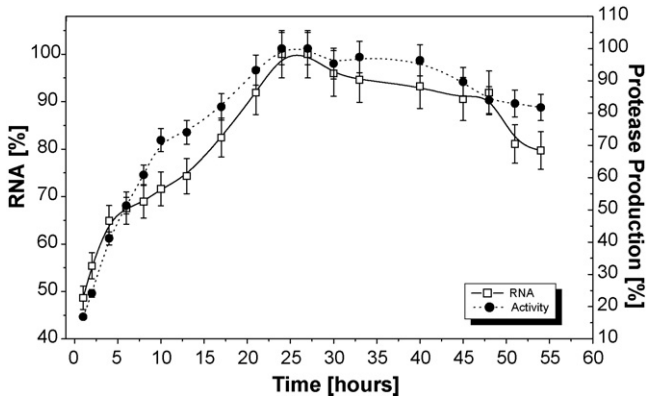

Fig. 2. Cell growth (RNA) and protease production of *B. cereus*. Cultivation of *B. cereus* was done in wool medium pH 7.0 at 30 °C. Standard deviation based on triplicate data.

2.16. Determination of the specificity of B. cereus protease

The proteolytic specificity of *B. cereus* protease was determined by analysis of the peptides obtained after incubation with the oxidized insulin B-chain as substrate.

Cleavage of oxidized insulin B-chain by the *B. cereus* protease at 37 °C was investigated using an enzyme-substrate in a molar ratio of 1:100. The insulin was dissolved in 10 mM Tris–HCl buffer pH 7.5, and then the protease was added. Samples were taken at different times, 30 min, 3, 6, 12 and 24 h. After incubation, the reaction 40 μl of 0.1% TFA was added to inactivate the enzyme. Identification of the cleavage products were analysed by MALDI-TOF mass spectrometry and identified from their corresponding molecular masses. The cleavage was determined according Schecher and Berger [22].

2.17. MALDI-TOF

The digest of oxidized insulin B-chain by *B. cereus* protease was diluted 1:10 in 50% (v/v) acetonitrile/0.1% trifluoroacetic acid and mixed 1:1 with a 5 mg ml^{-1} solution of α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) in 50% (v/v) acetonitrile/0.1% trifluoroacetic acid. One microlitre of the samples were spotted onto a stainless steel target MALDI plate and air-dried before analysis in the mass spectrometer. Identification of the cleavage products was performed on a matrix-assisted laser desorption ionization reflectron-type time-of-flight (MALDI-TOF) mass spectrometer (Waters® Micromass® MALDI micro MX, Manchester, UK), equipped with a 337 nm nitrogen laser. Spectra were acquired in the reflectron positive mode and calibration was carried out using a mixture of PEG oligomers. Cleavage sites were identified using the ExPASy tool findpept.

3. Results and discussion

3.1. Isolation and screening of microorganisms

From the 30 bacterial strains isolated from wool (Fig. 1), one strain was selected based on the proteolytic activity produced in skim milk agar plates and growth in a minimal medium with wool as the only source of carbon and nitrogen. Based on the relationship between the level of protease production and growth, the selected microorganism was identified as *B. cereus* (DSMZ, Braunschweig, Germany). *B. cereus* is a ubiquitous Gram-positive, spore-forming, motile rod, commonly found in soil, plant material, hay, raw and processed food. It is also frequently found in pasteurised milk, causing spoilage because of the production of lipases and proteases [23]. *B. cereus* grew and produced a protease using wool as sole of carbon and nitrogen (Fig. 2). Complete degradation of wool protein was observed after 55 h, indicating that wool was used as an inducer for protease production. Peptides or proteins have been reported to increase proteases synthesis in a number of microorganisms [24]. The maximum enzyme activity was reached after 24 h incubation and prolongation of the fermentation was accompanied with a gradual decrease of enzyme activity. From the growth curve of *B. cereus* based on the RNA content, it can be seen that protease production is correlated with growth. Secretion of proteases has been linked to the bacterial growth either associated with the growth or with stationary phase, with greatest expression of protease during the stationary phase, when sporulation also occurs [28]. To determine the number of proteases produced by *B. cereus*, an SDS-PAGE on a gel that was copolymerised with casein (zymogram) was performed (Fig. 3a). Extracellular proteases are common among *Bacillus* genus. However, in contrast to other *Bacillus* sp., *B. cereus* according to zymography, the presence of a single clear zone in the blue background suggests that the crude filtrate of *B. cereus* mainly contains a single protease.

B. cereus protease was purified to electrophoretic homogeneity by the combination of three chromatographic steps. In the first step using hydrophobic interaction chromatography, 98% of original protein was removed (Table 1). From the purification

Table 1
Summary of the purification of *B. cereus* protease from the lyophilized culture supernatant

Purification step	Volume [ml]	Total protein [mg]	Total activity [nKat]	Specific activity [nKat mg^{-1}]	Purification [fold]	Yield [%]
Crude lyophilized	200.0	669.6	1422.9	2.1	1.0	100.0
Hic phenyl	20.0	12.3	400.1	32.7	15.3	28.1
Desalting	10.0	2.3	267.7	116.4	54.7	18.8
Gel filtration	2.0	0.3	30.7	122.7	57.6	2.16

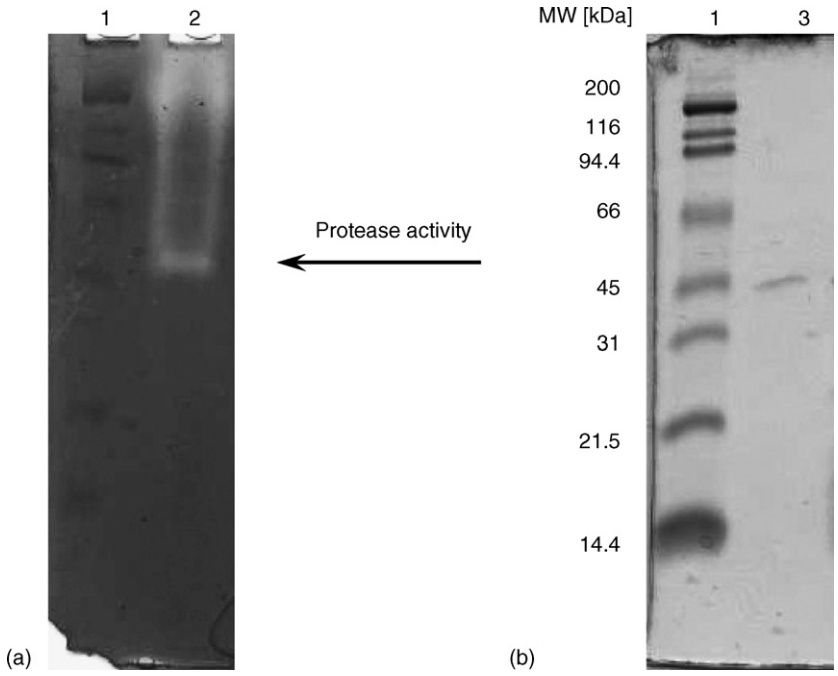

Fig. 3. (a) Zymogram analysis of proteases in the culture supernatant, in 12% polyacrylamide gel with copolymerized casein. The clear zone in the blue background is the result of enzymatic action. (b) SDS-PAGE with 12% acrylamide of the purified *B. cereus* protease. Lane 1, molecular weight standards (broad range), lane 2, culture supernatante and lane 3 and *B. cereus* protease with a molecular weight of 45.6 kDa.

table, the protease was purified to 57.6-fold with a 2.16% yield. The specific activity of the final enzyme preparation was estimated to be 122.7 nKat mg^{-1}. In gel filtration chromatography, proteolytic activity was detected in a single peak corresponding and SDS-PAGE showed the homogeneity of the purified enzyme with a molecular weight of 45.6 ± 1.7 kDa (Fig. 3b). The molecular weight of *B. cereus* protease is in agreement with data from the literature indicating the molecular masses of previously works found proteases are rarely more than 50 kDa [25–27].

3.2. Biochemical characterization

The optimum pH of *B. cereus* protease at 37 °C was determined to be 7.0. Bacteria of the *Bacillus* genus secrete mostly two types of extracellular proteases, a neutral or metalloprotease, and an alkaline protease which is functionally a serine endopeptidase, referred to as subtilisin. The first exhibit optimal pH at 7.0, whereas the latter have pH optima between 9 and 11 [31]. The protease from *B. cereus* belongs to the first group since the optimum pH was 7.0. However, this protease has new characteristics when compared to the others produced by *B. cereus* until now published.

The enzyme has a temperature optimum at 45 °C and retains 55% of activity at 50 °C after 2 h incubation (the half-life time at 50 °C was 2 h) and is stable in a wide range of pH, retaining 85% of its original activity between pH 6 and 9 after 1 h at 37 °C. At pH 5, the half-life time (37 °C) was 1 h.

In addition, the biochemical characterization and inhibition pattern provides clear results for the classification of the protease produced by *B. cereus*. The serine protease inhibitors (aprotinin and PMSF), cysteine protease inhibitor (E-64) and aspartic protease inhibitor (pepstatin) were ineffective as inhibitors. However, the metal chelators 1,10-phenantroline and EDTA caused 100% inactivation of enzyme. The strong inhibition observed against these inhibitors demonstrates that the purified protease is a metalloprotease (the results are summarized in Table 2). This was confirmed by studying the effect of EDTA on enzyme inhibition (data not shown) in which the enzyme is inhibited by even with low concentrations of EDTA, showing that 0.25 mM EDTA was needed to cause 50% drop (I_{50}) in *B. cereus* protease activity. This inhibition pattern is typical of met-

Table 2
Effect of inhibitors and metal ions on protease activity

Substance	Concentration	Residual activity [%]
Control		100
1,10-Phenantroline	1 mM	0
EDTA	1 mM	0
PMSF	1 mM	164.8
Aprotinin	10 μM	100
E-64	1 mM	153.7
Pestatin A	1 μM	98.1
Ca^{2+}	3 mM	117.9
Zn^{2+}		70.6
Cu^{2+}		5.5
Mg^{2+}		98.0
Mn^{2+}		104.6
Fe^{3+}		66.0
Li^{+}		95.4
K^{+}		62.0
Co^{2+}		93.4

The residual activity was measured after pre-incubation of *B. cereus* protease with the different substances.

alloproteases and suggests that in the *B. cereus*, unlike *Bacillus subtillis*, proteolytic activity when grown on wool is a result of neutral protease.

Calcium ions were found to enhance protease activity, achieving its maximum activity at a concentration of 10 mM, while Cu^{2+} decreased the activity to 5.5% (see Table 2). These results indicated that the enzyme require Ca^{2+} for its optimal activity. Since Ca^{2+} is the best inducer, the effect of its concentration on protease activity and the maximum activity was obtained with 10 mM Ca^{2+} (data not shown). The effect of Ca^{2+} on the protease under investigation may involve stabilization of the enzyme molecule structure. Calcium cations are known to be stabilizers of many enzymes, protecting them from conformational changes. The Ca^{2+} binding sites examined for some bacterial proteases contain a number of co-coordinating aspartate and glutamate residues [29].

Several reports show that proteolytic activity could be enhanced by the addition of organic solvents in the reaction mixture [30]. However, enzymes are usually inactivated or denatured in the presence of organic solvents. The protease showed good stability in the presence of DMSO (20 mM) and acetonitrile (1 mM), since the activity increase approximately 5 and 3%, respectively, when compared to the enzyme in the absence of an organic solvent (see Table 3). The protease activity was rapidly inhibited in presence of 2% SDS; however, showed some stability in presence of Triton X-100. Reducing agents like DTT inhibited the enzyme even at low concentrations, with β-mercaptoethanol and thioglycolate the enzyme retained 27 and 45% of its initial activity, respectively, above these concentrations the enzyme was rapidly inhibited. From our study, it can be concluded that the stability of *B. cereus* protease depends on the nature of organic solvents, as well as the nature of detergents. This means that replacement of some water molecules in an enzyme with organic molecules sometimes stabilizes the structure of the enzyme [31]. A similar increase in protease activity from *P. aeruginosa* PST-01 was also reported by Ogino et al. [32] when the enzyme was pre-incubated in the presence of water miscible organic solvents.

Table 3
Effect of solvents, detergents and reducing agents on *B. cereus* protease activity, after pre-incubation for 30 min at 37 °C

Substance group	Substance	Concentration [%]	Residual activity [%]
Control			100
Detergents	SDS	0.1	23.66
		0.5	11.56
		2.0	0.36
	Triton X-100	0.1	94.13
		0.5	91.81
		2.0	87.72
Organic solvents	DMSO	1.0	93.06
		5.0	100.00
		20.0	105.16
	Isopropanol	1.0	92.88
		5.0	87.37
		20.0	69.93
	Acetonitrile	1.0	103.20
		5.0	90.57
		20.0	91.81
Reducing agents	DTT	0.5	5.34
		2.0	1.07
		8.0	0.18
	β-Mercaptoethanol	0.5	27.40
		2.0	4.80
		8.0	0.89
	Thioglycolate	0.5	45.20
		2.0	2.85
		8.0	1.78

3.3. Hydrolysis of various protein substrates

The activity of *B. cereus* protease was tested with different soluble and insoluble proteinaceous substrates at 37 °C for 30 min. The enzyme hydrolysed azocasein and azocoll efficiently, exhibiting 216.04 and 355.63 nKat mg^{-1}, respectively, under similar conditions. The keratinolytic activity 12.45 nKat mg^{-1} was significantly lower than the obtained with the other protein substrates.

3.4. Determination of the cleavage specificity

The proteolytic specificity of *B. cereus* protease was determined by analysis of the peptides obtained after incubation with the oxidized insulin B-chain as substrate. Cleavage of oxidized insulin B-chain by the *B. cereus* protease at 37 °C was investigated using an enzyme-substrate in a molar ratio of 1:100. The reaction products after incubation from 30 min up to 24 h were analysed by MALDI-TOF mass spectrometry and identified from their corresponding molecular masses. From the peptides obtained after the hydrolysis of the oxidized insulin B-chain by *B. cereus* protease, the enzyme showed endopeptidase activity and a broad specificity, a total of seven peptide bonds in the B-chain of insulin were hydrolysed (Fig. 4). The complete hydrolysis was reached after 12 h incubation, however all larger cleavages were detected after 30 min incubation. The

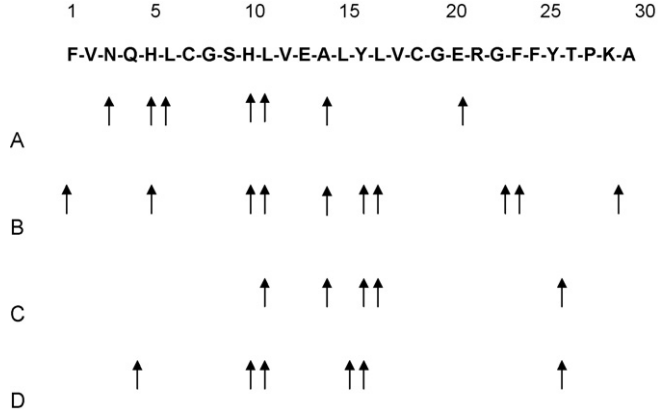

Fig. 4. Specificity of *B. cereus* protease (A) for oxidized insulin B-chain, in comparison to thermolysin (B) [37], camelysin (C) [12] and subtilisin (D) [36].

Table 4
Hydrolysis of peptide derivates by *B. cereus* protease

Substrate	Recommended use	Activity [nKat mg^{-1}]
L-Leucine-pNA	Leucine aminpeptidase	0.145
N-Succinyl-Ala-Ala-Pro-Phe-pNa	Chymotrypsin, Cathepsin G	0.216
N-Succinyl-Gly-Gly-Phe-pNA	Chymotrypsin, *S. griseus* protease B	0.010
N-Succinyl-Ala-Ala-Ala-pNA	Elastase	Negligible small
N-Succinyl-Ala-Ala-Pro-Leu-pNA	Elastase	0.069
N-Succinyl-Gly-Gly-Gly-pNA		0.015
N-(3-[2-Furyl]acryloyl)-Gly-Leu amide	Thermolysin	49.61
N-(3-[2-Furyl]acryloyl)-Ala-Phe amide	Thermolysin	203.45
Sar-Pro-Arg-pNA dihydrochloride	Thrombin	0.013
BAPNA	Trypsin	No activity
BAEE	Trypsin	No activity
BTEE		No activity
ATEE	Chymotrypsin	No activity
4-Nitrophenyl acetate	Esterase	No activity
4-Nitrophenyl butyrate	Esterase	No activity

major requirement is that a hydrophobic residue such as Leu, Val or Ala should participate with the N-amino group in the bond to be cleaved. However, hydrolysis of bonds at the N-terminal side of His, Cys and Glu was also observed. The residue in the bond by the carboxyl group may have a stimulating effect; the cleavage is favoured with polar groups at the N-terminal. This cleavage is different from other metalloproteases reported; the cleavage pattern is clearly different from that of thermolysin [37] and camelysin [12]. Only four cleavage sites were in common with thermolysin and two for camelysin (which is a metalloprotease from *B. cereus*). The peptide bond Leu^{15}-Tyr^{16} in the oxidized insulin B-chain, which is the major cleavage product obtained from the alkaline proteases are resistant to the attack of *B. cereus* protease and other neutral proteases [35,36].

3.5. Substrate specificities

Amino acids in the position P_1 have a strong influence in the catalytic action of the enzyme, see Table 4. The *B. cereus* protease showed preference for Leucine and Phenylalanine amino acids at the position P_1 and exhibited the highest activity for L-Leucine-pNA (0.145 nKat mg^{-1}), *N*-Succinyl-Ala-Ala-Pro-Phe-pNa (0.216 nKat mg^{-1}), L-Leucine-pNA and with *N*-Succinyl-Ala-Ala-Pro-Leu-pNA (0.069 nKat mg^{-1}). Low or very low hydrolysis was detected when Ala or Gly where the amino acids residues at position P_1 (*N*-Succinyl-Ala-Ala-Ala-pNA or *N*-Succinyl-Gly-Gly-Gly-pNA). However, for *N*-Succinyl-Gly-Gly-Phe-pNA, the rate of hydrolysis decreased indicating that the amino acid in position P_2 has influence in the catalytic activity.

B. cereus protease exhibited no esterolytic activity towards *p*-nitrophenyl esters. Shorter peptide substrates such as L-Leucine-pNA were good substrates for this enzyme.

To investigate the effects of the amino acid residues adjacent to the sensitivity for Leucine and Phenylalanine residues in peptide substrates more in detail, a kinetic study was undertaken using various synthetic peptides. These peptides were all split at the peptide bond containing the amino group of Leucine and Phenylalanine, see Table 5.

The nature of the amino acid in the position P_2 also influenced the specificity for the specificity in the position P_1. This can be seen from the difference of the kinetic parameters when comparing the substrates *N*-Succinyl-Ala-Ala-Pro-Phe-pNa (K_{cat}/K_m, 0.24 mM^{-1} s^{-1}) and *N*-Succinyl-Gly-Gly-Phe-pNA (K_{cat}/K_m 0.040 mM^{-1} s^{-1}); when Proline is substituted by Glycine, the K_{cat}/K_m decreases confirming the influence of amino acid residue present at position P_2. These characteristics of specificity have been found for thermolysin and for other neutral proteases from bacterial origin, which are optimally active at neutral pH and are inhibited by EDTA [37]. The results indicate that the *B. cereus* protease is very similar to thermolysin not only in the specificity against P_1 position but also with regard to the effects of amino acids residues neighbouring the site of cleavage. However, some differences where observed in the side chain specificity at P_2. At P_2 in *B. cereus* protease, Proline promotes the hydrolysis while for thermolysin this was not observed.

Table 5
Kinetic parameters of the BC protease with selected synthetic substrates

Substrate	V_{max} [nKat mg^{-1}]	K_m [mM]	K_{cat} [s^{-1}]	K_{cat}/K_m [mM^{-1} s^{-1}]
L-Leucine-pNA	0.158	0.187	0.079	0.42
N-Succinyl-Ala-Ala-Pro-Phe-pNa	0.371	0.785	0.19	0.24
N-Succinyl-Ala-Ala-Pro-Leu-pNA	0.244	1.253	0.12	0.097
N-Succinyl-Gly-Gly-Gly-pNA	0.129	2.626	0.065	0.025
N-Succinyl-Gly-Gly-Phe-pNA	0.115	1.335	0.058	0.040
N-(3-[2-Furyl]acryloyl)-Gly-Leu amide	262.73	2.363	131.30	55.60
N-(3-[2-Furyl]acryloyl)-Ala-Phe amide	406.50	0.858	203.15	236.84

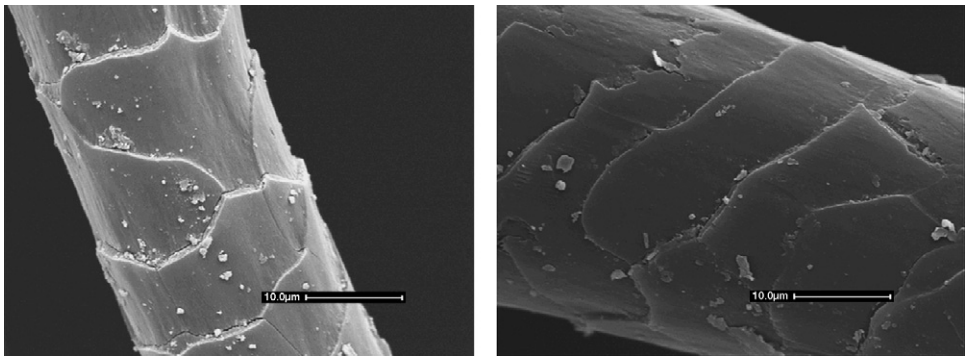

Fig. 5. High resolution scanning electron microscope image of wool surface after treatment with *B. cereus* protease (25,000× magnification).

Interestingly, Phe at the N-terminal side of the insulin B-chain did not show any effect on the hydrolysis while with the synthetic substrates the *B. cereus* protease show higher efficiency with Phe at this position. A possible explanation is due to the influence of other amino acids at positions P'_2, P'_3, etc., as well as at the positions P_2, P_3, etc. Based on its hydrolytic activity towards *N*-(3-[2-Furyl]acryloyl)-Gly-Leu amide and *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide, the *B. cereus* protease has identical substrate specificity as thermolysin, showing the highest kinetic for *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide (K_{cat}/K_m 236.84 $mM^{-1}\,s^{-1}$) and *N*-(3-[2-Furyl]acryloyl)-Gly-Leu amide (K_{cat}/K_m 55.60 $mM^{-1}\,s^{-1}$) as substrates. Except for its lower thermostability, the *B. cereus* protease has some similarity to thermolysin, with respect to substrate specificity and the presence of zinc (data not shown) in the molecule which is required for activity [33]. However, unlike thermolysin, it does not exhibit considerable elastolytic activity [34]. On the other hand, like thermolysin (pH optimum between 7 and 9) it shows a broad pH optimum between 6 and 8.5. This study somewhat clarified the effects of some several neighbouring amino acid residues surrounding the susceptible peptide bond on the appearance of the specificity of *B. cereus* protease.

The inhibition constant (K_i) was calculated with *N*-(3-[2-Furyl]acryloyl)-Ala-Phe amide as substrate at different inhibitor (EDTA) concentrations. This substrate was selected for the inhibition kinetics, due to the high affinity of the enzyme toward this substrate. One important consideration in this assay was that the substrate was kept constant by not allowing more than 5% of hydrolysis. The K_i value (0.971 mM) is almost the same as the K_m (0.858 mM) value of the corresponding substrate. On the other hand, the V_{max} (406.50 nKat mg^{-1}) significantly decreased (V_{imax} 213.93 nKat mg^{-1}), indicating non-competitive inhibition.

3.6. Hydrolysis of wool

Wool keratins are characterized by a high degree of cross-linked disulfide bonds, hydrophobic interactions and hydrogen bonds which stabilizes keratin filament structure [4]; which are very difficult to proteolytic attack. SEM analysis (Fig. 5) showed that *B. cereus* protease hydrolysed the cuticles on wool surface. An interesting effect is that somehow the enzyme "cracked" the wool surface.

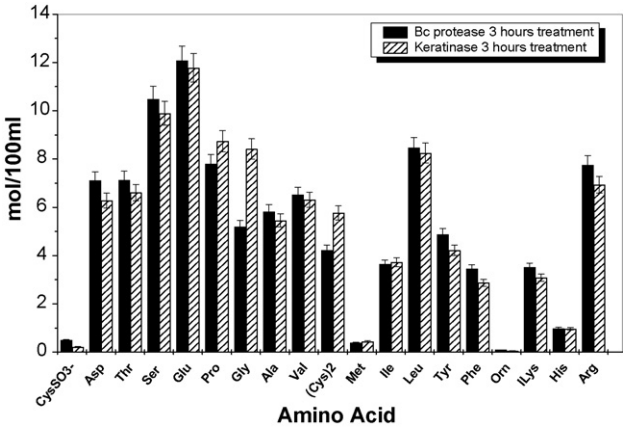

Fig. 6. Comparison of the amino acids composition of hydrolysed wool after 3-h treatment with *B. cereus* protease and keratinase. Standard deviation based on triplicate data.

The action of *B. cereus* protease on wool was done in comparison with a keratinase (Fig. 6), which are a group of proteolytic enzymes able to hydrolyse insoluble keratins more efficiently than other proteases. After the enzymatic treatment, the wool was completely hydrolysed and the amino acid analysed. Amino acid content analysis of wool after treatment with this protease shows a reduction of the cystine content, which is the main content of the wool cuticle, as well in the total amino acid content. The cystine content on wool after treatment with *B. cereus* protease is much lower after 3 h than with keratinase treatment. The amount of the total amino acids of wool after *B. cereus* protease treatment (75.3 nmol) is much lower than the one with keratinase treatment (88.9 nmol). This indicates that *B. cereus* protease has higher catalytic activity towards wool than keratinase. The results showed that there was no significant differences between *B. cereus* protease and keratinase-treated wool, which can be said that *B. cereus* protease is a keratinase-type.

4. Conclusion

In this work, several different bacteria growing on wool samples were isolated. *B. cereus* was selected based on the level protease production and growth toward wool.

B. cereus protease was purified to electrophoretic homogeneity using a combination of three chromatographic steps.

SDS-PAGE showed the homogeneity of the purified enzyme with a molecular weight of 45.6 ± 1.7 kDa.

The extracellular protease produced by *B. cereus* exhibits optimal pH at 7.0 and optimum temperature at 45 °C. This inhibition pattern is typical of metalloproteases. *B. cereus* protease has some similarity to thermolysin, with respect to substrate specificity, however the cleavage pattern of the oxidized insulin B-chain is clearly different from that of thermolysin.

Wool keratin is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin and papain. However, from SEM images of wool treated with *B. cereus* protease, it was clear that this enzyme is able to degrade the wool surface.

The results of the action of *B. cereus* protease toward wool showed that *B. cereus* protease is a keratinase-type.

The results presented here, including some thermostability and activity over a wide range of pH values, stability against some organic solvents and detergents, hydrolysis of various protein substrates (such as azocasein, azocoll, keratin azure and wool), endopeptidase activity and broad specificity, demonstrate that *B. cereus* protease will most probably play an important role for the application in the anti-shrinking process efficiently hydrolysing wool cuticles. However, as we have described before, increasing the molecular weight of this enzyme by chemical modification will be necessary to target its action to the wool surface [38].

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