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Characterization of a novel protease from *Bacillus cereus* and evaluation of an eco-friendly hydrolysis of a brewery byproduct

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Proteases and proteolytic enzymes constitute one of the most important groups of enzymes and are attracting worldwide attention in attempts to exploit their physiological and biotechnological applications. In this study, partial purifications and biochemical and antimicrobial characterizations of a protease from *Bacillus cereus* spp., originally isolated from fermented cabbage, were carried out. The crude extract obtained after purification, involving ammonium sulphate precipitation and dialysis, was designated as a partially purified protease (PPP). The obtained PPP had a specific activity of 0.395–2.539 U/g at 32 °C, with maximum activities for the fractions precipitated at 60 and 80% ammonium sulphate. The PPP activity ranged between 20 and 55 °C, with an optimum temperature at 40 °C. At 60 °C, the PPP retained more than 30% of its activity. The optimum pH for the PPP was achieved at pH 9, indicating the alkaline source of the enzyme. Protease production was specifically dependent on the calcium concentration in the culture medium. Also the robustness of the protease on brewer's spent grain hydrolysis was demonstrated. This suggests a potential eco-friendly application of the enzyme. Finally, it was found that the PPP inhibited the growth of *Escherichia coli* O157:H7. This novel property of the PPP liberated by the *B. cereus* spp. could provide important future benefits to industry. Copyright © 2015 The Institute of Brewing & Distilling

Keywords: antimicrobial activity; *Bacillus cereus*; partial purification; protease

Introduction

Proteases, also known as proteinases or proteolytic enzymes, constitute one of the most important groups of enzymes both industrially and academically. The global enzyme market is predicted to rise at a rate of 7% to US$8.0 billion in 2015 *(1)*. Proteases occur naturally in all organisms and belong to the hydrolase class of enzymes, with classification based on the source from which they are extracted, optimum activity, temperature, etc. They hydrolyse peptide bonds in aqueous solutions and synthesize them under non-aqueous conditions *(2)*. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications *(3)*.

Although proteases are produced by various plant, animal and microorganism sources, the latter are most widespread in nature, and are preferred owing to their fast growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. Microbial proteases are an important group of enzymes that have applications in various industries such as leather processing, food processing, pharmaceutical and bioremediation processes and the textile industry to removed protein-based stains *(4)*. Among the bacteria, *Bacillus* strains are the most important producers of commercial extracellular enzymes *(5)*, including proteases.

Although proteases generally work best under acidic conditions *(6)*, in an earlier study a *Bacillus cereus* strain, producing an alkaline protease, was isolated from fermented cabbage and the enzyme production was optimized under submerged conditions. Maximum enzyme production of the culture occurred at the mesophilic temperature of 32 °C and pH 8. No modifier additions were required in the fermentation medium *(7)*. Furthermore, the economic value of this isolate could be established after producing a high protease yield on an inexpensive medium consisting of brewer's spent grain, an agro-industrial by-product that stimulates high protease production *(8)*. While there have been numerous reports on the purification and characterization of extracellular proteases from bacteria *(1,2,6,9)*, very limited information has been published on the antimicrobial activity of these proteases. The scope for proteases, not only as hydrolytic enzymes, but also as an antibiotic for many infections and diseases, could have a huge impact in the field of food biotechnology. Mainly they could contribute to disinfection treatment of agro-industrial by-products or wastes in order to reduce competing microflora.

In view of the above facts, in the present investigation an attempt was made to partially purify and characterize the proteases produced by *B. cereus* spp. First, a selective precipitation with ammonium sulphate was performed to fractionate the crude enzyme of *B. cereus* spp., thus obtaining a partially purified protease

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(PPP). Then a conventional biochemical characterization was performed to find the optimal conditions (temperature, substrate concentration, pH, etc.). Also the ability of the protease fractions to hydrolyse brewer's spent grain was evaluated. Finally, the antimicrobial activity of these proteases on different human pathogens was studied. The novel properties of the proteases liberated by this strain could have important benefits for the biotechnology industry.

Materials and methods

Bacterial strain

The strain of *B. cereus* used in this study was isolated from fermented cabbage and has previously demonstrated high proteolytic activity *(10)*. The culture was routinely maintained on soft Brain and Heart Infusion (BHI) agar (35 g/L of agar) at −18 °C. Subcultures were performed every 6 months.

The strain was activated in two steps. First a loopful of B. *cereus* spp. was subcultured in 8 mL BHI and incubated at 32 °C for 24 h. Subsequent to which 2 mL of the culture was centrifuged at 1000 rpm for 3 min at 4 °C. The precipitate was then added to 25 mL of fresh BHI and incubated statically using the above described conditions.

Protease production

Five millilitres of an overnight culture of *B. cereus* strain was used to inoculate a 250 mL Erlenmeyer flask containing a 100 mL aliquots of Minimal Broth [consisting of (mg/L)–0.001 bacteriological glucose (Britania, lot no. 095, Buenos Aires, Argentina) and 0.0025 yeast extract (Acumedia, lot no. 66-22, Maryland, USA)] *(10)* buffered at pH 8 with Tris–HCl 0.2 M. The inoculated flask was incubated at 37 °C for 24 h. The bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The obtained supernatant, named crude extract (CE), was filtered using a 0.22 μm filter (Merck Millipore, Millipak 40) and this supernatant was used as the starting point for purification.

Evaluation of proteolytic activity

Proteolytic activity of the PPP samples was assessed using azocasein as the substrate. Briefly, 120 μL of the sample was incubated with 480 μL of 10 g/L azocasein in 0.2 M Tris–HCl buffer, pH 8, for 30 min at 32 °C. The reaction was stopped by the addition of 480 μL of trichloroacetic acid to a final concentration of 100 g/L and incubated for 30 min at 4 °C before being centrifuged at 10,000 rpm for 10 min. Then 800 μL of the supernatant from the centrifuged reaction was added to 200 μL of 1.8 M sodium hydroxide and the absorbance at 420 nm was measured using a Spectrum SP-2000 UV (Zhejiang, China) spectrophotometer.

In the control sample, the reaction was stopped with trichloroacetic acid immediately after the supernatant was added. One enzyme activity unit (U) was expressed as the amount of enzyme that causes an absorbance change of 0.01 at 420 nm under the assay conditions (30 min at 32 °C). Proteolytic activity values are expressed as U/mL. The specific activity is expressed in unit of enzyme activity/gram of protein (U/g).

Qualitative proteolytic activity assay was evaluated in Modified Basal Medium supplemented with 6.2 g/L protein of skim milk plates (5 g/L of casein) *(10)*.

Wells, 5 mm in diameter, were cut under sterile conditions into the agar plates. A 20 μL aliquot of the growth culture was placed into the well. The plates were incubated aerobically at 32 °C for 48 h and bacterial proteolytic activity was observed daily. Proteolytic activity from *B. cereus* spp. was detected on the basis of the appearance of clear zones around the bacterial colonies (hydrolysed zones). A clear zone (halo) in the medium surrounding the bacterial growth (6 mm or more, measured from the well centre) indicated positive proteolytic activity *(10)*. Proteolytic activity was negative when no visible clearing halo was present.

Partial purification of protease

The protease obtained from the *B. cereus* strain was purified through ammonium sulphate precipitation of the culture broth containing the extracellular enzyme, named CE.

Ammonium sulphate fractionation

The supernatant was first brought to 20% saturation by the gradual addition of solid ammonium sulphate while stirring at 4 °C in an ice bath. After a 30 min equilibration period, the precipitated protein was obtained by centrifugation for 15 min at 15,000 rpm at 4 °C. The obtained pellet was re-suspended in a 10 mL of ice-cold 0.2 M Tris–HCl buffer pH 8. Centrifugation was repeated and the pellet was re-suspended in Tris–HCl buffer (0.2 M, pH 8) and dialysed against the same buffer overnight at 4 °C in order to determine both the enzyme activity and the protein content. The remaining supernatant was supplied again with ammonium sulphate in order to achieve 40, 60, 80 and 100% saturation of ammonium sulphate precipitation. A sample–dialysed buffer ratio of 1:1000 (vv) was used at each step. Fractions named P_1, P_2, P_3, P_4 and P_5 were obtained by precipitation with 20, 40, 60, 80 and 100% ammonium sulphate, respectively. The enzyme activity and protein content were determined for each separate fraction, according to the procedures described below.

Dialysis against Tris–HCl buffer

The supernatant was transferred into a special cellulose membrane (Spectra/Por, 3500 Da MWCO) for dialysis, which was previously cut into the desired length, with some extra length for the knots. Two knots were tied at one end, close together, and then filled with the enzyme filtrate, and then the filled sack was covered with 0.2 M Tris–HCl buffer pH 8 in a jar. This external buffer was changed every 4 h during the dialysis process until the enzyme solution volume inside the plastic bag reached a constant value. After dialysis was completed, the sack was carefully punctured and emptied into a beaker.

Fermentation medium

Brewer's spent grain (BSG) (~32.5% protein on a dry basis) was purchased from Antares S.A. (Mar del Plata, Argentina). The BSG was pre-treated according to the procedure described in Kotlar et al. *(11)* in order to standardize the BSG and to prepare the fermentation medium (FM) comprising the following: 0.32 g/L pre-treated BSG in 0.2 M Tris–HCl buffer, pH 8. Briefly, the following protocol was set in order to obtain the FM: drying at 60 °C for 24–48 h; sieving; grinding; polyphenol extraction with an alcohol–water solution (30:100); and, finally autoclaving (121 °C for 15 min).

Preliminary characterization of protease

The optimum temperature for enzyme activity was determined using azocasein as the substrate by conducting the assay at a constant pH of 8 at various temperatures from 20 to 60 °C. Before the addition of the PPP, the substrate was pre-incubated at the respective temperatures. After precipitation with ammonium sulphate, the most active enzyme fractions were collected and called PPP. For temperature stability, PPP was pre-incubated in 0.2 mM Tris–HCl buffer, pH 8, at different temperatures (20–60 °C) for 30 min, then the residual proteolytic activity was determined as described below.

The pH effect on the protease activity was determined by incubating the reaction mixture for 10 min at pH values ranging from 4 to 12 in the following buffer systems: phosphate buffer (20 mM) from pH 4.0 to 6.0; Tris–HCl buffer (20 mM) from pH 7.0 to 9.0; Na_2CO_3–NaOH (20 mM) from pH 10.0 and 11.0; and glycine–NaOH buffer (50 mM) from pH 12.0. The proteolytic activity was measured.

To evaluate the effect of different substrate concentrations on the PPP activity, azocasein was applied at concentrations of 5–25 g/L. The proteolytic activity was then determined.

To evaluate the effect of different PPP concentrations on the proteolytic activity, PPP samples were assayed at concentration ranges from 10 to 500 mL/L. The enzyme activity was determined.

The PPP samples as holoenzymes were converted to apoenzymes by the method of Coolbear et al. *(12)*. The samples were dialysed against 25 mM Tris–HCl, pH 8, containing 10 mM EDTA for 12 h with one buffer change. This was followed by further dialysis against buffer 20 mM Tris–HCl, pH 8, made up in distilled water, for 24 h with three buffer changes. All dialysis steps were performed at 4 °C using 3500 Da cut-off dialysis tubing (Spectra/Pro, Spectrum Laboratories, Inc.).

The dialysed apoenzymes were preincubated with various metal ions: Ca^{2+}, Na^{+}, Fe^{3+}, Li^{+} and Zn^{2+} (from $CaCl_2$, NaCl, $FeCl_3$, LiCl, $ZnCl_2$) at 1 mM concentration in 20 mM Tris–HCl buffer pH 8, for 15 min. After that, 0.1 mL samples of enzyme solution were used for proteolytic activity determination. The proteolytic activity of the original holoenzymes was defined to be 100%. The effect of NaCl on proteolytic activity was determined in the presence of 0–4 M NaCl in the enzyme reaction mixture. The enzyme activity was determined.

Estimation of kinetic parameters

The steady state kinectic constants were determined by the Lineweaver–Burk transformation of the Michaelis–Menten equation *(13)*.

Enzyme inactivation parameters

Immediately after the enzyme reaction had taken place, the medium was heated at five temperatures (50, 80, 85, 90 and 95 °C) for 5 min in a water bath to inactivate the protease and then rapidly cooled to room temperature. The mixture was centrifuged at 2000 ***g*** at 4 °C for 10 min and the supernatant was collected. In the sonication test, the mixture (50 °C) was transferred into a sample bag and treated with an ultrasonic unit (Testlab S.R.L., Buenos Aires, Argentina) at 40 kHz, amplitude of 20 μm, and initial input power of 100 W.

A *D*-value, the time required to decrease the initial enzyme activity by 90%, was used in the kinetic studies, and was calculated from the negative reciprocal of the slope on an inactivation curve *(14)*, as given by:

$$\log\left(\frac{A}{A_0}\right) = -\frac{t}{D} \quad (1)$$

where A is the activity of protease at time t; A_0 is initial protease activity; and D is the D-value (decimal reduction time). A z-value, the increase in temperature to cause 90% reduction in D-value, is also used to describe the temperature sensitivity of the enzyme under different treatment conditions.

Evaluation of antimicrobial activity

The sensitivity of human pathogenic microorganisms to the protease produced by *B. cereus* spp. was determined by the agar diffusion method *(15)*. Five millimetre-diameter wells were made on the surface of the inoculated BHI agar, with 0.1 mL of inoculum containing the indicator microorganisms in the range of 10^6 CFU/mL (colony-forming units), using a sterile corer. The microorganisms used as indicators were *Listeria monocytogenes* (Gram-positive indicator) provided by Centro de Referencia para Lactobacillus and *Escherichia coli* O157:H7 isolated from meat (Gram-negative indicator). These organisms were grown individually in nutrient broth for 24 h at 37 °C.

Aliquots (20 μL) of partially purified protease were added into the wells. Distilled and sterile water was utilized as a control. The plates were pre-incubated without inverting at 4 °C for 3 h to allow the test material to diffuse into the agar, after which the plates were incubated at 37 °C for 24 h. After the incubation, the plates were examined for clearance zones around the individual wells. The sensitivities to the different PPP were classified by the diameter of the inhibition halos as follows: not sensitive (−) for diameters <8 mm; sensitive (+) for diameters 9–14 mm; very sensitive (++) for diameters 15–19 mm; and extremely sensitive (+++) for diameters >20 mm.*(15)*

Evaluation of soluble protein content

Total soluble protein content in the fermentation broth was determined according to the method of Lowry et al. *(16)*, using the Folin Ciocalteu phenol reagent (Sigma-Aldrich) and bovine serum albumin as standard.

Thermal properties

Thermal characteristics of hydrolysate BSG samples were determined with a Shimadzu DSC50 instrument (Shimadzu Corporation, Kyoto, Japan). BSG samples collected from the FM after enzymatic hydrolysis by P_1, P_2, P_3, P_4 and P_5 (about 1 mg each) were directly weighed into coated aluminium pans. The aluminium pans were hermetically sealed and heated from 30 to 220 °C at a rate of 10 °C/min. A sealed empty pan was used as the reference. Thermal denaturation temperature (T_d) and denaturation enthalpy were calculated from thermograms with the thermal analysis software (Thermal Analysis Instruments TA-60, MD, USA).

Statistical analysis

Each assay was performed in duplicate in three independent experimental runs and data were averaged. Data were analysed using SAS software version 8.0 (SAS 1999; PROC GLM, general

linear model procedure). Mean differences were judged at the 5% significance level. The Tukey–Kramer multiple comparison test was used for pair-wise comparison when significant differences were found.

For further characterization, the fractions that showed the highest specific enzyme activity (P_3 and P_4) were pooled and designated as PPP. In order to evaluate the optimal conditions for the PPP, composed of the fractions P_3 and P_4, a classical biochemical characterization was performed.

Results and discussion

Fraction proteolytic activities

The proteolytic activity of the crude protease enzyme was 312.92 U/mL with a specific activity of 33.28 U/mg (Table 1). After ammonium sulphate precipitation, the proteolytic activity and the soluble protein content of P_1, P_2, P_3, P_4 and P_5 were measured. Fractions P_3 and P_4 presented higher enzymatic activity (511.25 and 434.17 U/mL, respectively). Since the $(NH_4)_2SO_4$ precipitation process removed several other enzymes and proteins, these fractions also presented the maximum specific activity (2313.35 and 2539.01 U/g, respectively).

Taking into account the specific activity increase, the precipitation step reached a purification of almost 77-fold. Also these fractions (P_3 and P_4) together with P_5 presented the highest qualitative proteolytic activity with halo diameters ranging between 26 and 28 mm, without significant differences between them ($p < 0.05$).

Rao et al. *(3)* reported a lower degree of purification (2.2-fold) with 60% saturation using an extracellular protease from *Bacillus circulans*.

Temperature effect on proteolytic activity and stability

Temperature is a critical factor for maximum enzyme activity. For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme *(17)*. The temperature effect on the activity and the stability of PPP were analysed between 20 and 60 °C (Fig. 1). The results showed an optimal activity at 40 °C and also good stability between 40 and 50 °C. When the reaction temperature was >50 °C, there was a sharp activity decrease and <30% of the maximum activity was retained. Therefore the PPP can be classified as a mesophilic enzymatic fraction. High and low temperatures may affect structural and functional changes in proteins to modify biological behaviour by altering rates of enzyme activities, which may have important consequences for the integration of biochemical pathways; denaturation events could unfold proteins or cause subunit dissociation. The conformational stability of proteins results from a complex balance of individual forces (hydrogen bonding, electrostatic or hydrophobic interactions), which vary in different ways with temperature. Therefore, all proteins are stable only within a limited temperature range. Muthu and Christudhas found for a

Table 1. Ammonium sulfate fractionation pattern of peptidase by *Bacillus cereus* spp

Sample		$(NH_4)_2SO_4$ saturation	Qualitative proteolytic activity (mm)[a]	Proteolytic activity (U/mL)	Soluble protein (mg/mL)	Specific activity (U/mg)
Crude enzyme		0	18.6^{C}	312.92^{D}	9.404	33.28^{D}
Precipitated protein	P_1	0–20%	22.3^{B}	295.00^{D}	0.560	526.79^{B}
	P_2	20–40%	22.5^{B}	369.17^{C}	0.810	455.77^{C}
	P_3	40–60%	27.4^{A}	511.25^{A}	0.221	2313.35^{A}
	P_4	60–80%	28.3^{A}	434.17^{B}	0.171	2539.01^{A}
	P_5	80–100%	26.0^{A}	363.75^{C}	0.921	395.95^{C}

Values with different upper-case letters in each column are significantly different ($p < 0.05$).
[a]Mean diameter represents a qualitative measure of the proteolytic activity.

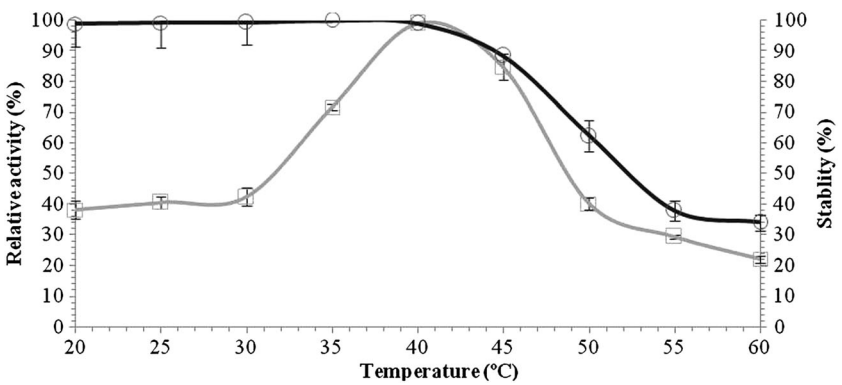

Figure 1. The temperature effect on the activity and the stability of partially purified protease between 20 and 60 °C.

partial purified protease from *Bacillus subtilis* that its optimum temperature was 37 °C and it remained stable within the range of 30–60 °C *(18)*.

As shown in Fig. 1, PPP had >90% of the initial activity at 20, 30 and 40 °C, and about 60% of the initial activity at 50 °C. After incubation at 60 °C for 30 min, PPP had 35% of its initial activity.

pH effect on proteolytic activity

The influence of pH on the enzyme activity of PPP is shown in Fig. 2. The enzyme was active over the pH range studied. The activity of PPP was very low at a pH between 4 and 6, and finally increased sharply beyond pH 7.

The PPP had its maximum activity at pH 9 (100%) and retained 50% of its maximum activity at pH ranges 7–8 and 10–11. Enzymes possess many ionizable groups so pH changes can alter the conformation of the enzyme *(19)* and the catalytic sites. The optimum pH for alkaline proteases of *Bacillus* sp. has been reported to vary from 8 to 11 and they are species specific *(20)*.

Effect of substrate concentration

Optimum substrate concentration for maximum PPP activity was determined in terms of the Michaelis–Menten kinetic constant (K_M) and the maximum velocity (v_{max}) using azocasein as a substrate. The v_{max} represents the maximum rate of enzymatic reaction when the enzyme is saturated with substrate and the K_M expresses the affinity of the enzyme for its substrate *(21)*. The K_M and v_{max} of PPP from *B. cereus* spp. were 14.5 g/L and 1250 U/mL, respectively. On the other hand, Ahmed et al. *(22)*. found from the catalytic properties that the K_M and v_{max} values of purified alkaline protease from *Bacillus subtilis* were 58 μM and 148 U/mL respectively. An enzyme with a low K_M has a greater affinity for its substrate. Adinarayana et al. *(23)*. reported that protease has a high level of hydrolytic activity against casein as substrate and poor to moderate hydrolysis of BSA and egg albumin, respectively. Patel et al. *(24)*. reported a K_M and v_{max} of protease of 0.153 g/100 mL and 454 U/mL respectively.

Effect of enzyme concentration

An experiment was carried out to determine the effect of enzyme protease concentrations on the PPP enzyme activity. The original pool was diluted five times, and the activity recorded (Table 2).

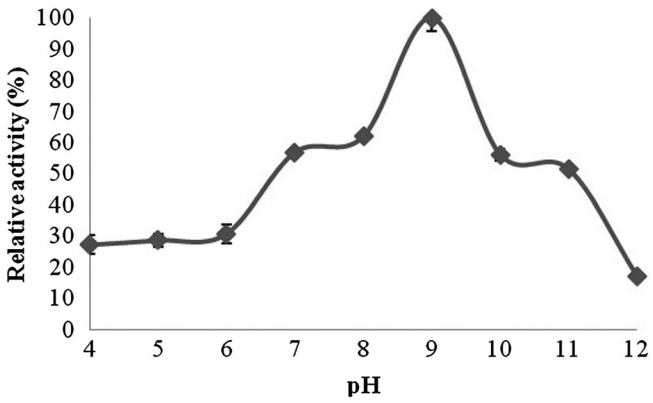

Figure 2. The influence of pH on the enzyme activity of partially purified protease between pH 4 and 12.

Table 2. Effect of enzyme concentration on the activity of the partially purified protease (PPP) produced by *B. cereus* spp

PPP concentration (%v/v)	Enzyme activity (U/mL)
10	502.17 ± 14.32^{D}
20	528.96 ± 12.97^{C}
30	643.17 ± 14.68^{B}
40	704.56 ± 12.32^{A}
50	718.80 ± 25.41^{A}

Values with different upper-case letters are significantly different ($p < 0.05$).

The enzyme activity was concentration dependent and a continuous increase in enzyme concentration increased the proteolytic activity to a concentration of 40% v/v PPP. After this point, the reaction rate no longer depended on the concentration. There was a significant decrease in the proteolytic activity with the increase in enzyme dilution and this result was in agreement with the study reported by Senthilraja and Saravanakumar *(25)* working with a purified protease of *B. cereus* from mangroves.

Effect of metal ions on proteolytic activity

The effects of metal ions on the proteolytic activity of PPP were determined by the addition of metal components to the apoenzyme (Table 3). The conversion of the holoenzyme to apoenzyme form was accompanied by a reduction in the proteolytic activity to 40.14%. The addition of the Na^+ metal ion had no effect on the apoenzyme activity, while Fe^{3+}, Zn^{2+} and Li^+ metal ions decreased this activity. However, Ca^{2+} addition restored the activity of PPP to about 80% of the initial holoenzyme activity. This indicates that Ca^{2+} has a functional role in the molecular structure of the enzyme and suggests that this metal ion is essential for the induction of protease production and/or enzyme stabilization after synthesis. This finding is in line with several earlier reports showing that the three-dimensional structure of the protease from *B. cereus* contains four Ca^{2+} binding sites *(26)*.

A reduction to about half the value of the apoenzyme activity was detected when adding Fe^{3+} and Li^+ ions. Also the addition of Zn^{2+} ions caused a higher inhibition effect (Table 3). Mehrnoush et al. *(27)*. also reported similar trends for an alkaline-thermostable protease enzyme from Pitaya (*Hylocereus polyrhizus*). The activity of the enzyme was inhibited by Fe^{2+} and Zn^{2+}, while protease activity was increased in the presence of Ca^{2+}. *(28)*. The inactivation of the

Table 3. Activity of partially purified peptidase with and without metal ions

Preparation	Relative activity (%)
Holoenzyme	$100\%^{A}$
Apoenzyme	$40.14 \pm 2.50\%^{C}$
Apoenzyme + 1 mM Ca^{2+}	$79.55 \pm 4.10\%^{B}$
Apoenzyme + 1 mM Na^+	$41.81 \pm 1.95\%^{C}$
Apoenzyme + 1 mM Fe^{3+}	$32.68 \pm 2.83\ \%^{D}$
Apoenzyme + 1 mM Li^+	$31.37 \pm 2.55\%^{D}$
Apoenzyme + 1 mM Zn^{2+}	$24.80 \pm 1.37\%^{E}$

Values with different letters are significantly different ($p < 0.05$).

enzyme by these metal ions may be due to their binding to the catalytic residues in the active site of the enzyme *(29)*.

Effect of NaCl concentration on proteolytic activity

The proteolytic activity measured in the presence of NaCl in the concentration range of 0–4 M represented the maximum activity retention (almost 100%) at 0–0.5 M. This result indicates that PPP differ from extreme halophylic proteases. At an NaCl concentration of 2 M, the proteolytic activity remained at 50%, and at higher NaCl concentrations the activity retention was lost. Gupta et al. *(29)* reported that haloalkaliphilic *Bacillus* sp. enzyme activity was slightly increased at 0.03 M NaCl. However, on increasing the salt concentration, reduction was observed up to 70 or 83%, depending on the enzymes; 392 were in their purified or crude state. On the other hand, the protease produced by *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* 321 101 still retained 65, 75 and 50% activity at 10% NaCl concentration and 21, 35 and 22% at 20% NaCl concentration, respectively *(30)*.

PPP inactivation

The residual PPP activity (A/A_0) after temperatue treatment is shown in Table 4. The data fit well to a first-order model. It can be seen that at 50 °C, enzyme inactivation caused by heating is negligible, as indicated by a *D*-value of 62.89 min. When the temperature increased from 60 to 95 °C, the time needed to achieve 90% reduction in PPP residual activity was also reduced. These results are in accordance with the reduction time reported for *B. cereus (31)*, which was in the range of 1.5–36.2 min. A *z*-value of 2.74 °C was found for *B. cereus* spp.

The ultrasound treatment combined with temperature (50 °C) effectively increased the PPP inactivation compared with a thermal treatment at the same temperature. Comparing thermal inactivation with the sonication test, it can be seen that sonication substantially decreases PPP activity at 50 °C. When ultrasound was applied, a significant decrease in the *D*-value was observed, indicating an 11-fold increase in inactivation. When ultrasound waves are passed through a liquid substance or a biological substance such as food material, alternating regions of high and low pressure, that is, compression and expansion, respectively, are created, which induce cavitation and form gas bubbles. The vigorous cavitation created in the sonicated medium and the simultaneous effect of heat may cause PPP denaturation, resulting in a decrease in the catalytic efficiency *(32)*.

Table 4. *D*-values from partially purified peptidase inactivation by thermal and ultrasound treatment

Treatment	Temperature (°C)	*D* (min)
Thermal	50	62.89
	60	41.72
	70	27.30
	80	7.62
	90	2.24
	95	1.69
Ultrasound	50	5.60

Antimicrobial activity

After ammonium sulphate precipitation and dialysis, the susceptibility of the human pathogens to the different protease fractions (P_1, P_2, P_3, P_4 and P_5), was determined by the agar diffusion method. P_4 was the protease fraction with the highest inhibitory effect against *E. coli*, with an inhibition halo of 34.17 ± 1.89 mm in diameter. The susceptibility of the pathogen to P_1, P_2, P_3, P_4 and P_5 was also appreciable, with *E. coli* inhibition zones with diameters of 10.17 ± 1.04, 8.33 ± 0.58, 10.17 ± 0.76, 8.17 ± 0.76 and 17.33 ± 1.53 mm, respectively.

On the other hand, *Listeria monocytogenes* was less susceptible to P_1, P_4 and P_5, with inhibition halo diameters <8 mm. Finally, no inhibition zones were detected for P_2 and P_3 against *L. monocytogenes*, indicating that these proteases do not have any apparent effect against this pathogen.

As shown in the results, both P_4 and P_5 presented the highest inhibition halos against *E. coli*, especially P_4, which is a fraction that presented both proteolytic activity and antimicrobial properties. Since P_5 presented only antagonistic effects, it is possible that the *B. cereus* strain generates proteases and biopeptides with antimicrobial properties. Probably these peptides are bacteriocins with antagonistic effects against some bacteria *(33)*. These features make this bacteriocin potentially interesting as an antimicrobial agent for the control of *E. coli* O157:H7 in foods.

The decrease of the antimicrobial activity in P_3 could be caused by the bacteriocin's relative sensitivity to extracellular proteases. Ghanbari et al. *(34)* reported a similar trend owing to a partial digestion of the antagonistic compound by proteolytic enzymes released from the cells, losing the antimicrobial properties.

It has been reported that *Bacillus* secretes many enzymes that degrade slime and biofilms, thus allowing the bacterium to penetrate the slime layer around Gram-negative bacteria *(35)*.

Proteolytic activity of PPP on FM

In previous work, the ability of *B. cereus* spp. and its crude extracellular protease to hydrolyse and transform a FM containing BSG to soluble protein through a submerged fermentation process was demonstrated *(11)*. In this study, the proteolytic activity of the fractions P_1–P_5 was established, as well as the activity on the pools containing the combinations of these fractions and on the CE.

Table 5 presents the content of the soluble protein and the proteolytic activity in FM. There was a positive relationship between protein content and proteolytic activity in FM.

The higher soluble protein content and proteolytic activity were achieved using the $P_3 + P_4$ combination. An ~74% higher soluble protein content and 200% higher proteolytic activity were found for the binomial $P_3 + P_4$ compared with the use of crude enzyme. This shows the advantage of using the purified enzymes over the crude enzymes in the fermentation medium.

Thermal characterization of hydrolysed BSG proteins

Differential scanning calorimetry (DSC) is a sensitive technique for studying thermal denaturation and conformational transitions of proteins and it has been used extensively in various food systems *(36)*. The DSC thermogram data for the BSG hydrolysated fractions is presented in Table 6.

An endothermic denaturation transition was observed in the DSC thermograms. Therefore, the result appears to suggest that

Table 5. Soluble protein content and proteolytic activity of P_1, P_2, P_3, P_4 and P_5 and combinations of these fractions and crude extract (CE) on a fermentation media containing brewer's spent grain

Sample	Proteolytic activity (U/mL)	Soluble protein (mg BSG/mL)
CE	396.42 ± 10.08^{I}	1206.42 ± 100.78^{D}
P1	472.28 ± 36.88^{H}	947.39 ± 36.21^{F}
P1 + P2	467.98 ± 28.16^{H}	961.09 ± 12.36^{F}
P1 + P2 + P3	507.92 ± 47.83^{G}	976.58 ± 76.34^{E}
P1 + P2 + P3 + P4	680.24 ± 55.71^{E}	1341.32 ± 102.64^{C}
P2	416.97 ± 35.08^{I}	725.69 ± 71.46
P2 + P3	540.71 ± 20.18^{F}	1029.50 ± 91.48^{E}
P2 + P3 + P4	761.32 ± 21.00^{D}	1680.71 ± 69.49^{B}
P2 + P3 + P4 + P5	801.24 ± 75.65^{D}	1678.56 ± 167.8^{B}
P3	991.63 ± 72.81^{B}	1041.66 ± 71.22^{E}
P3 + P4	1190.42 ± 59.64^{A}	2194.00 ± 15.76^{A}
P3 + P4 + P5	963.21 ± 32.24^{B}	2097.55 ± 148.67^{A}
P4	860.91 ± 78.94^{C}	1176.30 ± 41.78^{C}
P4 + P5	950.73 ± 64.65^{B}	1329.08 ± 64.11^{C}
P5	512.16 ± 21.32^{F}	1010.64 ± 67.71^{E}

The numbers marked with different superscript letters in the same row indicate a statistical difference at a significance of level 5%.

Table 6. Data obtained from differential scanning calorimetry (DSC) curves

	Peptidase fraction				
	P_1	P_2	P_3	P_4	P_5
Moisture content (%)	12.00	13.25	13.19	12.64	12.22
T_{peak} (°C)	107.25	110.69	104.89	102.87	102.68
T_{onset} (°C)	72.22	79.84	74.87	61.49	64.21
T_{endset} (°C)	152.02	144.68	140.93	144,28	152.56
ΔH (J/g)	−167.92	−120.25	−113.94	−197.44	−152.48

the BSG after enzymatic hydrolysis had a unique protein fraction, corresponding to the 11S fractions of barley protein.

Increasing severity of enzymatic treatment resulted in the disappearance of the 7S fraction, while the globulin 11S was still present after hydrolysis. Also, the greater stability of the 11S fraction could have contributed to a greater resistance to unravelling of the polypeptide chains, and consequently to enzymatic attack, which could explain the presence of this fraction in the BSG hydrolysated samples. A possible explanation for the different enthalpy values found could be associated with the effectiveness of the protease fractions to hydrolysate the BSG proteins. Higher values of enthalpy are associated with higher contents of native or little denatured proteins. However, protease fraction P_3, which had the lower enthalpy, was the most single efficient fraction in the BSG enzymatic hydrolysis treatment.

Conclusions

A PPP, from *B. cereus* spp. isolated from fermented cabbage, was obtained after several purification steps involving ammonium sulphate precipitation and dialysis. The most active enzyme fractions were obtained at 40–60 and 60–80% ammonium sulphate concentration, with specific activities from 0.395 to 2.539 U/g at 32 °C. The optimum temperature for PPP activity was 40 °C, while the activity retention was about 30% at 60 °C. The extracellular protease produced by *B. cereus* exhibits optimal activity at pH 9.0 indicating the alkaline source of the enzyme. The protease production was dependent on the calcium concentration in the culture medium; however a reduction to about half the value of the protease activity was detected when adding Fe^{3+} and Li^{+} ions. Also the addition of Zn^{2+} ions caused a higher inhibition effect. The novel protease was able to hydrolyse the main by-product of the brewery industry, indicating the potential application of the enzyme with recyclable capacity.

The results presented here, including some thermostability and activity over a wide range of pH values, hydrolysis of BSG and broad antimicrobial activity against *E. coli*, also demonstrated that the *B. cereus* protease could probably play an important role in the application in the food industry process owing to the efficient hydrolysis of BSG. Further purification steps and studies are required and are in progress.

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