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Two-Step Purification and Partial Characterization of Keratinolytic Proteases from Feather Meal Bioconversion by *Bacillus* sp. P45

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Abstract: This study aimed to purify and partially characterize a keratinolytic protease produced by *Bacillus* sp. P45 through bioconversion of feather meal. Crude protease extract was purified using a sequence of an aqueous two-phase system (ATPS) in large volume systems (10, 50, and 500 g) to increase obtaining purified enzyme, followed by a diafiltration (DF) step. Purified protease was characterized in terms of protein profile analysis by SDS-PAGE, optimum temperature and pH, thermal deactivation kinetics at different temperatures and pH, and performance in the presence of several salts (NaCl, $CaCl_2$, $MnCl_2$, CaO, $C_8H_5KO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeCl_3$) and organic solvents (acetone, ethanol, methanol, acetic acid, diethyl ether, and formaldehyde). ATPS with high capacities resulted in purer protease extract without compromising purity and yields, reaching a purification factor up to 2.6-fold and 6.7-fold in first and second ATPS, respectively, and 4.0-fold in the DF process. Recoveries were up to 79% in both ATPS and reached 84.3% after the DF step. The electrophoretic analysis demonstrated a 25–28 kDa band related to keratinolytic protease. The purified protease's optimum temperature and pH were 55 °C and 7.5, respectively. The deactivation energy (E_d) value was 118.0 kJ/mol, while *D* (decimal reduction time) and *z* (temperature interval required to reduce the *D* value in one log cycle) values ranged from 6.7 to 237.3 min and from 13.6 to 18.8 °C, respectively. Salts such as $CaCl_2$, CaO, $C_8H_5KO_4$, and $MgSO_4$ increased the protease activity, while all organic solvents caused its decrease. The results are useful for future studies about ATPS scale-up for enzyme purification and protease application in different industrial processes.

Keywords: microbial protease; purification; stability; thermal deactivation

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1. Introduction

Proteases are hydrolytic enzymes that act as biocatalysts for breaking protein into smaller peptides and amino acids [1]. They hold a market share of about 60% of the global enzyme owing to their versatile application in different corporate sectors, including the detergent, textile, leather, food, feed, waste management, and pharmaceutical sectors [2,3]. Unlike plants and animals, microorganisms stand out as protease sources because of their rapid growth, minimum space required for cultivation, and ease of being genetically manipulated [1,2]. Among different producers of proteases, the *Bacillus* genus is a prominent source capable of producing high yields of proteases even when low-cost agro-industrial substrates are used in cultivation [4–6]. Additionally, several *Bacillus* proteases present remarkable properties, such as high stability at extreme temperatures and pH and in organic solvents, detergents, and oxidizing compounds [7].

The strain *Bacillus* sp. P45, isolated from the Jaraqui fish (*Piaractus mesopotamicus*) intestine and recognized as safe (GRAS) [8], produces proteases with high keratinolytic activity [9] and good applicability in keratin-rich hydrolysis substrates [10] and as a technological agent in food products [11,12]. Previous studies have demonstrated that *Bacillus* sp. P45 can produce keratinolytic proteases in media containing low-cost substrates, such as feather meal [5,9], a by-product of poultry processing generated inevitably in the millions of tons annually. Other studies focused on the purification of *Bacillus* sp. P45 proteases by aqueous two-phase systems (ATPS) [13], a simple, selective, and scalable method formed by mixing two incompatible aqueous solutions [14]. Both low-cost production and scalable purification are important for the further application of protease in biotechnological processes. Characterizing purified proteases is the next step that gives information about their industrial performance, including protein profile analysis, overall stability at different pH values, temperatures, and response to regulatory molecules that increase or decrease their activity [15–17].

This study aimed to purify and partially characterize a keratinolytic protease produced by *Bacillus* sp. P45 through bioconversion of feather meal. First, a sequence of two ATPS steps from large volume systems, followed by diafiltration (DF), was used to purify the protease extract. Then, the influence of temperature, pH, salts, and organic solvents on the purified enzyme's activity was evaluated. The protein profile, purified protease's thermal stability, and deactivation kinetics at different temperatures and pH values were also investigated.

2. Materials and Methods

2.1. Materials

The feather meal was supplied by a poultry processing plant located in the southern region of Rio Grande do Sul, Brazil. All of the chemicals used in the investigation were of analytical grade: NaCl, $C_8H_5KO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, acetone, ethanol, trichloroacetic acid, NaOH (Synth, São Paulo, Brazil), $FeCl_3$, methanol, diethyl ether, (Vetec, Rio de Janeiro, Brazil), $CaCl_2$ (Alpratec, Curitiba, Brazil), $MnCl_2$ (Isofar, Rio de Janeiro, Brazil), CaO (Qeel, São Paulo, Brazil), acetic acid (Dinâmica, Indaiatuba, Brazil), formaldehyde (Merck, Rio de Janeiro, Brazil), and azocasein (Sigma-Aldrich, Saint Louis, MO, USA).

2.2. Microbial Enzyme Production

Keratinolytic proteases were produced by submerged cultivation using a *Bacillus* sp. P45 strain (GenBank accession number AY962474) and feather meal as substrate. Enzyme production was performed at 30 °C and 125 rpm for 48 h, as described by Daroit, Corrêa, and Brandelli [9], using a mineral medium composed of (g/L) NaCl (0.5), KH_2PO_4 (0.4), K_2HPO_4 (0.3), NH_4Cl (1.9), and feather meal (43.0). At the end of cultivation, the whole culture was centrifuged (5000× *g*, 4 °C, 20 min), and the supernatant (crude extract) was utilized in the purification steps.

2.3. Enzyme Purification by Integrated Strategies in Large Volume Systems

The crude extract containing keratinolytic proteases was purified using a sequence of ATPS with the below composition integrated into the DF process to remove polyethylene glycol (PEG) [13]. The first ATPS was composed (m/m) of PEG 1500 Da (3%), potassium phosphate pH 7.0 (23%), NaCl (8%), enzyme extract (20%), and deionized water (46%). The second ATPS was formed by the addition (m/m) of the top phase from the first ATPS (36%), 100 mM tris-HCl buffer pH 7.0 (36%), and $(NH_4)_2SO_4$ (28%). PEG was removed by diafiltration at 15 °C using a 10 kDa regenerated cellulose membrane, five diafiltration cycles, and 1.5 kgf/cm^2 of pressure.

Enzyme purification was also evaluated using a sequence of ATPS at large-volume systems (10, 50, and 500 g). The systems were made as described above. After the sequential ATPS, the purified fraction was diafiltered to remove residual PEG, lyophilized, and stored at 4 °C for further characterization assays. The purification efficiency was determined by

the purification factor (PF) and enzymatic recovery (REC), according to Equations (1) and (2). Yields in volume and enzymatic activity (U total) were calculated based on the complete processing of 1000 mL of crude keratinase extract and the final characteristics of the purified enzyme (volume and activity), considering the particularities of the purification process.

$$PF = \frac{A_{sf}}{A_{si}} \quad (1)$$

$$REC\ (\%) = \frac{A_f \times V_f}{A_i \times V_i} \times 100 \quad (2)$$

A_{sf} and A_{si} were the respective specific activities (U/mg) of final and initial (crude extract) fractions. The specific activity is defined as the ratio between the activity (U/mL) and the total protein concentration (mg/mL); A_f and A_i were the respective enzymatic activities (U/mL) of final and initial (crude extract) fractions; and V_f and V_i were the volume (mL) of final and initial (crude extract) fractions, respectively.

2.4. SDS-PAGE

The electrophoresis was performed using the Laemmli [18] method in a polyacrylamide gel (5% stacking, 12% separating, 0.75 mm thickness) for both crude and purified keratinase extracts. Samples were mixed in a ratio (1:1) with sample buffer containing β-mercaptoethanol, heated to 100 °C for 5 min, and applied to the gel. Electrophoresis was performed at 150 V for 80 min (Bio-Rad, Hercules, CA, EUA), and a standard protein sample (Bio-rad, USA) with molecular weight ranging from 15 to 250 kDa was used as a marker. The gel was stained overnight using Coomassie Blue R-250 solution and then destained using two solutions. The first solution consisted of 30% methanol and 7% acetic acid, and the second was 5% methanol and 7% acetic acid. The gel was destained until protein bands appeared against a clear background for adequate visualization of the bands.

2.5. Characterization of Purified Protease Extract

2.5.1. Optimum pH and Temperature for Purified Protease Activity

The optimum pH was determined by assaying the proteolytic activity at 40 °C and pH values ranging from 6.0 to 6.5 (0.1 mol/L sodium phosphate buffer) and 7.0 to 11 (0.1 Tris-HCl buffer). The optimum temperature was established by performing the enzyme activity at optimal pH and temperatures between 40 °C and 65 °C. Relative activity (%) was expressed as the protease activity that remained after incubation at each temperature or pH compared to the maximum activity observed [19].

2.5.2. Thermal Stability of Purified Protease and Deactivation Kinetics

Thermal deactivation kinetics of purified keratinolytic protease was investigated using the protocol described by Braga et al. [20] by incubating the enzyme at different temperatures (40 to 60 °C) and pH values (7.0 to 9.0) in the absence of substrate with a time of up to 130 min, adjusted for each operating condition. Aliquots were withdrawn at periodic intervals, cooled in an ice water bath, and analyzed regarding protease activity as described in Section 2.5.4. The stability of the purified protease was expressed as percent residual activity (%).

Thermal inactivation rate constants (k_d, h^{-1}) for each temperature were determined from the slopes of the semi-natural logarithmic plot of the residual activity vs. time. At the same time, the half-life ($t_{1/2}$, h) was calculated through Equation (3). The half-life was defined as the time the enzymatic activity took to reach 50% of the initial value.

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (3)$$

The temperature dependence of k_d was analyzed by Arrhenius plot, whereas the deactivation energy (E_d) was calculated by the Arrhenius equation (Equation (4)), where

E_d is the deactivation energy of the transition state of enzyme deactivation, A is a constant, T is the absolute temperature in Kelvin and R is the universal gas constant (8.314 J/mol.K).

$$\ln\ k_d = \ln A - \frac{E_d}{R}\left(\frac{1}{T}\right) \quad (4)$$

The *D* value (min, decimal reduction time) was calculated by Equation (5). The *z* value (°C, temperature interval required to reduce the *D* value in one log cycle) was determined from the slope of the semi-logarithmic plot of *D* value vs. temperature. The calculation of the parameters above was based on the regression obtained with a minimum of 5 points, and only a coefficient of determination (R^2) above 0.93 was accepted.

$$D = \frac{2.3026}{k_d} \quad (5)$$

2.5.3. Effects of Salts and Solvents on Proteolytic Activity

The effects of NaCl, $CaCl_2$, $MnCl_2$, CaO, $C_8H_5KO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeCl_3$ on proteolytic activity, as well as their concentrations (1, 5, and 10 mM), were evaluated by pre-incubation of the purified enzyme with respective salts at 25 °C for 30 min [21]. The effects of solvents were determined by resuspension of the freeze-dried enzyme in organic solvents (acetone, ethanol, methanol, acetic acid, diethyl ether, and formaldehyde). The concentration of organic solvents was 50% of the reaction mixtures incubated for 1 h at 10 °C [22,23]. After incubation, the protease activity was determined as described in Section 2.5.4 and compared with the one without solvents and salts (control), which was incubated in the same conditions and considered 100%.

2.5.4. Enzyme Assay and Protein Quantification

Proteolytic activity was determined by a method described in Section 2.5.4 with azocasein as substrate [8]. The reaction contained 100 µL of 100 mM Tris-HCl buffer (pH 7.5), 300 µL of 10 mg/mL azocasein (in Tris-HCl buffer), and 100 µL of the enzyme. After incubation (40 °C, 30 min), the reaction was stopped by adding 600 µL of 10% (*w*/*v*) trichloroacetic acid (TCA). Then, this mixture was centrifuged (10,000× *g* for 10 min). The supernatant (800 µL) was mixed with 1.8 M NaOH (200 µL), and the absorbance at 420 nm was measured with a spectrophotometer. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase in 0.1 absorbance unit in the defined assay conditions.

Soluble proteins in crude and purified extracts were quantified according to Bradford [24], taking bovine serum albumin (BSA) as standard. The specific activity (U/mg) was expressed as the ratio between the proteolytic activity (U/mL) and the soluble protein content (mg/mL).

2.6. Statistical Analysis

The mean values were calculated based on three independent experiments described above and triplicate assays of enzyme activity and protein quantification. Data were statistically evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. The statistical significance was established at 0.05 ($p < 0.05$).

3. Results

3.1. Protease Purification through Integrated Strategies in Large Volume Systems

In ATPS, the partition coefficient for soluble substances is often independent of the total system volume and substance concentration to be partitioned. In this sense, partition behavior in large volume systems can be predefined with great accuracy from small volumes, where mixing and rapid phase equilibrium are easily achieved. Some studies have already demonstrated the efficacy of upscaling ATPS to recover proteins [25]. Here, we used the purification protocol previously established in [13] and evaluated the scale-up

of the process between 5- and 50-fold to purify the crude keratinolytic proteases produced from *Bacillus* sp. P45 strain using the conditions described in Section 2.2 "Microbial Enzyme Production".

As shown in Table 1, using ATPS with high capacities allowed the purification of proteases without significantly compromising enzyme yields and purity (Table 1). The purification factor was up to 2.6-fold in the first ATPS, 6.7-fold in the second ATPS, and 4-fold in the diafiltration step. Enzymatic recovery values were up to 70% in both ATPS stages and reached 84.3% after the diafiltration step. According to these results, the small-scale system can be extrapolated to purify protease efficiently.

Table 1. Purification factor (PF) and enzymatic recovery (REC) for *Bacillus* sp. P45 protease purified by integrated strategy in large volume systems. The statistical analysis was conducted by ANOVA followed the Tukey's test. Equal lowercase (1st ATPS) or uppercase letters (2nd ATPS) in the same column indicate no statistical difference at the 5% level for the purification factor and enzymatic recovery for each of the two-phase aqueous systems studied.

ATPS	Capacity (g)	Specific Activity (U/mg)	Purification Factor (fold)	Enzymatic Recovery (%)
1st	10	2494.4 ± 96.4	2.4 ^{a} ± 0.1	73.6 ^{a} ± 2.6
	50	2747.1 ± 300.1	2.6 ^{a} ± 0.3	78.0 ^{a} ± 1.3
	500	2477.7 ± 82.8	2.6 ^{a} ± 0.1	75.3 ^{a} ± 1.1
2nd	10	7820.0± 84.2	6.2 ^{A} ± 1.4	72.9 ^{A} ± 1.8
	50	7534.5 ± 137.4	6.4 ^{A} ± 2.1	73.1 ^{A} ± 2.1
	500	7668.2 ± 102.4	6.7 ^{A} ± 1.1	75.1 ^{A} ± 1.5
Diafiltration		25,795.00	4.0	84.3

Regarding the yield, for each 1000 mL of the produced crude enzyme (cell-free culture medium showing the following characteristics: enzymatic activity of 738.94 U/mL; protein of 0.765 mg/mL; and specific activity of 965.9 U/mg), it was possible to produce 275 mL of the purified enzymatic extract using ATPS sequence coupled to diafiltration with an enzymatic activity of 3611.3 U/mL, protein of 0.14 mg/mL, and specific activity of 25,795 U/mg, which results in a volume yield of 27.5% and enzymatic activity yield of 134.5%. Yield values greater than 100% are expected since the diafiltration process removes contaminants that inhibit enzyme activity and eliminate PEG and salts that cause interference in activity and protein determinations (commonly reported in the literature) [26,27].

Crude proteases from *Bacillus* sp. P45 exhibited good performance in enzymatic hydrolysis to produce peptides and other bioactive compounds, as described by Cunha, Brandelli, Braga, Sala, and Kalil [10]. In addition, previous studies have also demonstrated the applicability of two-step purified protease P45 in the hydrolysis of eight keratinous substrates (i.e., bovine serum albumin, casein, whey, native and heat-treated chicken feather, fish meal, feather meal, and blood meal). In detail, the purified enzyme hydrolyzed all protein substrates more efficiently than the crude enzyme, demonstrating the beneficial effect of enzyme purification on its catalytic activity [13].

In food segments, purified protease P45 was applied as milk clotting to develop cream cheese with chia and quinoa flour [12]. The protocol used to purify *Bacillus* proteases was the same as presented here and in large volume systems. Previous findings of our research group indicated that crude and purified enzymes had similar milk clotting capability as the commercial coagulant. Although the milk clotting activity was similar for both extracts, the purified ones are more advantageous regarding sensory aspects of the final product and consumer acceptance [12].

The results presented here align with the previous studies carried out by our research group and described above and reveal the two-step purification procedure's relevance to obtaining more pure proteases for industrial purposes. Studies on upscaling ATPS could

enable future microbial protease purification in large volume systems and their application in strategic industrial segments.

Crude and purified enzymatic extracts from feather meal bioconversion by Bacillus sp. P45 were analyzed through the SDS-PAGE stained using Coomassie Blue R-250 solution (silver-stained electrophoresis is also available in the Supplementary File). The electrophoretic analysis showed protein bands in both enzymatic extracts (Figure 1). Lane 2 demonstrated several bands with lower intensities related to the concentration and diversity of proteins in the crude extract. On the other hand, Lane 3 showed two bands well highlighted in the purified extract. The band around 25–28 kDa is related to keratinolytic protease, similar to other proteases from bacteria, as shown in Table 2. It is important to highlight that the characteristics of the protease will be different due to the strain used, cultivation conditions, type of fermentation, and substrates, among others. Furthermore, the extract's specific activity and purity depend on the purification protocol applied in the downstream step [28] (Table 2).

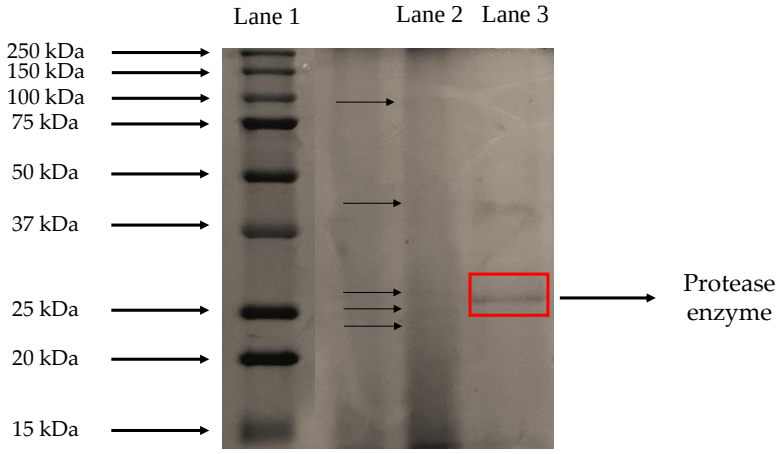

Figure 1. Electrophoresis analysis (SDS-PAGE) of crude and purified keratinolytic enzyme produced from feather meal bioconversion by *Bacillus* sp. P45. Lane 1: standard protein markers. Lane 2: crude extract enzyme. Lane 3: purified keratinolytic enzyme.

The efficiency of protease purification using ATPS coupled with diafiltration was previously demonstrated in our studies through zymogram and SDS-PAGE analyses [13]. In this case, the enzyme with 28 kDa showed more intense gelatinolytic activity in the purified extract, indicating the efficiency of the integrated process to purify proteases.

In another study by our research group, we already related a protease with a molecular mass of around 26 kDa. The enzyme was purified using high-resolution techniques (ammonium sulfate precipitation + gel filtration + ion exchange chromatography) and then identified by UPLC (ultra-performance liquid chromatography) system coupled to a Q-TOF micro tandem mass spectrometer with nano-ESI. The analysis of tryptic peptides revealed sequence homologies that characterize the keratinase as a subtilisin-like serine protease. The eight peptides identified from trypsin digestion and their amino acid sequences covered approximately 50% of the mature sequences from the comparative subtilisins [29].

In the present work, we proposed applying two simple purification techniques (ATPS coupled with diafiltration) which are less expensive and easily scaled up. In general, the methods used in the purification of enzymes influence extract characteristics, including the operational aspects of the enzyme and, mainly, the costs of the process, demonstrating the need to use appropriate techniques for applying the enzyme [28,30,31]. It should be mentioned that when obtaining enzymatic extracts, their stability concerning different temperatures, pHs, salts, and solvents commonly used in target industrial processes must be considered, which allows their proper application and the viability of the processes. In some applications, high purity is unnecessary; therefore, it is possible to use a few purification techniques, reducing the costs and time to obtain the enzyme [32].

3.2. Optimum Temperature and pH

The enzyme activity was determined at different temperatures and pH values. The purified keratinolytic protease from *Bacillus* sp. P45 was active at pH values between 6.0 and 11.0 and temperatures between 40 and 65 °C. Maximum activity was observed at pH 7.5, in which the enzyme showed a typical bell-shaped curve (Figure 2A). Regarding the temperature, the enzyme had optimum activity at 55 °C, in which it is possible to check the increase in the activity up to the maximum, followed by a drastic decrease at higher temperatures (Figure 2B), thus indicating rapid denaturation. An increase in the temperature reflects in the reaction rate since heat increases the proportion of groups that react and reach the transition state at a given time. However, the heat results in enzyme deactivation when the temperature rises above a certain point. Since most proteins are only marginally stable, denaturation occurs, resulting in a sharp drop in enzyme activity [33].

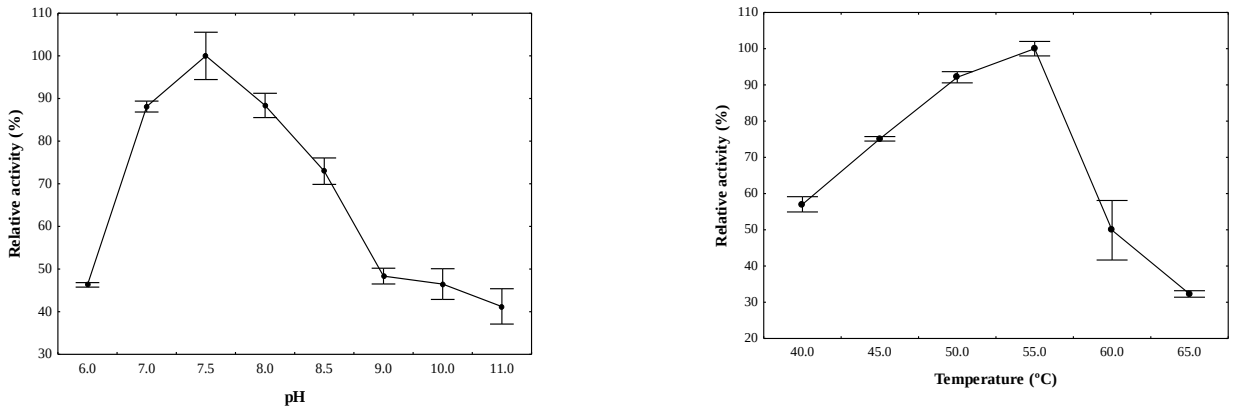

Figure 2. Optimum pH (**A**) and temperature (**B**) for the activity of purified protease from *Bacillus* sp. P45. Enzymatic activity was determined with the use of azocasein as substrate by incubation for 30 min at different pH and temperature values.

Optimum conditions of pH and temperature for the purified protease from *Bacillus* sp. P45 are in the range reported for purified protease from mesophilic *Bacillus* sp. [7] and most alkaline proteases of different microbial species, whose optimum temperature values range from 40 to 70 °C and optimum pH values range from 7.0 to 10.0 (Table 2). Comparatively, purified proteases from *Bacillus* sp. CL 18 showed optimal activity at 51–59 °C and pH 7.4–8.8 [16], and maximal activity of purified proteases from *Bacillus licheniformis* LBA 46 was achieved at 50–60 °C and pH 8.5 [4]. The highest activity of purified protease from *Bacillus subtilis* FTC02PR1 occurred at 60 °C and pH 8.0 [15]. It should be highlighted that each enzyme has optimum pH and temperature for operation in which its activity is maximal. However, since it does not ensure that these conditions are those that favor its stability, proper evaluation is required.

Table 2. Optimum temperatures and pH values for some proteases from fungi and bacteria.

	Source	Temperature	pH	Specific Activity (U/mg)	Molecular Weight (kDa)	Reference
Bacterium	*Bacillus* sp. P45	55 °C	7.5	25,795.00	28.0	This study
	Bacillus safensis RH12	60 °C	9.0	37,000.00	28.0	[34]
	Bacillus sp. *CL33A*	55 °C	8.0	13.40	N.D.	[6]
	Bacillus licheniformis K7A	70 °C	10.0	1923.00	30.3	[35]
	Bacillus luteus H11	45 °C	10.5	115.20	37.0	[36]

Table 2. *Cont.*

	Source	Temperature	pH	Specific Activity (U/mg)	Molecular Weight (kDa)	Reference
	Bacillus stearothermophilus	65 °C	10.0	59,022.00	28.0	[37]
	Geobacillus SBS-4S	60 °C	9.0	14,910.25	30.0	[38]
	Streptomyces sp. GS-1	40 °C	8.5	N.D.	30.0	[39]
	Chryseobacterium sp.	37 °C	8.0	100.00	22.2	[40]
Fungus	*Aspergillus ochraceus BT21*	50 °C	8.0	111,379.50	59.0	[41]
	Aspergillus oryzae	40 °C	9.0	632.60	29.0	[42]
	Aspergillus niger WA 2017	60 °C	10.0	715.20	N.D.	[43]
	Neurospora crassa	55 °C	9.0	5518.37	36.0	[44]
	Penicillium chrysogenium	80 °C	10.0	78,500.00	43.0	[45]

This phenomenon has been described by Ricca, et al. [46]. The authors emphasize that "optimum pH and temperature" is generally used for indicating the temperature of maximum enzyme activity, where the curve of activity vs. temperature or pH often shows a maximum point. Thus, the optimum temperature is an indirect measure of enzyme thermostability, where the higher the optimum temperature, the more stable the enzyme. On the other hand, the optimum temperature is found experimentally by initial velocity tests at different temperatures and pH values. Under these conditions, the effect of irreversible thermal deactivation is negligible. If the optimal pH or temperature were used as the operating conditions, a rapid enzyme deactivation would occur, and the process would not be optimized since higher temperatures favor kinetics but hinder activity retention by increasing the deactivation rate. Considering the best enzyme application, working at a lower temperature could be convenient for a slower but more enduring process. In addition, stability tests described hereafter are recommended to determine which value best compromises both requirements.

3.3. Kinetic Parameters for Thermal Deactivation of Keratinolytic Protease

Denaturation occurs when a protein's structure (secondary, tertiary, or quaternary) is altered without breaking covalent bonds. It implies a loss of the efficiency of enzymatic processes, making it necessary to evaluate such properties [20]. Determining the kinetic parameters for thermal deactivation is essential to understand the stability of the enzyme and also allows the evaluation of its potential for industrial application. Furthermore, estimating kinetic parameters helps understand the denaturation mechanism, which is important for enzyme application at high temperatures [47].

The deactivation of enzymes, commonly employed in the food industry, is generally expressed in terms of D and z values. The z values calculated for purified keratinolytic protease P45 were 15.3, 16.7, 13.6, 17.5, and 18.8 °C to pH values of 7.0, 7.5, 8.0, 8.5, and 9.0, respectively, within a temperature range from 40 to 60 °C (Table 3). Parameter D was consistently higher at lower temperatures and decreased drastically with high incubation temperatures. It shows rapid enzyme inactivation at higher temperatures and the necessity for more prolonged incubation at lower temperatures to reach 90% enzyme inactivation. D values ranged from 237.1 min at 40 °C to 6.7 min at 60 °C. The deactivation rate constants (k_d) for purified keratinolytic protease were inversely proportional to the half-life ($t_{1/2}$) (Table 3), which indicates that the enzyme has its stability reduced as the temperature increment. Under the temperature of greater thermal stability (40 °C), the enzyme was more stable at pH values between 7.0 and 7.5.

Table 3. Values of parameters *D*, *z*, k_d, and half-life ($t_{1/2}$) obtained by the thermal denaturation of purified protease from *Bacillus* sp. P45.

pH	Temperature (°C)	*D* (min)	*z* (°C)	k_d (min)	Half-Life ($t_{1/2}$) min
7.0	40	232.3	15.3	0.0099	70.0
	45	89.8		0.0256	27.1
	50	30.9		0.0744	9.3
	55	23.9		0.0959	7.2
	60	11.5		0.1999	3.5
7.5	40	237.1	16.7	0.0097	71.4
	45	60.7		0.0379	18.3
	50	24.5		0.0937	7.4
	55	17.0		0.1351	5.1
	60	15.1		0.1518	4.6
8.0	40	196.6	13.6	0.0117	59.2
	45	45.2		0.0509	13.6
	50	31.8		0.0722	9.6
	55	17.8		0.2289	3.0
	60	6.7		0.3449	2.0
8.5	40	145.5	17.5	0.0158	43.9
	45	43.2		0.0532	13.0
	50	34.0		0.0676	10.2
	55	17.3		0.1324	5.2
	60	10.4		0.2192	3.2
9.0	40	132.9	18.8	0.0173	40.1
	45	86.14		0.0267	25.9
	50	31.6		0.0728	9.5
	55	23.8		0.0968	7.2
	60	11.5		0.1999	3.5

The average deactivation energy value (E_d) was 118.0 kJ/mol for temperatures ranging from 40 to 60 °C and represented the energy barrier to be overcome to inactivate the enzyme. Therefore, the higher the E_d values, the higher the energy barrier to be transposed for enzyme inactivation, indicating increased stability. The E_d value obtained for the protease purified in large volume systems in this study was compatible with others of various *Bacillus* proteases [4,16,48–50].

Daroit et al. [51] obtained lower $t_{1/2}$ values than those obtained by this study for protease from *Bacillus* sp. P45 purified by a strategy involving a concentration step with ammonium sulfate (30–60% saturation), followed by gel filtration (Sephadex G-100) and ion exchange (DEAE-Sepharose) chromatography. The $t_{1/2}$ and *D* values reported at 40 °C were nearly 88 min and 26 min, which were reduced to 1.06 min and 3.51 min, respectively, by increasing the temperature to 50 °C at pH 8.0. Similarly, Narwal, Bhushan, Pal, Panwar and Malhotra [49] purified protease from *Bacillus subtilis* MTCC 10422 using a sequential procedure with ammonium sulfate precipitation (30–70%), gel filtration (Sephadex G-100), and ion exchange (DEAE-Sepharose) chromatography; however, the $t_{1/2}$ and *D* values at 40 °C for purified protease were higher than those observed in the current study at the same temperature under all pH values.

Data obtained here and by other studies demonstrate the importance of the knowledge of protease kinetic parameters in different conditions since, depending on the enzymes, their properties can be very different. In addition, this study showed that information about protease deactivation kinetics might help to determine the temperature at which the enzyme can be applied without compromising the bioprocess viability. An example is the previous suitable performance of two-step purified protease P45 at 40 °C in the milk clotting process, as described in prior work by our research group [12].

3.4. Effects of Salts and Solvents on Proteolytic Activity

The influence of NaCl, $CaCl_2$, $MnCl_2$, CaO, $C_8H_5KO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeCl_3$ (Figure 3) on the proteolytic activity was verified since positive effects on the enzyme performance have been reported by adding these salts [6,17,35,52–54]. Initially, the effects of different salts on enzyme activity were investigated (Figure 3). Then, the effect of salt concentration (1 to 10 mM) was checked for the salts that influence enzyme activity equal to or significantly higher ($p < 0.05$) than the control assay (Figure 4).

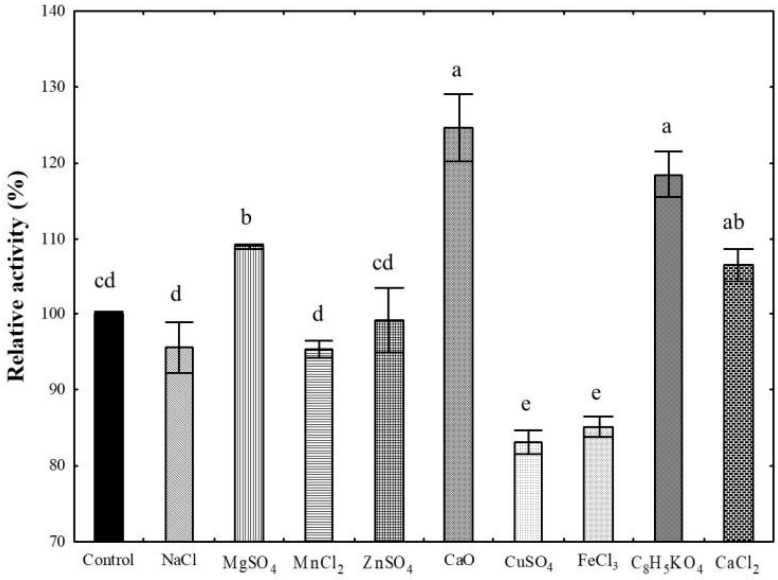

Figure 3. Effect of different salts (1 mM) on the activity of purified protease from *Bacillus* sp. P45. Bars are the means ± standard deviations of three independent experiments. The statistical analysis was conducted by ANOVA, followed by Tukey's test. Equal lowercase letters represent no significant differences at the 5% level.

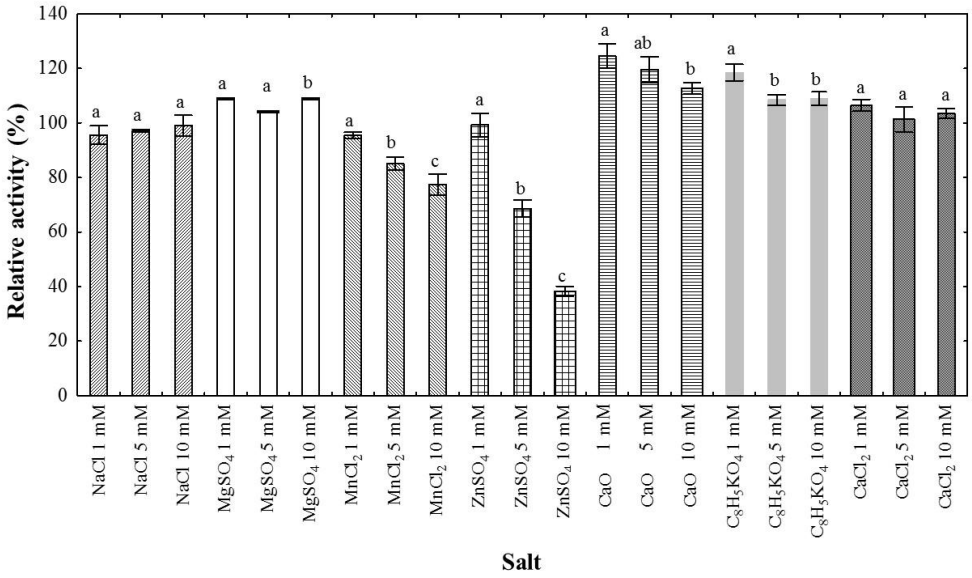

Figure 4. Effect of salt molarity on the activity of the purified protease from *Bacillus* sp. P45. Bars are the means ± standard deviations of three independent experiments. The statistical analysis was performed through ANOVA, followed by Tukey's test. Equal lowercase letters represent no significant differences at the 5% level.

The purified protease had high activity in the presence of some salts. Proteolytic activity increased significantly ($p < 0.05$) by CaO, $C_8H_5KO_4$, $CaCl_2$, and $MgSO_4$ (112.7%, 108.9%, 103.6%, and 108.9%, respectively) salts. Positive effects of Ca^{2+} and Mg^{2+} ions on *Bacillus* protease activity have also been reported [17,35,52,53]. On the contrary, the enzyme activity was inhibited by $CuSO_4$ and $FeCl_3$ (21.3% and 9.8%, respectively), which can also be attributed to the interaction with the charged side-chain groups of accessible amino acids, thus influencing the conformation and stability of the enzyme [55]. Both salts interfere with enzyme catalysis by slowing the enzymatic reaction; therefore, they were excluded from molarity assays. Other studies have demonstrated a decrease in protease activity in the presence of Cu^{2+} and Fe^{2+} ions [6,52,54].

An increment in activity is due to the ability of some ions to stabilize the binding of the enzyme–substrate complex, keeping the protein conformation and protecting against thermal denaturation [56,57]. Furthermore, the increase in enzyme activity when using salts containing divalent ions, e.g., Ca^{2+}, is associated with enhanced conformational stability of the protein [58,59] and stabilization of the ternary enzyme complex [60], which, in some cases, involves two or more substrate molecules that bind to the enzyme and participate in the reaction. The protective effect of Ca^{2+} has been continuously reported [34,35,37], but its mechanism of action is unclear. It is suggested that the salt causes an increase in water activity on the protein surface, which leads to increased hydrophobicity of the nucleus, thereby resulting in rigidity and structural enzyme stability [61]. Additionally, the Ca^{2+} was reported to act in a switch-like mechanism, which shifts protease between stable and unstable forms as appropriate to the biological need. Under some circumstances, the protease is stable, wholly folded or partially unfolded, and susceptible to degradation. This change in protease stability is regulated by the binding of calcium ions [62].

The following salts, which resulted in proteolytic activity higher ($p < 0.05$) than the control assay, were evaluated in higher concentrations: NaCl, $CaCl_2$, $MnCl_2$, CaO, $C_8H_5KO_4$, $MgSO_4$, and $ZnSO_4$. An increase in molarity led to a significant reduction in enzyme activity, except for NaCl, $CaCl_2$, and $MgSO_4$ (Figure 4). $MnCl_2$ and $ZnSO_4$ were also found to affect enzyme activity strongly when the concentration increased, resulting in an inhibition of 22.6 and 61.8%, respectively (10 mM). The inhibition of keratinolytic proteases by Zn^{2+} at the millimolar range has been demonstrated by other studies [51]. Several peptidases are inhibited by excess Zn^{2+}, particularly from neutral to alkaline pH through a bridge between zinc monohydroxide ($ZnOH^+$) and catalytic zinc ions at the active site [63].

Evaluating the protease behavior in the presence of salts is required for enzyme use since a lack of knowledge of their interference can make application difficult. Furthermore, some salts may be found in biotechnological processes or raw materials to which the enzyme can be applied. Some relevant processes include: (i) the enzymatic coagulation of milk for dairy processing, whose main primary material contains significant amounts of calcium; (ii) the bioconversion process of feathers, in which the action of the keratinase enzyme is made easier by reducing agents, such as potassium biphthalate ($C_8H_5KO_4$), responsible for the degradation of the disulfide bonds of keratin in feathers; (iii) protease formulations that contain NaCl in their composition, developed to soften meat; and (iv) leather treatment which uses NaCl for storage to further application of enzymes related to the depilation process, which can reduce the use of chemical compounds, such as sulfides [64].

The proteolytic activity of the purified enzyme was also evaluated in the presence of organic solvents (Figure 5). The solvents evaluated here were selected because of their promising results regarding other proteases and their use for developing processes/products involving protease not only in the preparation of cosmetics, chemicals, and personal toiletries but also in protease immobilization processes [65,66]. Other solvents may also be applied to prepare detergents and peptide biosynthesis processes [67].

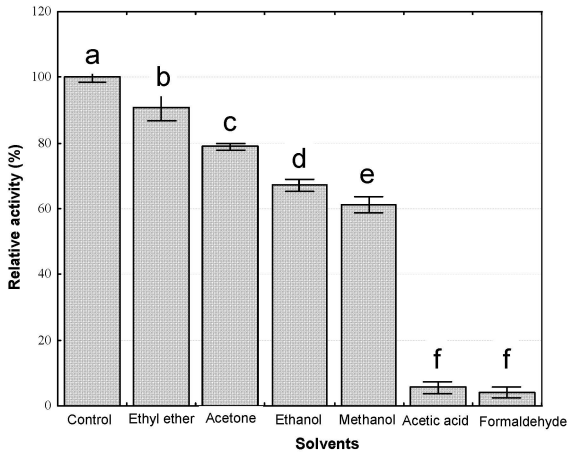

Figure 5. Solvent effect on the activity of the purified protease from *Bacillus* sp. P45. Bars are the means ± standard deviations of three independent experiments. The statistical analysis was performed through ANOVA, followed by Tukey's test. Equal lowercase letters represent no significant differences at the 5% level.

The use of organic solvents caused a significant ($p < 0.05$) reduction in enzyme activity (Figure 5) using solvents at a concentration of 50% and incubating the enzyme for 1 h at 10 °C. Acetic acid and formaldehyde strongly inhibited enzyme activity with residual activity below 5%. Ethyl ether, acetone, ethanol, and methanol showed inhibition percentages of 9.2%, 21.2%, 32.8%, and 38.8%, respectively. The negative effect may be related to the ability of the solvents to withdraw an essential layer of water from the enzyme's active site [68] and water around the molecule [69], causing a loss of catalytic properties. Similar results were found for other proteases from different microbial sources [21,22,67,70]. Although methanol is toxic to humans, the literature reports that it increases protease activity by comparison with purified enzymes without any solvents [71]. In this study, however, the keratinolytic protease from *Bacillus* sp. P45 showed 61.2% relative activity in the presence of methanol.

Although organic solvents often reduce enzyme activity, they can be beneficial to reactions and should be used to make the enzymatic process more straightforward, particularly when the substrate has a greater solubility in organic solvents. In addition, organic solvents may help reduce the risk of microbial growth, improve thermal stability, enhance purification efficiency and recovery using volatile solvents, and control the specificity of the reaction substrate [71]. Proteases that show tolerance to organic solvents have attracted attention, and several benefits are associated with the enzyme application to hydrolysis processes. It was demonstrated that peptide production could be enhanced by adding organic solvents to the reaction mixture; thus, protease application to produce these compounds has been a viable alternative to chemical hydrolysis [58]. Maintaining proteolytic activity in organic solvents is important because proteases are generally inactivated before reaching the reaction conditions applied to peptide synthesis [70].

4. Conclusions

Keratinolytic proteases from bioconversion of feather meal by *Bacillus* sp. P45 were purified using an integrated procedure and partially characterized. Using ATPS with high capacities resulted in a purer protease extract without compromising purity and yields. In the electrophoretic analysis, a band of keratinolytic protease already reported in our previous studies (25–28 kDa) was detected. Purified protease from *Bacillus* sp. P45 showed optimum temperature and pH at 55 °C and 7.5, respectively. The half-life ($t_{1/2}$) was approximately 237.3 min at 40 °C and pH 7.5; the higher the incubation temperature, the more this parameter decreased. Additionally, the thermal denaturation constant (K_d) was inversely proportional to half-life ($t_{1/2}$). The deactivation energy (E_d) value was 118.0 kJ/mol, and z and D values ranged from 13.6 to 18.8 °C and from 6.7 to 237.3 min,

respectively. Adding salts $CaCl_2$, CaO, $C_8H_5KO_4$, and $MgSO_4$ increased enzyme activity in opposition to all organic solvents tested.

The results presented and discussed here could collaborate with advances in bioprocess and enzymatic technology in the following aspects: (i) the use of a by-product to obtain low-cost proteolytic enzymes; (ii) the possibility of scaling up the purification process and obtaining partially purified extract in large volume systems and; (iii) relevant information about the application of proteases, contributing to its future use in industrial purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11030803/s1. Supplementary Figure S1: Electrophoresis analysis (SDS-PAGE) of crude and purified keratinolytic enzyme produced from feather meal bioconversion by *Bacillus* sp. P45 using silver staining. Lane 1: Standard Protein Markers. Lane 2: Crude extract enzyme; Lane 3: Purified keratinolytic enzyme.

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