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ORIGINAL ARTICLE

An Aspartic-metalloprotease from an Endemic Plant Tuber (*Burnatia enneandra* micheli): Purification and Biochemical Characterization

Ngangoum Eric Serge[1,2], Mezajoug Kenfack Laurette Blandine[1], Sanjit Kumar[2], Tchiegang Clerge[1], Mookambeswaran Vijayalakshmi[2]

[1]Department of Food Sciences and Nutrition, Bioprocess Laboratory, Institute University of Technology, University of Ngaoundere, Ngaoundéré, Cameroon, [2]Centre for Bio-Separation Technology, VIT University, Vellore, Tamil Nadu, India

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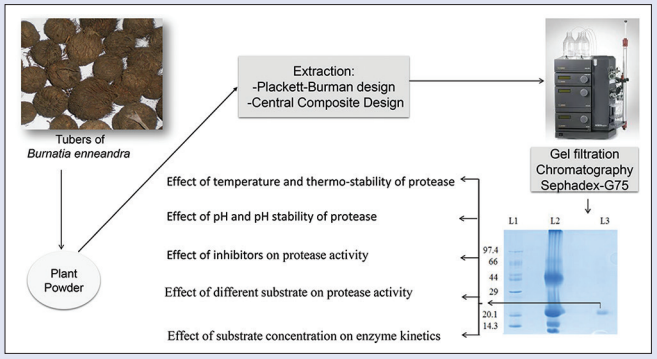
ABSTRACT

Background: The objective of this work was to isolate, optimize, and characterize protease from Burnatia enneandra which is an endemic plant found abundantly in the Far-Nord Region of Cameroon. The optimum condition to extract maximum quantity of protease from B. enneandra with respect to pH, the ratio (m/v), and agitation frequency was defined as 5.1%, 4%, and 100 rpm, respectively. **Materials and Methods:** The enzyme was purified using ammonium sulphate precipitation, double gel filtration chromatography sephadex G200 followed by sephadex G75 and the purified protease was further characterized. With an apparent molecular weight of 23 kDa on SDS-PAGE, the purified protease showed maximum activity at 5.1 and 40°C respectively for pH and temperature. Its activity was enhanced by metal ions such as Ca2+ and Ni2+, while Fe2+and Zn2+ showed significant inhibition. **Results:** B. enneandra protease activity was not affected by proteases inhibitors such as phenylmethylsulfonyl fluoride, aprotinin, and iodoacetamide but was strongly inhibited by Pepstatin A and ethylenediaminetetraacetic acid which allowed to classify this new protease as aspartic-metalloproteases. Using casein as substrate, protease from B. enneandra had a maximum rate of reaction (Vmax) and Michaelis-Menten constant (Km) of 64.935 (U/mL) and 373.941 (µg/mL), respectively.

Key words: Classification, extraction, gel filtration chromatography, optimization, plant, protease

SUMMARY

- Purification of an Aspartic-metalloprotease from an Endemic Plant Burnatia enneandra micheli
- Biochemical characterization of metalloprotease
- Effect of different substrates on metalloprotease activity
- Effect of metal ions on metalloprotease activity



Abbreviations used: CCD: Central composite design; Km: Michaelis–Menten constant; Vmax: Maximum Velocity; PBD: Plackett–Burman design; PMSF: Phenylmethylsulfonyl floride; AAD: Absolute Average Deviation; AF: Accuracy Factor; BSA : Bovine serum albumin; BF: Bias factor; EDTA: Ethylene diamine tetraacetic acid; RSM: Response surface methodology; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Correspondence:

Prof. Tchiegang Clerge,
Université de Ngaoundéré, Laboratoire des Bio-Procédés, Unité de Recherche en Biochimie-Technologie Alimentaires et Nutrition, BP 455 Ngaoundéré, Cameroun.
E-mail: tclerge@yahoo.fr

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INTRODUCTION

Proteases are a group of enzymes which are distributed in plants, animals, and microorganisms where they play a significant role in the physiological process.[1-13] Proteases isolated from various organisms are commercially used in various industries such as in food, pharmaceutical, leather, waste treatment, detergent, and textile industries.[4,14,15] Most of the commercial proteases are either isolated from microorganisms or animals.[6,14,16,17] There are very few proteases which are isolated, characterized from plants.[2] The purification and characterization of new promising proteases from plant sources have also incurred greater attention by various food and biotechnology-based industries due to their properties being significant active in a broad range of pH and temperature.[3] Hence, plant-based proteases have been extracted from various species as well as from all parts of the plant.[2,12,18-20] However, due to higher demand for protease globally, it is necessary to continue searching for newly available sources with low prices. In Cameroon, some plants are used conventionally according to their potential. *Burnatia enneandra* which is one of them is an endemic plant belonging to *Alismataceae* family largely found in Far-Nord Region of Cameroon. Its tubers are used in food system of the local population, especially in local beverages production. In 2014, Mezajoug-Kenfack *et al.* reported

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that *B. enneandra* tubers could be an important source of proteases for industries after purification and characterization Mezajoug-Kenfack, Ngangoum, Tchiégang, and Linder.[21]

The main objective of this work was to determine the optimal condition to extract protease from B. enneandra tubers and also to characterize the purified enzyme using various biochemical methods and to determine its applicability in industrial application. Therefore, the Plackett–Burman design was investigated to select the main parameters which can significantly influence protease extraction from *B. enneandra* tubers.[22] These significant parameters were there after optimized using response surface methodology (RSM) through center composite experimental design.

MATERIALS AND METHODS

The tubers of *B. enneandra* used in this work were collected from a farm at Yagoua Far-Nord Region of Cameroon (10°20′ 34″ N, 15°14′ 26″ E). The tubers were treated according to the method described by Mezajoug-Kenfack, Ngangoum, Tchiégang, and Linder. They were peeled, cleaned, cut in the small disk, and dried into the convective dryer (CKA 2000-AUF) at 40°C for 48 h. Dry tubers were pounded in mortar immediately after drying to avoid humidity, then ground in a micro hammer mill Culatti and sifted. Powder samples with particles size lower than 400 µm were retained for proteases extraction.

Extraction and purification

Plackett-burman design

Protease extraction from plants can be influenced by many factors. The Plackett–Burman design was used to study the effect of the ratio m/v, pH, temperature, extraction time, stirring speed, and NaCl concentration on the protease extraction from the powder of *B. enneandra* tubers, taking protease activity as a response. All the factors were studied at two levels: high (level + 1) and low (level-1) [Table 1]. Statgraphics Centurion XVI was used as software to generate and analyze the experimental matrix of Plackett–Burman [Table 2]. The relation between code and real values is expressed by the following equation:

$$a_i = \frac{(A_i - A_0)}{\Delta A} \quad (1)$$

Where a_i is the coded value of the parameter A_i, A_0 is the value of A_i at the center point, and ΔA is the step change of an independent parameter.

Central composite design

The significant parameters selected through Placket–Burman design were optimized using RSM method with central composite design (CCD). Seventeen experiments were carried out to study each selected parameters in five levels: two alpha levels (−1.35313, +1.35313), low (−1), medium (0), and high (+1) levels. The measured responses were subjected to analysis of variance (ANOVA) using Statgraphics Centurion XVI as software. Based on the experimental data, an empirical second order polynomial equation was established to evaluate the relationships between the independent variables and the process dependent on Giovanni.[22] A second order polynomial equation is express as follows:

$$y = \alpha_0 + \sum_{i=1}^{k} \alpha_i x_i + \sum_{i=1}^{k} \alpha_{ii} x^2 + \sum_{i<j}^{k} \alpha_{ij} x_i x_j + \varepsilon \quad (2)$$

where Y is the predicted protease activity defined as response, α_0: Is the intercept term, α_i: Is the linear coefficients; α_{ii}: Is the quadratic term coefficients, and α_{ij}: Is the cross product coefficients, x_i and x_j are independent variables.

Table 1: Minimum and maximum levels for the parameters screened in Plackett-Burman design

Parameters	Units	Symbols	Levels	
			Low (-)	High (+)
pH		X_1	5.5	8
Ratio w/v	%	X_2	0.5	3.5
Temperature	°C	X_3	5	37
NaCl concentration	%	X_4	0.5	3
Extraction time	min	X_5	30	90
Shaker speed	rpm	X_6	30	100

Table 2: Experimental matrix and experimental data according to Plackett-Burman design

N°	X_1	X_2	X_3	X_4	X_5	X_6	AE (mU/mL)	SD
1	1	−1	1	−1	−1	−1	18.148	1.571
2	1	1	−1	1	−1	−1	39.815	0.002
3	−1	1	1	1	−1	1	41.667	0.005
4	−1	−1	−1	−1	−1	−1	20.926	0.078
5	1	1	1	−1	1	1	35.555	0.000
6	1	−1	−1	−1	1	1	15.370	0.262
7	−1	1	−1	−1	−1	1	40.555	0.262
8	−1	−1	−1	1	1	1	13.148	0.262
9	−1	−1	1	1	1	−1	22.592	1.047
10	−1	1	1	−1	1	−1	48.518	1.571
11	1	−1	1	1	−1	1	13.518	0.262
12	1	1	−1	1	1	−1	43.518	0.786
13	0	0	0	0	0	0	32.037	0.786
14	0	0	0	0	0	0	32.037	0.261
15	0	0	0	0	0	0	31.851	0.524

Where X_1 is pH; X_2: Ratio m/v; X_3: Temperature; X_4: Salt concentration; X_5: Extraction time, X_6: Stirring speed; AE: Enzyme activity; SD: Standard division

The acceptation of the given model was evaluated by calculating the determination coefficient (R^2), the absolute average deviation (AAD), the accuracy factor (AF), and the Bias factor (Bf) which the formula was given as follows:

$$R^2 = \frac{\sum_{i=1}^{N}\left(Y_{ical} - \overline{Y}\right)^2}{\sum_{i=1}^{N}\left(Y_{i\exp} - \overline{Y}\right)^2} \quad (3)$$

$$\mathrm{AAD} = \frac{\sum_{i=1}^{N}\left(\frac{\left|Y_{i\exp} - Y_{cal}\right|}{Y_{i\exp}}\right)}{N} \quad (4)$$

$$Bf = 10^{\frac{1}{N}\sum_{i=1}^{N}\log\left(\frac{Y_{ical}}{Yi_{\exp}}\right)} \quad (5)$$

$$Af = 10^{\frac{1}{N}\sum_{i=1}^{N}\log\left|\left(\frac{Y_{ical}}{Y_{i\exp}}\right)\right|} \quad (6)$$

Where N is the number of trials in the experimental design, $\overline{Y}$ is the response average, Y_{ical} and Y_{iexp} are calculated and experiment responses, respectively.

Protease activity assay

The protease activity of the purified protease as well as crude extract was measured according to the modified method described by Yezli *et al.*[23] Briefly, 1 mL of enzyme solution was mixed with 5 mL of 0.65% w/v casein prepared in 50 mM phosphate buffer solution (pH 6.0). The test tube containing the enzyme and substrate was incubated into the shaker

for 30 min at 37°C. After an incubation period, the reaction was stopped by adding 0.7 mL of 10% trichloroacetic acid and allowed to stand at room temperature for 20 min. The blank tube was made by substituting the enzyme solution with MilliQ water and used as a control. Both test and blank were centrifuged at 8000 rpm for 25 min, and the supernatant was collected. A volume of 200 µL of the supernatant was mixed with sodium bicarbonate solution 0.5M, and Folin–Ciocalteu reagent (0.67N) and the absorbance was measured at 660 nm.[24]

One unit of enzyme activity was defined as an amount of enzymes that release 1.0 µmole of L-tyrosine per min, in the assay conditions. The proteolytic activity was calculated using the following equation:

$$\text{Activity (U/mL)} = \frac{T \times V_t}{V_u \times t \times V_c} \quad (7)$$

Where, T: Mass (µg) of tyrosine released during hydrolysis; V_t: Total volume of the assay, V_u: Volume of enzyme solution used, t: Time of hydrolysis, V_c: Volume used in color development.

Protein determination

Protein content was estimated in the crude extract, and after each purification step, using the method described by Bradford.[25] The bovine serum albumin (BSA) was used as a standard.

Purification of protease

The crude extract obtained in the optimal defined conditions from *B. enneandra* tubers was used as the starting material for protease purification. The crude extract was subjected to ammonium sulfate precipitation at different concentrations of saturation (20%, 40%, 60%, and 80%) at 4°C. The precipitate was collected by centrifugation at 8000 rpm for 25 min at 4°C, and dissolved in 50 mM sodium phosphate buffer (pH 7.2) and dialyzed to remove the salt against the same buffer at 4°C for 24 h with continuous stirring. The dialyzed suspension was concentrated and subjected to gel filtration chromatography column Sephadex G-200 (1.5 cm × 140 cm) previously equilibrated with sodium phosphate buffer (50 mM, pH 6) containing 0.1 M NaCl. The protease was eluted with the same buffer and fractions of 1 mL were collected at the flow rate of 250 µL/min. The active fractions containing some impurities were pooled concentrated and loaded in Sephadex G-75 (1.6 cm × 67 cm) column previously equilibrated with sodium phosphate buffer (50 mM, pH 6). The protease was eluted with the same buffer-containing 0.1 M NaCl and fractions of 1 mL were collected at the flow rate of 0.50 mL/min. Protein concentration, proteolytic activity was determined in each of the fractions and the enzyme purity was verified by electrophoresis sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Molecular-weight determination

The molecular weight of the purified protease from *B. enneandra* was evaluated by SDS-PAGE[26] in 12% resolving gel as described by Laemmli. Electrophoresis was carried out using PowerPac, Basic™ from BIO-RAD as apparatus and the protein bands were stained with 0.1% Coomassie Brilliant Blue R-250 solution, then destained with destaining solution which was the mixture of milliQ water (50%), methanol (40%), and acetic acid (10%).The molecular mass of purified protease was determined by using a low-molecular-weight protein maker calibration.

Effect of pH on protease activity and stability

The effect of pH on protease activity was evaluated by incubating the protease at 37°C for 30 min at different pH ranging from 2 to 12. The appropriate pH was obtained with various buffer systems: glycine-HCl (pH 2–3), sodium acetate (pH 4–5), sodium phosphate (pH 6–8), Tris-HCl (pH 9–10), and glycine-NaOH (pH 11–12). For the evaluation of pH stability, the protease was preincubated at different pH for 1 h at 40°C, and the residual activity was then determined as described above.

Effect of temperature on protease activity and thermal stability

The influence of the temperature on protease activity was determined at different temperatures ranging from 10°C to 80°C with 10°C interval. One milliliter of protease solution and 5 mL of casein 0.65% w/v prepared in phosphate buffer (50 mM, pH 7.5) were added, and the mixture was incubated for 30 min at different temperatures. The enzyme activity was determined after the incubating period as described above. The thermal stability of purified protease was evaluated by preincubating the enzyme for 1 h at different temperatures vary from 10°C to 80°C, respectively, and the residual activity was determined.

Effect of protease inhibitors on protease activity

To determine the nature of the purified protease from *B. enneandra,* the enzyme was incubated with different proteases inhibitors. The inhibitors in which the effect was studied were phenylmethylsulfonyl fluoride (PMSF), aprotinin, Pepstatin A, iodoacetamide, and ethylenediaminetetraacetic acid (EDTA). The purified protease was preincubated in 5 mM and 10 mM of the inhibitors solutions and then incubated with casein solution for 30 min at 40°C and the remaining protease activity was determined. A control tube of the protease activity was done without inhibitors, and its activity was taken as 100%.

Effect of metal ions on protease activity

The effect of different metal ions ($MgCl_2$, $ZnCl_2$, $NiCl_2$, $SnCl_2$, $FeCl_2$, $CaCl_2$, KCl, and NaCl) on protease activity was evaluated. The mixture of protease and substrate were incubated with metal ions solution, and the remaining protease activity was determined according to the method described above. A control tube of the protease activity was done in the absence of metal ions, and its activity was taken as 100%.

Effect of different substrates on protease activity

The effect of the natural substrate which was protein from defatted flours of *Ricinodendron heudelotii* (*Euphorbiaceae*) as well as different synthetics substrates such as hemoglobin, casein, gelatin, and BSA on purified protease activity was evaluated. The enzyme activity was determined after incubating each substrate with protease solution at 40°C for 30 min.

Determination of kinetic parameters

The Michaelis–Menten constant (Km) and the maximum velocity (Vmax) of the purified protease were calculated by fitting the data of protease activity obtained from the increasing casein concentrations to a linear regression after Lineweaver–Burk transformation.

RESULTS AND DISCUSSION

Screening of the variables affecting protease activity

The Plackett–Burman design (PBD) was investigated to screen and select the main significant parameter which influences protease extraction from *B. enneandra*. The experimental matrix along with response is presented in Table 2.

Base on the experimental data and the related independent variables, a first order polynomial equation representing protease activity (Y_1) as the response was established.

$$Y_1 = 29.950 - 1.790 \times X_1 + 12.160 \times X_2 + 0.555 \times X_3 - 0.401 \times X_4 + 0.339 \times X_5 - 2.807 \times X_6$$

The ANOVA of the PBD experiments was done to evaluate the significance of the parameters. They were judged statistically significant or not if their $P \leq 0.05$ or ≥0.05, respectively [Table 3]. The effect of pH, the ratio (w/v), and stirring speed was found to be significant, while the effect of time, temperature, and NaCl concentration was not. In fact, their *P* value that are 0.6315, 0.4397, and 0.5754 were higher than 0.05, which demonstrating that the three variables produced not significant effects on proteases extraction from *B. enneandra*. The pH, ratio (w/v), and stirring speed were, therefore, chosen for further optimization of protease extraction.

Optimization of the selected variables

A total of 17 experiments were carried out using CCD to study interactions between the pH, ratio (w/v), and stirring speed and also to determine their optimal values. The experimental design which contains the different combinations is presented in Table 4, along with the experimental (Y_{2Exp}) and the calculated (Y_{2Cal}) responses.

Base on the experimental data, a second order polynomial equation was established to predict protease extraction from the powder of *B. enneandra* tubers.

$$Y_2 = 145.163 + 73.474 \times X_1 - 16.697 \times X_2 + 10.6609 \times X_3 - 10.317 \times X_1^2 - 8.6805 \times X_1 \times X_2 + 7.523 \times X_1 \times X_3 + 19.2667 \times X_2^2 + 15.0463 \times X_2 \times X_3 + 12.3135 \times X_3^2$$

To determine the accuracy of the polynomial model, the coefficient of determination Joglekar and May, Ross, the AAD Bas and Boyaci,[27] the bias factor, and the AF Dalgaard and Jørgensen were calculated and the results are presented in [Table 5].

All calculated parameters values were in range with the norms found in the literature Bas and Boyaci, Dalgaard, and Jørgensen.[27,28] To confirm this accuracy, protease was extracted in the optimal defined condition. The protease activity obtained was 311.954 mU/mL which was close to the predicted value 308.145 mU/mL. These results suggest that the established model was acceptable and can be used to predict protease extraction from *B. enneandra,* this with the respect of the independent variables range.

The ANOVA at the level of 5%, of the center composite design (optimization) experiments is given in Table 6. Base on this ANOVA, the means parameter to consider for protease extraction from *B. enneandra* were ratio (m/v), pH, interaction between pH, stirring speed, and the quadratic effect of pH, this because of their $P < 0.05$ which was the analysis level. To better understand, the effect of pH and ratio (w/v), the three-dimensional curve was plotted [Figure 1] using Sigma plot software version 11.0. From this Figure 1 we can see that protease activity increase when the pH value increase. However, up to about pH 6 any further increasing of pH decrease the protease activity. This can be due to the fact that, in solution protein behavior depends on pH condition of the environment. The optimal condition for protease extraction from *B. enneandra* was found to be: 4%, 5.1 and 100 rpm, for the ratio (m/v), pH, and stirring speed, respectively. The implementation of this condition gave 308.145 mU/mL as protease activity which was closed to the predicted value 311.954 mU/mL calculated with the regression polynomial second order model.

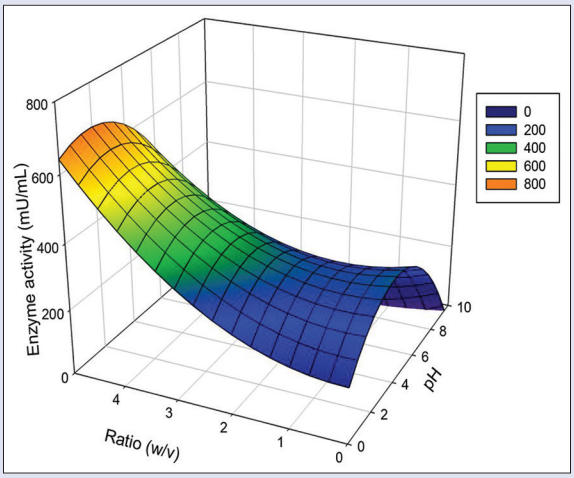

Figure 1: Three-dimensional plot presenting the effect of pH and ratio (w/v) on protease extraction

Purification and molecular weight of protease

Protease was extracted from *B. enneandra* tubers and subjected to three steps purification all carried out at 4°C. The purification procedure is summarized in Table 7. Ammonium sulfate fractionation was the first step in purification and the protease of interest was obtained maximally at 60% of the saturation of salt with 10.69 U/mg as specific activity and 3.12 folds purification compare

Table 3: Analysis of variance for the screening variables

Source	Sum of squares	Degree off reedom	Mean squares	*F*	*P*
pH	38.456	1	38.456	6.870	0.0306
Ratio (w/v)	1774.530	1	1774.530	317.160	0.0000
Temperature	3.703	1	3.703	0.660	0.4395
NaCl concentration	1.931	1	1.931	0.350	0.5731
Extraction time	1.382	1	1.382	0.250	0.6326
Shaker speed	94.663	1	94.663	16.920	0.0034
Pure error	44.760	8	5.595		
Total	1959.430	14			

Table 4: Experimental matrix for central composite design

Run	X_1	X_2	X_3	Y_2 Exp	Y_2 Cal
1	−1	−1	1	97.222	89.060
2	0	−1.35313	0	194.444	203.033
3	−1.35313	0	0	30.555	26.853
4	0	0	−1.35313	152.778	153.283
5	0	0	1.35313	196.759	182.134
6	1	−1	−1	273.148	262.140
7	−1	−1	−1	111.111	112.877
8	1	1	−1	166.667	181.292
9	1	−1	1	259.259	268.415
10	0	1.35313	0	180.555	157.846
11	−1	1	1	85.648	103.120
12	1.35313	0	0	236.111	225.693
13	1	1	1	243.055	247.753
14	−1	1	−1	69.444	66.751
15	0	0	0	138.889	145.163
16	0	0	0	138.889	145.163
17	0	0	0	141.203	145.163

X_1: pH; X_2: Ratio (w/v); X_3: Stirring speed

Table 5: Parameters for central composite design model validation

Parameters	Values	Norms	References
ADD	0.006	0-0.3	Baş and Boyac
Af	1.006	0.75-1.25	Dalgaard and Jørgensen
Bf	0.996	0.75-1.25	Dalgaard and Jørgensen
R^2 (%)	97.589	≥80	Joglekar and May, Ross

Af: Accuracy factor; Bf: Bias factor; ADD: Absolute average deviation

to crude extract [Table 7]. The fraction obtained at 60% saturation was dialyzed, concentrated, and loaded on Sephadex G200 column and two Peaks I and II [Figure 2] were eluted and analyzed for protein concentration as well as protease activity. The second peak of elution [Figure 2] showed activity but was not pure according to gel electrophoresis analysis [Figure 2]. Fractions constituting this peak were pooled, concentrated, loaded on Sephadex G75 column, and eluted with phosphate buffer (50 mM, pH 6). During this last step of purification, one single peak was obtained from Sephadex G75 column [Figure 3] with 37.09 folds purification and the specific activity was enhanced to 127.23 U/mg.

The homogeneity of the eluted protease through the second gel permeation chromatography was confirmed by polyacrylamide gel electrophoresis with the presence of one single protein band. The standard protein markers used were phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

The purified protease from *B. enneandra* showed single band and the molecular weight was evaluated at 23 kDa [Figure 4].

Effect of pH on protease activity and stability

The effect of pH on protease activity was evaluated at different pH range 3–12 using casein as substrate [Figure 5]. Protease activity increased with increasing pH value and the maximum activity was found at pH 5. Above this value, any increasing of pH lead to decrease protease activity. The stability of protease from *B. enneandra* was also done after preincubating the enzyme for 1 h at the same range of pH. The purified protease was stable at pH range 3–10 and retained more than 60% of its original activity after preincubating the protease for 1 h. Similar results were reported by Balqis and Rosma who purified protease from *Artocapus. integer* leaves.[29]

Effect of temperature on protease activity and thermal stability

The temperature profile of the purified protease was studied at pH 5 by incubating the enzyme at different temperatures ranging from 10°C to 80°C. From Figure 6, it is obvious that protease from *B. enneandra* showed its highest activity at 40°C, and then, the activity rapidly decreased with increasing temperature. This result is in accordance with some reported from plants proteases which exhibited their

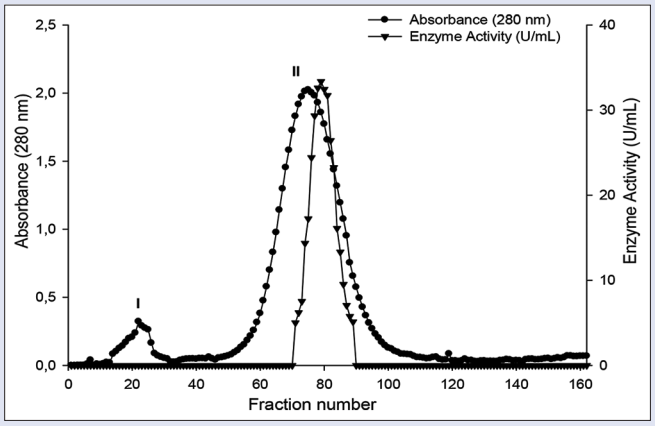

Figure 2: Elution profile of protease from *Burnatia enneandra* on Sephadex G200 gel filtration chromatography

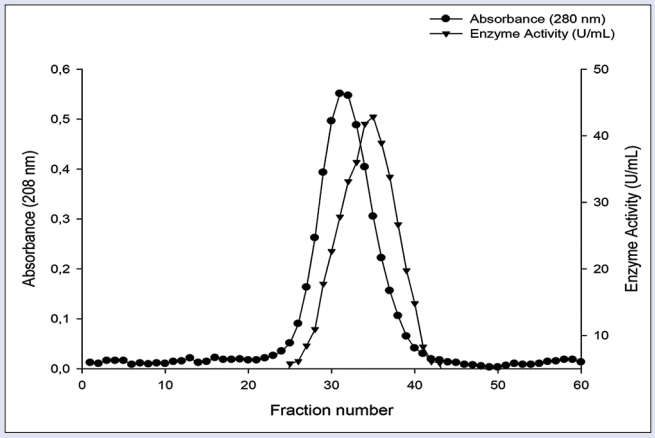

Figure 3: Elution profile of protease from *Burnatia enneandra* on Sephadex G75 gel filtration chromatography

Table 6: Analysis of variance for the second order polynomial model

Source	Sum of squares	Degree of freedom	Mean squares	*F*	*P*
X_1:Ratio (w/v)	62956.00	1	62956.00	239.05	0.0000
X_2:pH	3251.24	1	3251.24	12.35	0.0098
X_3: Stirring speed	1325.42	1	1325.42	5.03	0.0598
X_1^2	713.66	1	713.66	2.71	0.1437
X_1*X_2	602.81	1	602.81	2.29	0.1741
X_1*X_3	452.76	1	452.764	1.72	0.2312
X_{22}	2488.86	1	2488.86	9.45	0.0180
X_2*X_3	1811.12	1	1811.12	6.88	0.0343
X_3^2	1016.60	1	1016.60	3.86	0.0902
Total error	1843.48	7	263.35		
Corr. total	76462.00	16			

Table 7: Purification scheme of protease from *Burnatia enneandra* tubers

Steps	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	1222.50	4190.92	3.43	1	100
Ammonium sulfate precipitation 60%	356.10	3807.75	10.69	3.12	90.96
Gel filtration chromatography G200	44.05	2750.48	62.44	18.2	65.63
Gel filtration chromatography G75	14.69	1903.36	127.23	37.09	45.42

maximum activity in the temperature range 40°C–50°C Mohamed and Habbani; Devi, and Hema-Latha. However, the results found here are in disagreement with those of Sanatan *et al.* Sanatan, Lomate, Giri, and Hivrale, who reported protease with optimal temperature at 60°C from *Periplaneta americana.*[30]

Protease from *B. enneandra* was stable up to 60°C after 1 h incubation and more than 50% of its original activity remained at this temperature [Figure 6].

Effect of protease inhibitors on protease activity

To determine the class in which belongs the protease from *B. enneandra,* the effect of many proteases inhibitors on protease activity was investigated. Protease inhibitors such as serine protease inhibitor (Aprotinin, PMSF) and cysteine protease inhibitor (iodoacetamide) displayed no significant effect on protease activity. This result involved that *B. enneandra* protease is not belong to the class of serine or cysteine protease. At the concentration of 1 mM, peptatin A which is the inhibitor of aspartic proteases shows no great inhibition (1.2%) on protease activity. However, at 10 mM concentration 42.05% inhibition was observed with the same inhibitor proved that the protease is an aspartate protease Kumar, Sharma, Saharan and Singh, Raposo and Domingos. *B. enneandra* protease activity was also inhibited up to 48.59% and 90.18% following incubation with EDTA at 1 mM and 10 mM concentration, respectively [Figure 7]. These results consistent with previous findings demonstrating that *B. enneandra* protease also belonging to metalloprotease class are strongly inhibited by EDTA.[31] Protease extract from *B. enneandra* tubers was inhibited both by aspartic and metalloprotease inhibitor which permitted to classify this protease as an aspartic-metalloprotease. This protease can be used in dairy industry, meat tenderization as well as protein hydrolysates production.

Effect of metal ions on protease activity

Effect of different metal ions either monovalent (K^+, Na^+) or divalent (Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2}+, Ni^{2+}, Li^{2+}, Co^{2+}, and Sn^{2+}) on protease activity was determined using casein as substrate. Metal ions such as Li^{2+}, Na^+, K^+ Sn^{2+}, and Co^{2+} have no effect on protease activity, while Mg^{2+} showed slightly increasing enzyme activity. Ca^{2+} and Ni^{2+} ions significantly enhanced the protease activity when Zn^{2+} and Fe^{2+} ions greatly shown inhibition [Figure 8].These results are in line with those reported by some authors Mahajan, Nayak, and Lele.[32] However, Anandharaj *et al.* Anandharaj, Sivasankari, Siddharthan, Rani and Sivakumar found that Ni^{2+} ion inhibited metalloprotease from *Bacillus alkalitelluris* TWI3 Isolated from Tannery Waste.

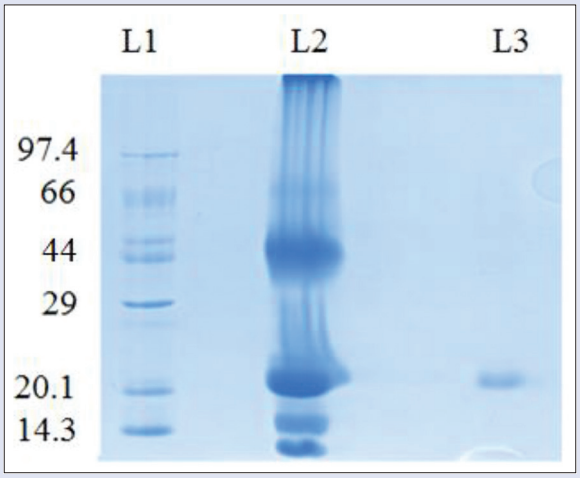

Figure 4: .Sodium dodecyl sulphate polyacrylamide gel electrophoresis profile of the purified metalloprotease protease from *Burnatia enneandra.* Where, L1: Protein Marker, L2: Crude extract and L3: Purified protease

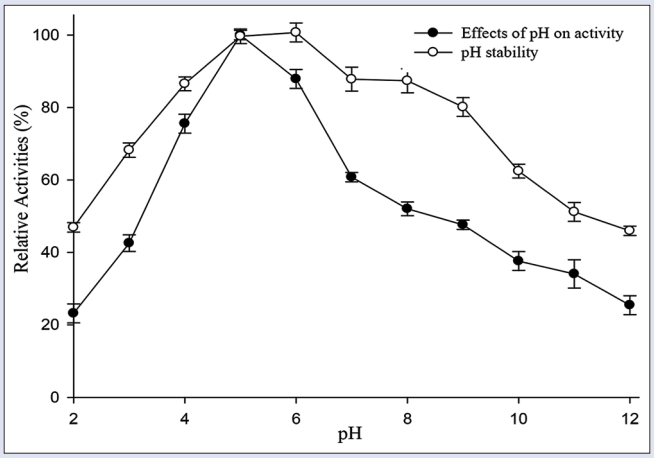

Figure 5: Optimum pH and stability of the purified protease from *Burnatia enneandra*

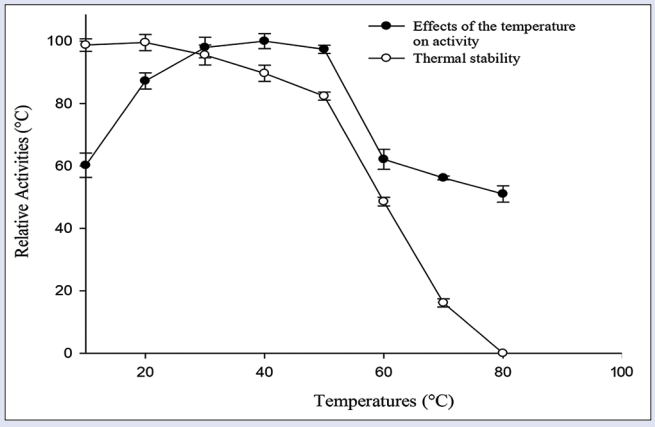

Figure 6: Optimum temperature and thermal stability of the purified protease from *Burnatia enneandra*

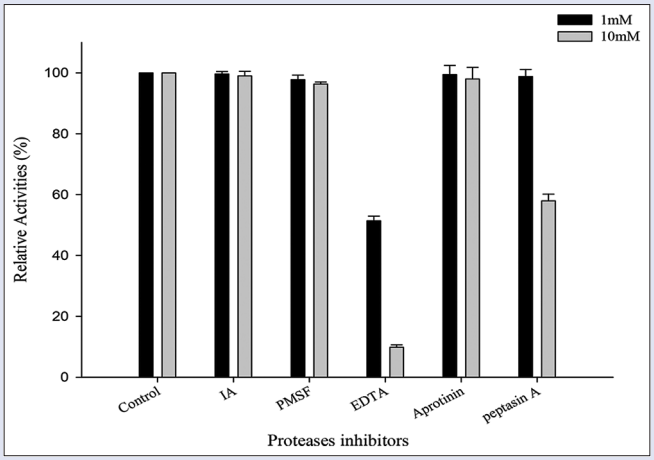

Figure 7: Effect of some inhibitors on protease activity

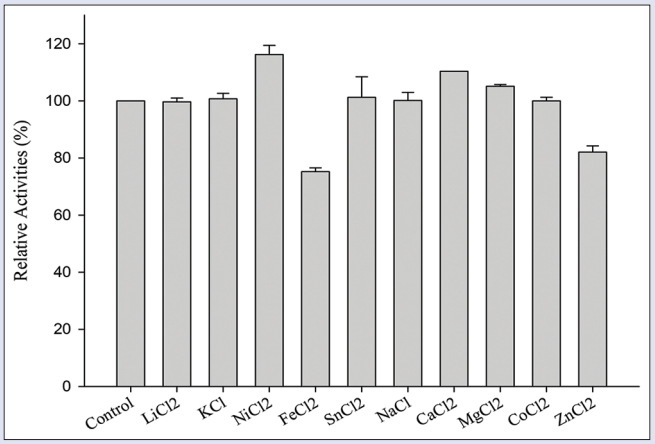

Figure 8: Effect of metal ions on protease activity

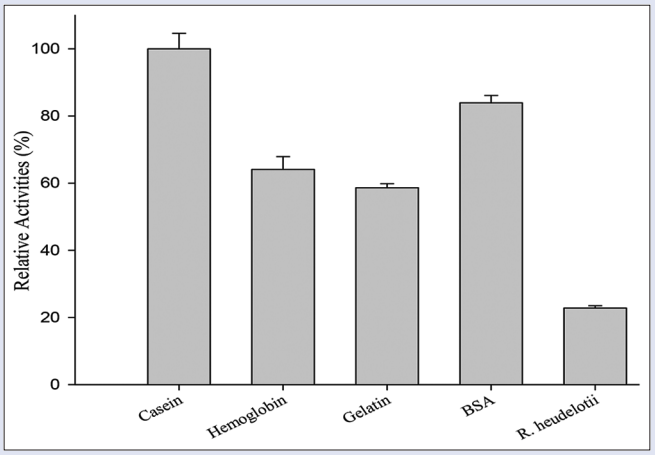

Figure 9: Effect of some substrates on protease activity

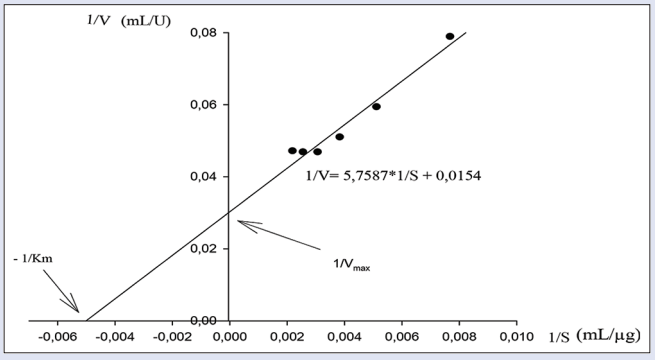

Figure 10: Lineweaver-Burk plot of *Burnatia enneandra* protease activity as a function of casein concentration

Effect of different substrates on protease activity

The protease activity of purified extract from *B. enneandra* tubers was assayed using both natural (protein from defatted flours of *R. heudelotii)* and synthetic substrates (casein, BSA, gelatin and hemoglobin). The enzyme was incubated with different substrates and its optimal condition and activities were determined compared to casein, which was taken as 100%. The protease activity decreased from synthetic substrates BSA (83.91%), hemoglobin (64.10%) and gelatin (58.63%) to natural substrate protein from *R. heudelotii* defatted flours (45.56%) [Figure 9]. Purified protease from *B. enneandra* is more active on BSA and gelatin than that extracts from the leave of *A. integer,* but in revenge this protease was more active on hemoglobin. In fact, Balqis and Rosma Balqis and Rosma reported 138, 81 and 24% as a relative activity of protease from the leave of *A. integer* on hemoglobin, BSA and gelatin, respectively, compared to casein.

Determination of kinetic parameters

The effect of increasing casein concentration was investigated and the protease activity was calculated as described above. The maximum rate of reaction Vmax and Km were evaluated graphically from regression Lineweaver-Burk plot [Figure 10]. The results revealed that purified protease from *B. enneandra* tubers had Vmax and Km of 64.935 (U/mL) and 373.941 (µg/mL), respectively.

CONCLUSION

The optimal conditions to extract protease from *B. enneandra* tubers were found to be 5.1, 40°C and 100 rpm for pH, temperature and agitation frequency, respectively. Crude enzyme extract was purified successively using ammonium sulfate precipitation, gel filtration chromatography Sephadex G200, and gel filtration chromatography Sephadex G75. Characterization of the purified protease showed that its activity was inhibited both by pepstatin A and EDTA proved that it is an aspartic-metalloprotease. The purified protease has optimal pH in acid zone that is 5.1 and its activity is enhanced by Ca^{2+} ion which suggested that this protease may be useful in the industries for milk clotting. However, to conclude this outcome, this enzyme needs more detailed study on milk clotting properties.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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