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Original Research Article

ISOLATION AND CHARACTERIZATION OF PROTEASE FROM *WISTERIA SINENSIS* AND *CHLORIS BARBATA*

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Abstract: *Enzymes, as biocatalysts, have extensive industrial applications. Protease enzymes, in particular, are widely used in industries such as leather, detergents, food, pharmaceuticals, and diagnostics. Alkaline proteases, which require a neutral to alkaline pH for activity, are notably sourced from plants.* ***Objective:*** *This study aimed to isolate and partially purify protease enzymes from the leaves of Wisteria sinensis and Chloris barbata, and to characterize their enzymatic properties, particularly their caseinolytic activity in alkaline conditions.* ***Methods:*** *Proteases were isolated and partially purified from the leaves of Wisteria sinensis and Chloris barbata. Enzyme kinetics were assessed to determine the optimal caseinolytic activity of the proteases. The study focused on evaluating the enzymes' stability at specific pH levels and temperatures. Statistical analysis was conducted to compare the enzymatic activities and to validate the findings.* ***Results:*** *The isolated proteases from both Wisteria sinensis and Chloris barbata demonstrated optimal caseinolytic activity in alkaline pH. The enzymes exhibited significant stability across specific pH levels and temperatures, confirming their alkaline nature. These findings highlight the potential industrial applications of these proteases.* ***Conclusion:*** *The study provides valuable insights into the isolation and characterization of proteases from Wisteria sinensis and Chloris barbata. The identified alkaline proteases show promise as industrial biocatalysts due to their stability and significant protease activity. Further research is warranted to purify these enzymes fully and explore their specific industrial applications, emphasizing the biotechnological potential of diverse plant sources.*

Keywords: Protease, *Wisteria sinensis, Chloris barbata,* caseinolytic

Introduction

Enzymes are notable biocatalysts that are industrially utilized in cleansers, food, pharma, diagnostic, and various other industries. Protease enzyme is a catalyst that is generally utilized in leather and silver recuperation (1). These proteins might be named either acidic, neutral, or alkaline protease, as per their ideal pH responses (2). Plants, microorganisms, viruses, and archaea are the primary hotspots for protease creation (3). Alkaline proteases need an Asp-His-Ser triad (serine protease) or metal ions (metalloprotease) to act on the substrate. Owing to their ability to sustain under alkaline pH without losing the action specificity, they have long been used in various industries, such as the detergent industry (4). Most proteases lose their activity under harsh conditions but the demands are increasing extensively, hence there is a need to extract and develop alkaline proteases for use in various industries (5).

Plants are the rich wellspring of protease compounds. Greenberg and Winnick recorded eleven plant proteases. Protease enzyme is a notable plant catalyst, utilized generally for medicinal and industrial applications. For example, it is used in the leather industry to improve quality, the meat industry for preparation and tenderization of meat, milk-coagulation in dairy farms, nutraceutical and pharmaceutical industry, cancer treatment, diagnostics, and silver recovery. Aside from this, plant proteases have numerous organic jobs, for example, they are effective in treating malignant tumors, turning away edema, improving gut health, and have procoagulant action (6). Moreover, this enzyme is commercially valuable and is expected to have a 15-billion-dollar industry by 2025, only alkaline proteases have about 35% sales among all protease enzymes (7).

Wisteria sinensis, a plant in the Fabaceae family, incorporates 10 types of deciduous woody veins. Chinese (W. sinenses) and Japanese wisteria (W. floribunda) have become two of the most well-known blossoming veins in United States science. *Chloris barbata* is a plant belonging to the Poceae family and has multiple restorative and medicinal uses (8). We have used *Wisteria sinensis* and *Chloris barbata* here for isolation and partial purification of protease from their leaves. Enzyme kinetics found that the optimal caseinolytic activity of protease was demonstrated in alkaline pH.

Methodology

Plants, belonging to the families of Leguminose and Graminae, which were available from nearby areas of Pakistan were used as a source for protease screening. Diverse plant expressions including leaves, bloom, and stem were used for this study.

New leaves weighing 25 g from the developed plant were mixed with 100 mL 15% sodium chloride, 100 0.2% (w/v) sodium hydroxide, 100 0.05 M hydrochloric acid, 100 mL 0.2 M phosphate buffer (pH 7), and water. After complete washing in refined water, the samples were air-dried. The concentrate was set up in a 500 mL Erlenmeyer flask by homogenizing 25 g of plant materials in 100 mL of extractant using an electrical blender. The homogenate was further additionally blended by incubating the contents at

room temperature in a rotating shaker for 30 min at 150 rpm. Subsequently, the slurry was sifted through cheesecloth, and the filtrate underwent centrifugation at 10,000 rpm for 15 min to remove any cell debris that remained in the preparation. A clear supernatant was obtained and securely stored at -4°C.

For partial purification of protein, Ammonium sulfate precipitation was employed. Different concentrations ranging from 30% to 80% were used so that the best possible concentration that yields protease in high concentration can be found. The whole process was carried out at 0°C to avoid possible denaturation of proteins. The beaker containing the protein sample was transferred to a container containing ice, Subsequently, the setup was placed on a magnetic stirrer. While the sample was undergoing stirring for approximately 30 minutes, a gradual addition of ammonium sulfate ensued, reaching final concentrations of 50%, 55%, 60%, and 70% saturation (refer to the table). Following this, the resultant mixture was transferred into falcon tubes and subjected to centrifugation at 6000 rpm for reconstituted in 1 ml of phosphate buffer with a pH of 7. A protease assay was used for 30 minutes. The supernatant was meticulously removed, and the pellet was determined concentration by each concentration and the sample was stored at -4°C for long-term usage.

In a sterile petri dish, we added 10g of skimmed milk and 100ml of distilled water. A second solution containing 2g of bacteriological agar in 100 ml of distilled water was also prepared. Both solutions were covered with aluminum foil before autoclaving. After sterilization, both solutions were mixed in a fume hood close to the burner to avoid contamination. Agar plates were prepared and wells were made in the plates and marked for identification.1ml of the supernatant was poured into each petri dish and incubated for 24 hours at 37 0C.

The effect of pH on protease activity was quantified using different buffers having pH values on a scale between 4 and 9. 0.2M Sodium acetate buffer (pH 4,5), 0.2M sodium phosphate buffer (pH 6,7), and Tris-Base (pH 8,9) were used for this purpose. The pH of these buffers was adjusted to 4, 5, 6, 7, 8, and 9 respectively.

The impact of changing time on proteases was tested by enzyme assay at different time gaps such as 10mins, 20mins, 30mins, 40mins, and 50mins.

The impact of changing temperature on enzyme activity was tested by providing multiple temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 45°C, 50°C, 55°C, 60°C) using enzyme assay.

Casein was used as a substrate at different concentrations for carrying out enzyme activity assay.

The effect was observed by continuously changing the concentration of casein ranging from 0.5 to
2.5 in enzyme assay which detects the activity of protease at specific concentrations.

Sodium 1,2-naphthoquinone-4-sulfonate also known as Folin reagent was used as a derivatizing agent to measure the levels of amines and amino acids. The reagent provides a fluorescent material in response to an alkaline solution. Folin & Ciocalteus Phenol, or Folin reagent produces a blue-shaded chromophore by interacting with free tyrosine, which was quantified and measured using a spectrophotometer.

Results

In this research, proteases were isolated from various parts of the plant body such as leaves, shoots, stems, flowers, and seeds. Our aim of studies was to examine the characterization of proteases extracted from selected plants. Proteases are important drug targets; they are extensively used as anti-hypertensive and antivirals. Proteases assist in various processes in the human body such as digestion, blood clotting, and cell division.

The proteolytic properties of all extractants were measured using skim milk along with bacteriological agar utilizing zone checking by agar plate method and showed as distance across of clear zone (Fig 1)

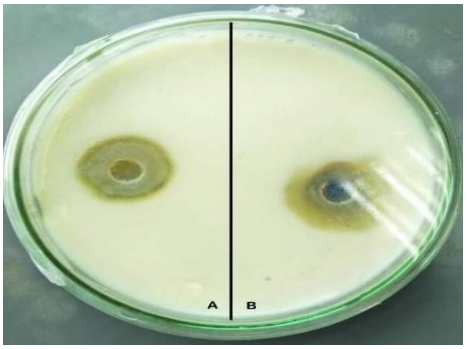

Fig 1: Zone checking by agar plate method (A*) *Chloris barbata*, (B) *Wisteria sinensis*

Proteolytic activity

We investigated the protease activity obtained from two different plants; Wisteria sinenses (Fabeacae) and Chloris barbata (Poaceae) by establishing an enzyme assay using casein as substrate.

To quantify the chemical activity, the tyrosine standard curve was plotted. The standard factor was calculated as 221.46(Table 1, Fig 2).

Table 1: Standard Curve of tyrosine

Concentration (mg/ml)	Absorbance at 280nm	Conc./Abs
40	0.105	380.95
80	0.301	265.78
120	0.501	239.52
160	0.708	225.98
200	0.901	221.97
240	1.111	216.02
Standard factor		221.46

Enzyme activity shows the amount of enzyme present in a plant extract. These values suggest that these plants can be used for further analysis of proteases. During the chemical test, protease digests casein and the amino acid tyrosine is freed alongside other amino acids and peptides, the more the tyrosine freed higher the action of protease (Table 2).

Table 2: Protease activity of Plants

Plant	Protease (IU/ml)
Wisteria sinensis	639.13
Chloris barbata	650.42

Folin reagent or sodium 1,2-naphthoquinone-4-sulfonate is a chemical reagent used as a derivatizing agent to quantify levels of amines and amino acids. The reagent responds in an alkaline solution to produce a fluorescent material. Folin & Ciocalteus Phenol, or Folin reagent reacts with free tyrosine to produce a blue-shaded chromophore, which is quantifiable and measured as an absorbance on the spectrophotometer.

For estimation of total protein content produced from plant extract, the BSA standard curve was plotted. The standard factor was calculated as 0.755 (Table 3, Fig 3).

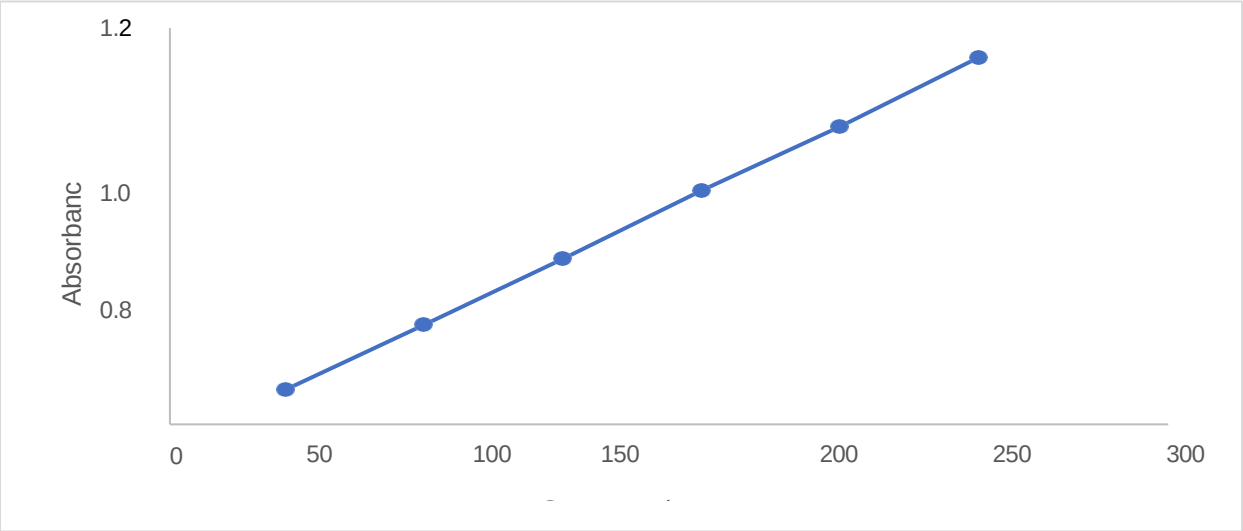

Fig 2: Tyrosine standard curve

Table 3: BSA Standard Curve

BSA (ml)	Water (ml)	Sample conc.(mg/ml)	Sample volume (ml)	Alkaline reagent (ml)	Folin reagent (ml)	Abs. (280nm)	Con./Abs
0.25	4.57	0.05	0.5	5	0.5	0.178	0.280
0.5	4.5	0.1	0.5	5	0.5	0.217	0.460
1	4	0.2	0.5	5	0.5	0.310	0.645
2	3	0.4	0.5	5	0.5	0.447	0.894
3	2	0.6	0.5	5	0.5	0.636	0.943
4	1	0.8	0.5	5	0.5	0.752	1.063
5	0	1.0	0.5	5	0.5	1	1
Standard factor							0.755

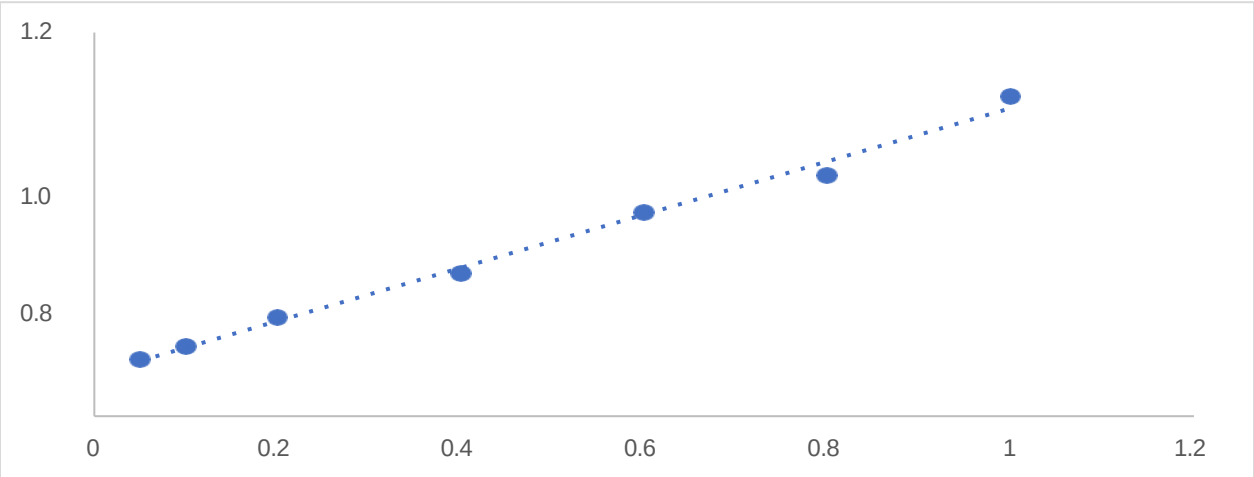

Fig 3: BSA Standard Curve

For the estimation of protein content in plants, absorbance is measured by the protease activity contrasted with a standard curve. More chromophores created means that more tyrosine is discharged from casein, which is directly proportional to the activity of protease (Table 4).

Ammonium sulfate precipitation was performed to find the ideal concentration. (Table 5; 6)

Table 4: Estimation of Protein in plants

Plant	Protease (IU/ml)
Wisteria sinensis	252.46
Chloris barbata	411.25

Table 5: Protease activity assay & Folin-Lowry values of stored pellets for protein concentration after partial purification for *Wisteria sinensis*.

Percentage of (NH4)2SO4	Protease activity assay	Folin-Lowry method
30%	487.21	377.14
40%	547.89	394.19
50%	591.29	401.28
60%	596.61	419.44
70%	610.56	578.23
80%	626.73	635.14

Table 6: Protease activity assay & Folin-Lowry values of stored pellets for protein concentration after partial purification for *Chloris barbata.*

Percentage of (NH4)2SO4	Protease activity assay	Folin-Lowry method
30%	29.89	91.46
40%	214.37	253.12
50%	226.08	268.40
60%	238.29	389.54
70%	512.01	504.7
80%	530.62	518.2

The pH 9 which was mildly basic was identified as optimal pH, which implies that maintenance of pH in this range would yield maximum enzyme production. Both plants show stability at alkaline pH. Therefore, these enzymes are alkaline proteases. (Table 7;8, Fig 4;5)

Table 7: Protease activity at different pH for *Wisteria sinenses*

pH	Absorbance at 280 nm	Protease (IU/ml)
4	2.751	609.23
5	2.643	585.31
6	2.801	620.30
7	2.461	545.01
8	2.352	520.87
9	2.95	653.307

Table 8: Protease activity at different PH for *Chloris barbata*

pH	Absorbance at 280 nm	Protease (IU/ml)
4	1.893	**419.22**
5	2.488	**550.99**
6	1.971	**436.49**
7	1.705	**377.58**
8	2.749	**608.79**
9	**2.801**	**620.30**

Temperature effect on protease activity

Protease assay was carried out at pH 9 to determine the effect of temperature on protease activity. At 37^0C the enzyme shows more stability and activity for both plants. (Table 9;10, Fig 6;7)

Table 9: Protease activity at different temperatures for *Wisteria sinenses*

Temperature (0C)	Absorbance at 280 nm	Protease (IU/ml)
37	2.866	**634.70**
42	1.270	**281.25**
47	2.244	**496.95**
52	1.791	**396.63**
57	**0.464**	**102.75**

Table 10: Protease activity at different temperatures for *Chloris barbata*

Temperature (0C)	Absorbance at 280 nm	Protease (IU/ml)
37	2.937	**650.42**
42	2.142	**474.36**
47	2.294	**508.02**
52	0.626	**138.63**
57	**0.301**	**66.65**

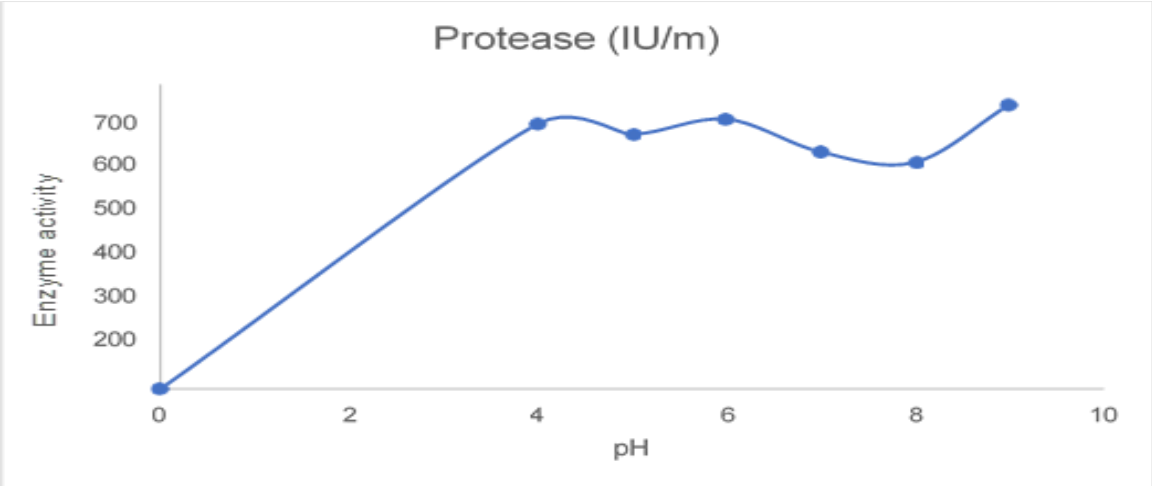

Fig 4: Impact of pH on the activity of the enzyme in Wisteria

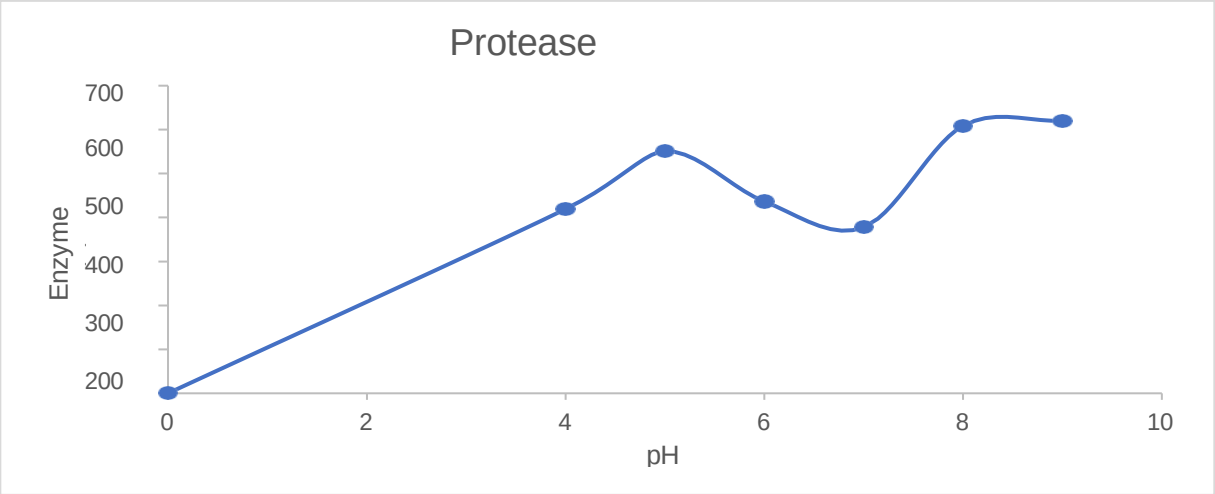

***Fig 5: Impact of pH on the activity of the enzyme in* Chloris barbata**

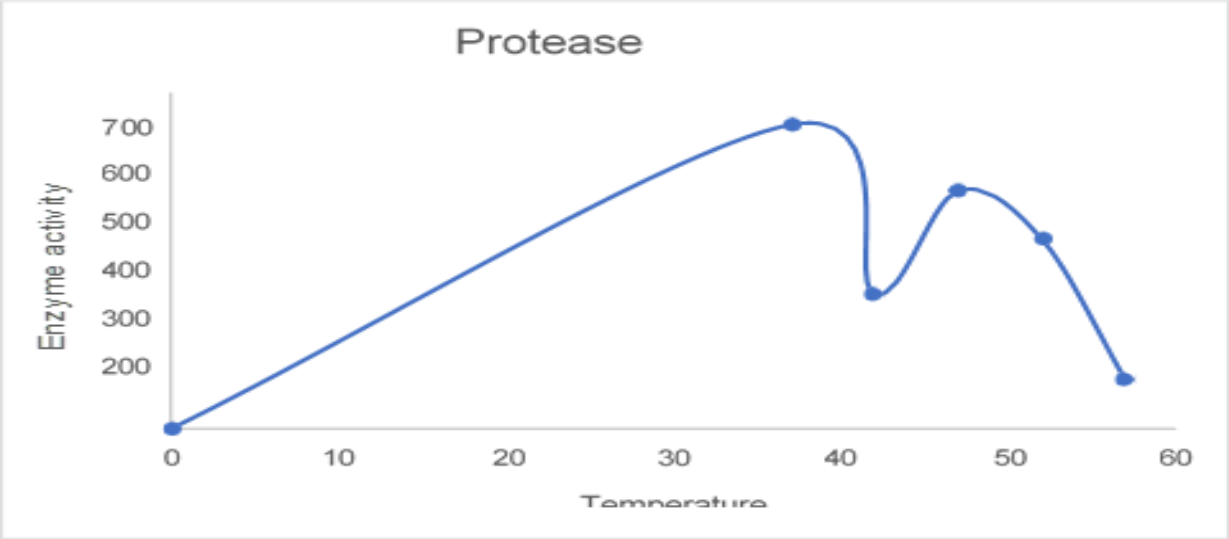

***Fig 6: Effect of temperature on enzyme activity in* Wisteria**

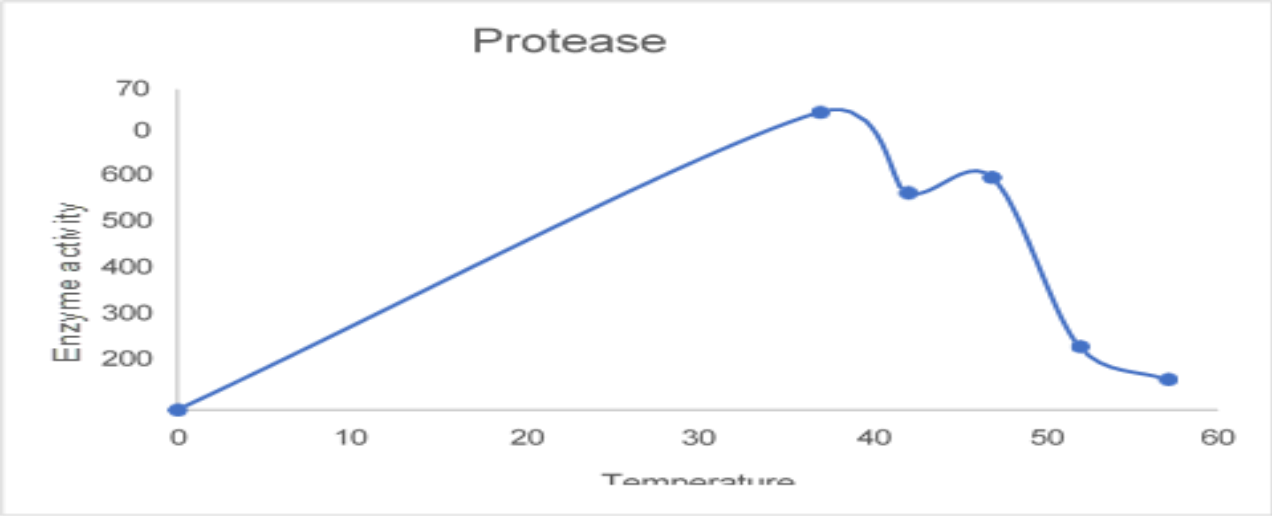

Fig 7: Effect of temperature on protease in *Chloris barbata*

[Citation: Qadir, N., Ali A. (2024). Isolation and characterization of protease from wi*steria sinensis* and *chloris barbata*. *Biol. Clin. Sci. Res. J.*, **2024***: 1130*. doi: https://doi.org/10.54112/bcsrj.v2024i1.1130]

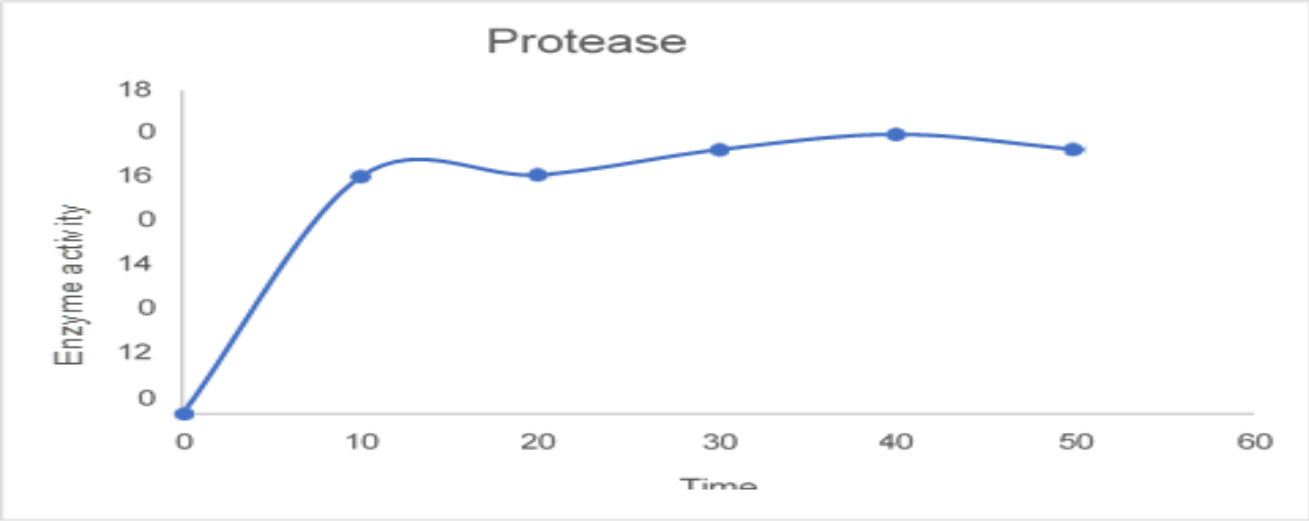

***Fig 8: Impact of time on enzyme activity in* Wisteria**

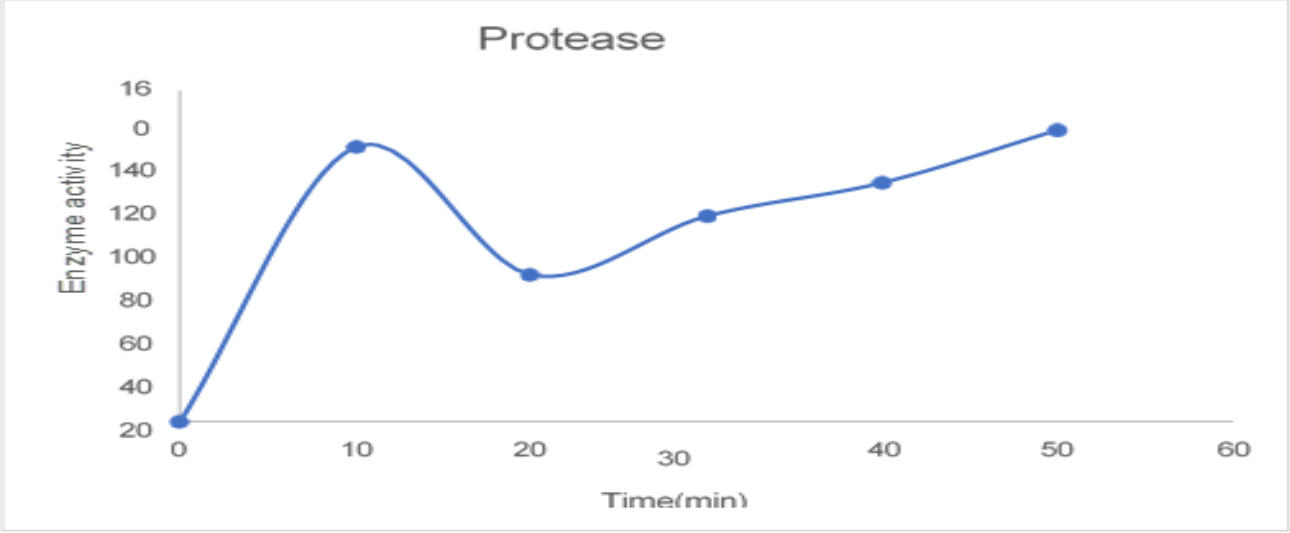

Fig 9: Impact of time on enzyme activity in *Chloris barbata*

Impact of time on functioning of protease

The crude enzyme was subjected to incubation for varying periods, at optimum conditions such as at 37^0C, in a 0.2M Tris-Base buffer pH 9 for both plants. Protease activity was measured by enzyme assay. The optimum time for *Wisteria sinensis* was 40 minutes and for *Chloris barbata* was 50 minutes (Table 11;12, Fig 8;9)

Table 11: Protease activity at different periods for *wisteria sinenses*

Time (min)	Absorbance at 280 nm	Protease (IU/ml)
10	0.597	132.21
20	0.601	133.09
30	0.664	147.04
40	0.703	155.68
50	0.665	147.27

Table 12: Protease activity at different periods for *Chloris barbata*

Time (min)	Absorbance at 280 nm	Protease (IU/ml)
10	0.60	132.87
20	0.321	71.08
30	0.449	99.43
40	0.522	115.60
50	0.637	141.07

Effect of concentration on protease activity

To check the activity of enzymes with different concentrations of casein, samples were incubated at optimum PH, temperature, and time. It is evident that Wisteria has efficient activity of enzyme with 2g of casein at pH 9 in tris-base buffer, required 40 minutes and optimum temperature is37 0C whereas Chloris barbata shows more enzyme activity with 1g of casein concentration at pH 9, required 50 minutes and optimum temperature 37 0C. (Table 13;14, Fig 10;11)

Table 13: Protease activity at different concentrations of casein for *Wisteria sinenses*

Concentration (g)	Absorbance at 280 nm	Protease (IU/ml)
0.5	0.301	66.65
1	0.280	62.00
1.5	0.208	46.06
2	0.321	71.08
2.5	0.081	17.9

Table 14: Protease activity at different concentrations of casein for Chloris barbata

Concentration (g)	Absorbance at 280 nm	Protease (IU/ml)
0.5	0.028	**6.20**
1	0.296	**65.52**
1.5	0.235	**52.04**
2	0.164	**36.31**
2.5	**0.072**	**15.94**

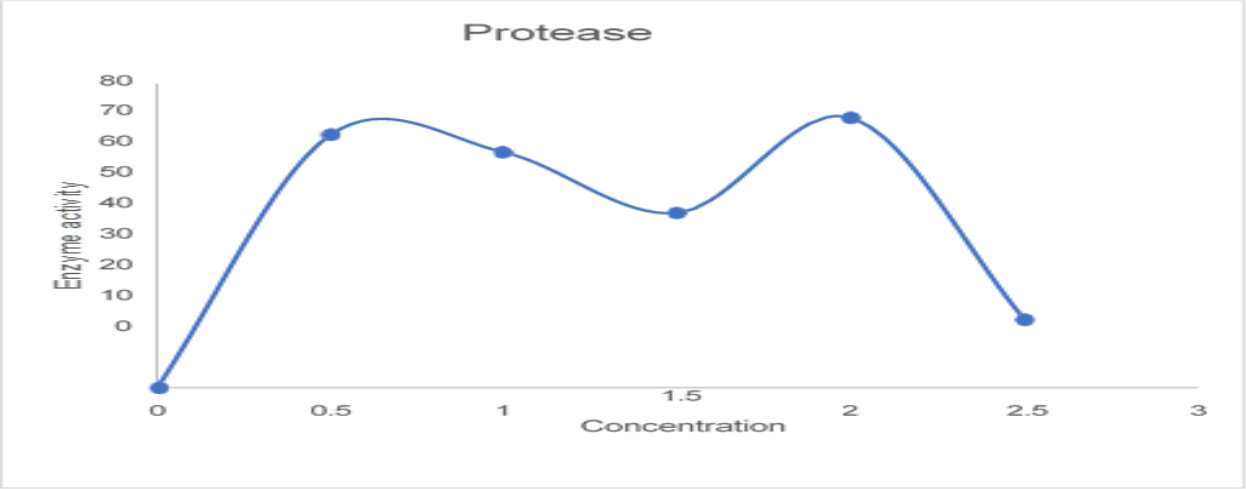

Fig 10: Effect of concentration on enzyme activity of *Wisteria*

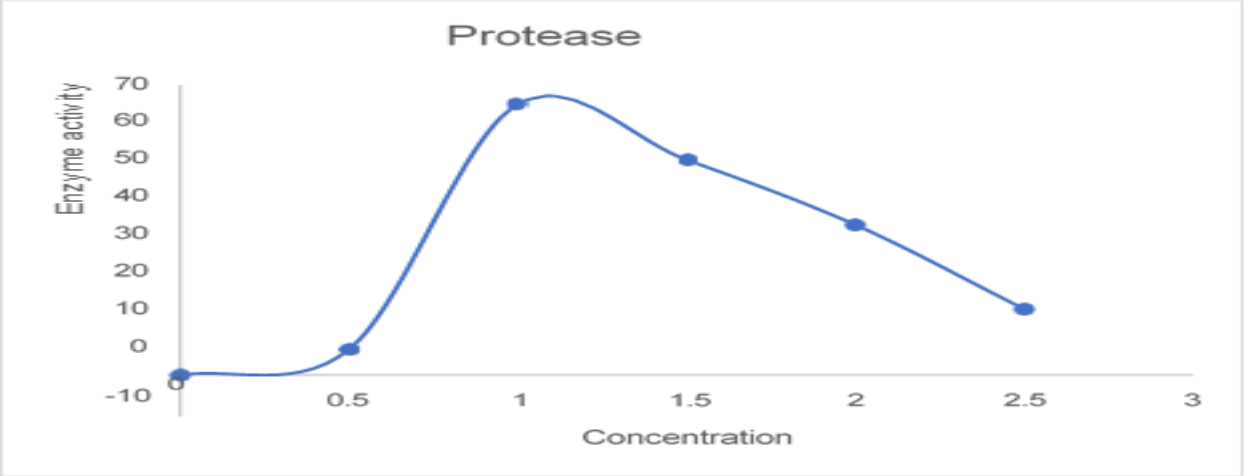

Fig 11: Effect of concentration on enzyme activity of *Chloris barbata*

Discussion

Enzymes are significant biocatalysts widely employed in detergents, food processing, pharmaceuticals, diagnostics, and fine chemicals (9). These encompass a variety of proteins categorized as either acidic, neutral, or alkaline protease, as per their ideal pH responses. Particularly the neutral protease makes up the biggest extent of enzymes utilized around the globe (7). Neutral proteases are created fundamentally by microorganisms, animals, and plants. Those created by microorganisms are most usually applied in industry, as a result of their solid hydrolytic capacity and wide versatility to synergist response conditions, which make them increasingly versatile to downstream processing than those proteases acquired from plants or animals. Moreover, these present a degree of higher peptidase action contrasted with other commercial enzymatic arrangements. The principle normal for this chemical is its affinity towards hydrophobic amino acids, which is invaluable for its utilization as a debittering agent (10).

The current study concerns with activity of the proteases that were extracted from two different plants (11). The results of this study showed significant resemblance with some recent studies on protease enzymes. Proteases from these two plants show maximum activity at 80% saturation of ammonium sulfate. The optimum pH was found to be slightly basic (pH 9), suggesting the maintenance of pH at

this point to gain maximum enzyme production and activity. The activity of protease from the leaves of Moringa oleifera is maximum at pH 8.0 (9). The pH profile of proteases showed significant results with the maximum action at pH seven to nine with one fundamental top at pH 9.0. The result related to the pH is insignificant with the seeds, Bengal gram indicated the most noteworthy explicit action (0.007659 U/mg of protein) at pH 7.5 however least action was seen in pea bean (0.001681 U/mg of protein) at pH 9.0 that relates with this examination (11). Protease from senesced leaves of the plant Carica papaya was partially purified by ammonium sulfate purification, at optimum conditions it showed a 15-fold purification. Protease activity was found in alkaline range 9 (12).

To investigate the temperature effect, the protease assay was performed at the different temperature ranges at pH 9. At 37°C the enzyme shows more stability and its activity for both plants is significantly related to the temperature versus reactant action of all protease relationships exhibited a symmetrical distribution with one main peak, optimum at 37°C aside from dark gram which indicated two principal tops at 37° Celsius and 70° Celsius individually. The results were not quite significant with 70°C of the black gram (10). The activity of protease from the leaves of Moringa oleifera is maximum at a temperature of 37°C which is also significantly related to the results of this study (10). That the Wisteria sinensis have efficient activity of enzyme with 2g of casein at pH 9 in tris-base buffer, required 40 minutes and optimum temperature is 37°C whereas Chloris barbata shows more enzyme activity with 1g of casein concentration at pH 9, required 50 minutes and optimum temperature 37°C. The research contributes valuable insights into the potential industrial applications of proteases isolated from Wisteria sinensis and Chloris barbata, showcasing their significance in fields such as pharmaceuticals, diagnostics, and biotechnology. Overall, the findings from this study underscore the importance of exploring diverse plant sources for protease enzymes and provide a foundation for further research on the purification, characterization, and applications of these enzymes in various industries.

Declarations

Data Availability statement
All data generated or analyzed during the study are included in the manuscript.

Ethics approval and consent to participate
Approved by the department Concerned.

Consent for publication
Approved

Funding
Not applicable

Conflict of interest

The authors declared absence of conflict of interest.

Author Contribution

NATASHA QADIR & ASMA ALI
Contributed equally

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