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Three Phase Partitioning of Serine Protease from Ash Gourd (*Benincasa hispida*), Its Characterization and Application on Whey Protein Hydrolysis

Dambar Bahadur Khadka[12]*, Laxmi Adhikari[1], Pragya Khadka[3], Alina Sharma[1], Abhishek Sharma[2], Dhan Bahadur Karki[1]

[1]Central Department of Food Technology, Tribhuvan University, Dharan, Nepal
[2]Central Campus of Technology, Tribhuvan University, Dharan, Nepal
[3]Dharan Multiple Campus, Tribhuvan University Dharan, Nepal

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Correspondence
Dambar Bahadur Khadka
e-mail: dambar.khadka@cct.tu.edu.np

ABSTRACT

Protease from the ash gourd was purified and concentrated in a one step, applying the three phase partitioning (TPP) methods after optimizing the partitioning factors: saturation level ammonium sulfate, ratio of extract: *t*-butanol, pH and temperature. The recovered protease (AGP) was characterized and further evaluated for whey protein (0.6% w/v) hydrolysis ability at varying enzyme concentrations (0-8% v/v). The ammonium sulfate (50% saturation), the ratio of crude extract: *t*-butanol (1:1v/v), 8 pH and 25°C temperature allowed achievement of 122.67 % activity recovery and 5.17 purification fold. The AGP was mainly partitioned at interfacial phase and was serine protease, had a molecular weight of 67.39 kDa. The enzyme performed optimally at temperatures of 60-70°C and pH ranges of 7-9 and demonstrated stability across pH ranges of 5-11 and temperatures up to70°C. The K_m and V_{max} value were 1.48 mg/mL and 5.28 µmol tyr/mL/min respectively. Additionally, AGP displayed effective hydrolysis of whey protein, exhibiting higher DPPH radical scavenging and ABTS inhibition activities than un-hydrolyzed and trypsin hydrolyzed whey protein concentrate. This work suggested TPP as a low-cost effective alternative for purification and concentration of AGP, and it could be applied for whey protein hydrolysis to improve the antioxidant activity.

1. INTRODUCTION

Proteases are the second-biggest category of hydrolase that hydrolysis the amide bonds present in the proteins or peptides (David Troncoso et al., 2022; Khadka et al., 2024). Protease can be derived from animal, microbial and plant sources. Plant proteases currently representing more than 7% total protease market, and are arising as a cheap substitute to microbial and animal protease (Mazarro-Manzano et al., 2017). Plant protease retains various benefits over the microbial and animal protease because of their elevated proteolytic action and substrate specificity, stability across the wider temperature and pH ranges (David Troncoso et al., 2022). The demand for plant proteases is further driven up by increasing ethical and religious worries regarding the use of natural enzymes, limited availability of livestock, and regulatory issues (Ben Amira et al., 2017; Matkawala et al., 2022). In food industries, plant proteases can be applied to clot the milk for cheese preparations, hydrolyze protein to generate bioactive/functional peptides, tenderize meat and ameliorate dough characteristics (David Troncoso et al., 2022; Matkawala et al., 2022). Plant proteases, so far applied in the food processing were belong to major three types: aspartic (e.g., cardosin, cynarase, onopordosin, arctiumisin), cysteine (e.g., papain, bromelain, actinidin, ficin) and serine (e.g., cucumisin, pomiferin, millin, carnein, nerifolin, dubiumin, religiosin, streblin, latex glycoprotein) (David Troncoso et al., 2022; Matkawala et al., 2022).

Plant serine protease among the other plant derived proteases, has an added advantage of being stable even in presence of the oxidizing agents, which make them more feasible and economical to use (Tripathi et al., 2011). A variety of plant species; including their fruits, seeds, stems, flowers and latex, have been discovered to contain remarkable amount of serine proteases (Antão & Malcata, 2005). Serine proteases have been divided into major six clans, representing two major clans; chymotrypsin like (SA) and substilisin-like (SB) (Rawling & Barrett, 1994). Subtilisin family is the second largest family of the serine protease is belong to the clan SB. Subtilisin family further grouped into two sub family: subtiisin like (S8A) family and kexin (S8B) family (Rawling & Barrett, 2013). The most of plant subtilisins also known as cucumisin or cucumisin like proteases are included in the subtilisin (S8A) family and are homologs of bacterial subtilisins (Gagaoua et al., 2017). Cucumisin from the *Cucumis melo* is the well-studied plant serine protease till now. A number of plants from *Cucurbitaceae* family were reported as an appreciable source of plant serine protease(Asif-Ullah et al., 2006; Gagaoua et al., 2017; Kaneda & Tominaga, 1975, 1977; Madhu & Sharada, 2019; Uchikoba et al., 1993; Uchikoba et al., 1998), accounting nearly 50% of total protein extract (Mazarro-Manzano et al., 2017).

Ash gourd (*Benincasa hispida*) belongs to the *Cucurbitaceae* family and the class *Magnoliopsida* of the plant kingdom (USDA, 2025). *B. hispida* is a nutritionally important vegetable indigenous to South and Southeast Asia. It is also traditionally used for medicinal purposes particularly in treatment of diarrheal, obesity, ulcer, oxidative stress and diuretic related problems (Singh et al., 2024). From *B. hispida*, a serine protease (Mw: 67 kDa) having greater homogeneity in N-terminal sequences with cucumisin, has been isolated and purified earlier, applying chromatographic techniques (Uchikoba et al., 1998). Cucumisin or cucumisin like proteases were shown to have potential in various food processing applications such as milk coagulation (Gagaoua et al., 2017) and producing hydrolysate that have improve functional properties (Alavi et al., 2019; Mazarro-Manzano et al., 2020; Mazarro-Manzano et al., 2017) .

Isolation and purification of the protease are crucial steps as it account nearly 70% of the total production expenses (Hafid et al., 2020). Several methods that applied before for isolation and purification of plant protease required various successive steps and were mainly based on chromatographic techniques applied individually or in combination (Amid et al., 2014; Asif-Ullah et al., 2006; Li et al., 2018; Tokonami et al., 2023; Tomar et al., 2008; Uchikoba et al., 1998). These techniques despite providing more resolution, are often expensive (require high-cost equipment), sophisticated, and difficult to scale up (Gagaoua, 2018; Yan et al., 2018). It emphasizes the need of the economic and efficient down streaming process for enzyme recovery and purification, which could be more feasible for industrial applications (David Troncoso et al., 2022). Recently, three phase partitioning (TPP), a non-chromatographic technique was mentioned as an easy, fast (one step), scalable and cost-effective for enzyme isolation, and purification (Eyssen et al., 2021; Gagaoua, 2021; Yan et al., 2018). In essence, TPP is the formation of three phases (upper *t*-butanol, lower-aqueous and middle-interfacial) in response to the addition of *t*-butanol (organic-solvent) and ammonium sulfate [$(NH_4)_2SO_4$] salt to the crude extract (Patil & Rathod, 2021). The upper *t*-butanol phase retained most of phenolic pigments, lipids and inhibitors; the lower phase consist of mainly polar compounds like saccharides, while the target protein is primarily concentrated and partitioned on the interfacial phase(Patil & Rathod, 2021; Yan et al., 2018). A protein-enriched interfacial phase often retains higher activity of the enzyme (Gagaoua et al., 2017; Maskey & Karki, 2023). Using TPP, various plants (Hafid et al., 2020; Maskey & Karki, 2023), microbial (Garg & Thorat, 2014), and animal (Ketnawa et al., 2014) proteases have been successfully purified and characterized. To date, the plant serine protease that have been purified using TPP include cucumisin from *C. melo* (Gagaoua et al., 2017), protease from stem of *Wrightia tinctoria* (Rajagopalan & Sukumaran, 2018), and trypsin-like protease from *Crotalaria stipularia* (Dos Santos et al., 2024).

This work was performed primarily to isolate protease in a one-step from ash gourd crude extract using the TPP method at optimized parameters (ammonium sulfate saturation level, *t*-butanol ratio, pH and temperature), and to characterize the purified protease. Furthermore, the study also illuminated ability of partitioned protease for whey protein hydrolysis, and assesses the antioxidant activities of generated hydrolysate.

2. MATERIALS AND METHODS

2.1 Materials

Whey protein concentrate (WPC-80%) was procured from Medizen Labs Pvt. Ltd., India. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH); 2, 2'-Azinobis-3-ethylenebenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma Aldrich. Porcine trypsin (1000-1500 BAEE units/mg); Casein; N, N, N′, N′-Tetramethylethylenediamine (TEMED); N-N′-Methylene-bis-acrylamide, 2- Mercaptoethanol, Ammonium sulfate [$(NH_4)_2SO_4$], Ammonium persulfate, Trichlroacetic acid (TCA), Bovine serum albumin (BSA), Gelatin, Phenyl methyl sulforyl fluoride (PMSF) were obtained from HiMedia laboratories Pvt. Ltd, India. Acrylamide, Pre-stained protein ladder -26619, *t*-butanol were procured from Thermo Fisher Scientific Pvt. Ltd, India. Sodium dodecyl sulfate (SDS) and Ethylenediamine tetraacetic acid (EDTA) were bought from Merck, India. Every chemical procured was of the analytical grade.

2.2 Preparation of crude protease extract

A well-matured unripe ash gourd (*B. hispida)* was procured from a local grower of Dharan, Nepal in the harvesting season (September to October). The crude extract of protease from ash gourd was prepared using the process as described by Gagaoua et al. (2017) with some modifications. Ash gourd was peeled and its pulp was chopped with a stainless-steel knife into small pieces after removing the seeds. The chopped sample was ground in mortar and pestle

and extracted with sodium phosphate buffer (0.1M, pH 6.8) at 1:1 (w/v) ratio, stirred in a magnetic stirrer (at 250 ± 50 rpm) in the refrigerated condition for 15 min and then filtered through a muslin cloth. The filtrate was centrifuged (5000 rpm at 4 °C for ten minutes) using the centrifuge (Sigma, 3-30KS). The supernatant was collected after muslin cloth-filtration, precipitated adding $(NH_4)_2SO_4$ at 60% saturation, and centrifuged (13000 rpm at 4°C for ten minutes) to obtain the pellet. After dissolving in a minimum amount of the same buffer, the pellet was dialyzed (Dialysis tube; MWCO: 14kDa, HiMedia LA395) at the refrigerated condition with four exchanges of buffer to get the ash gourd crude protease extract (AGP-CE). The AGP-CE was stored at -20° until used for the optimization study of TPP.

2.3 Three phase partitioning (TPP) of AGP-CE

The TPP of AGP-CE was performed following the process outlined by Gagaoua et al. (2017) with some modification. In short, AGP-CE was first saturated with $(NH_4)_2SO_4$ to a level of 30% and *t*-butanol in an equal volume was added. The mixture was lightly vortexed, let to stand for 45 min, and centrifuged for five minutes at 4°C and 4000 rpm to facilitate the separation of three phases. Using a Pasteur pipette, the upper phase was withdrawn, the lower aqueous (AP) and intermediate phases (IP) were carefully collected. The recovered IP fraction was solubilized with small amount of sodium phosphate buffer (pH 6.8). Both solubilized IP and AP were dialyzed whole-night, subjected to evaluation for protein and protease activity to estimate purification profile (specific activity, purification fold and % activity recovery). In contrast to AP; specific activity and purification fold of the IP was found to be extremely high (**Figure 1B**). Subsequently, only the IP fraction was taken for further TPP optimization process.

2.3.1 Optimization of $(NH_4)_2SO_4$ saturation level

In order to optimize the salt concentration, the AGP-CE was saturated at room temperature with varying concentrations (30–80%) of $(NH4)_2SO_4$, keeping the same ratio of AGP-CE: *t*-butanol (1:1 v/v). The $(NH4)_2SO_4$ saturation level was estimated following the table provided by Duong-Ly and Gabelli (2014).

2.3.2 Optimization of t-butanol ratio

Secondly, maintaining a constant optimized $(NH4)_2SO_4$ saturation level at room temperature, the effect of *t*-butanol was examined by varying its level at different ratios (AGP-CE: t- butanol; 1.00: 0.50, 1.00: 0.75, 1.00: 1.00, 1.00: 1.25, 1.00:1.50, and 1.00:2.00).

2.3.3 Optimization of pH

To optimize pH, salt was added first to AGP-CE to the optimized saturation level, the pH was adjusted to 4, 5, 6, 7, 8, 9, and 10 with addition of 1% NaOH or 1% HCl, and *t*-butanol was subsequently added to the optimized ratio.

2.3.4 Optimization of Temperature

Finally, TPP system's temperature optimization was carried out by varying incubation temperature between 10°C and 40°C, keeping the levels of the salt, *t*-butanol and pH at their optimal settings.

After optimization of all the four parameters (salt, *t*-butanol, pH, and temperature), the AGP-CE was purified and concentrated in a single step utilizing TPP process. Three repetitions were performed to verify the purification outcomes (total purification fold and % activity recovery) of the enzyme. The dialyzed enzyme was stored at -20°C for the characterization and further hydrolysis studies.

2.4 Protein Determination

The protein content of the crude and TPP partitioned fractions were measured by applying the dye binding method of Bradford (1976). The sample (0.1 mL) was added to Bradford reagent (3 mL), kept at 37°C for 30 min for development of color, and then absorbance was read at 595 nm by spectrophotometer (Carry UV-VIS, Agilent). Protein concentration was estimated by using BSA reference curve ranging from 0–1000 µg/mL and was reported in mg/mL.

2.5 Protease Activity Determination

Protease activity was determined using a modified version of Cupp-Enyard (2008) method. 100 µL of crude extract and enzyme solution (after suitable dilution) was mixed with 2.2 mL casein (5 mg/mL) solution prepared in TRIS-HCL buffer (50 mM, pH 9). After incubation (20 min at 40°C), the reaction was ceased by adding 3.5 mL of 5% TCA. To prepare the blank, TCA was added to the substrate solution before addition of enzyme. The reaction mixture was placed in the refrigerator for fifteen minutes, centrifuged for ten minutes at 5000 rpm to collect the supernatant (TCA filtrate). 1 mL of supernatant was added to 2% Na_2CO_3 reagent, and let to remain for 10 min at room temperature. Thereafter, 0.5 mL of folin-ciocalteu (diluted with equal part of distilled water) was added, incubated at 37°C for 30 min, and absorbance was recorded at 700 nm. A standard curve of tyrosine (0–100 µg/mL) was used to estimate the release of amino acids. "One unit of protease activity (U) was defined as the amount of enzyme that liberate 1µmol of tyrosine per mL per min under assay conditions" (Cupp-Enyard, 2008) .

$$\text{Protease Activity (U/mL)} = \frac{\mu\text{mol tyrosine equivalent released} \times V_t \times DF}{V_e \times t \times V_c}$$

Where, V_t; total assay volume (mL),
V_e, volume (mL) of enzyme
t; time (min) of hydrolysis
V_c; TCA supernatant volume (mL)
DF; Enzyme dilution factor

2.6 Characterization of AGP

2.6.1 SDS-PAGE analysis and substrate activity staining

Using 4% stacking and a 12% separation gel, tricine sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE) was performed as stated by Laemmli (1970) to assess the pattern of isolated proteases. The protease extract and TPP fractions were combined with reducing sample buffers in a 1:1 ratio followed by heat treatment (85°C for 5 min) to perform the electrophoresis. Samples (15 µg protein) were loaded into the well and ran for electrophoretic separation at constant 100V. After separation, the gel-staining was

performed over-night using solution containing 0.1% CBBR -250, acetic acid (10%) and methanol (45%). Then, gel was destained two times with acetic acid (7.5%) and methanol (50%) containing solution. Pre-stained protein ladder (Thermo Scientific) of known molecular weight (10-250 kDa) as a standard was used. The molecular weights of separated protein were calculated by applying the plot of logarithmic molecular weight against relative movement of markers.

Casein-substrate zymography was executed to confirm the activity of separated AGP by SDS-PAGE employing the method of Garciacarreno et al. (1993). Samples (at 2 µg and 5 µg protein) were loaded to the gel after combined with non-reducing sample buffer. SDS-PAGE was run in similar condition as mentioned before. The gel obtained after electrophoresis was kept in a renaturing buffer consisting of Triton X-100 (2.5%) for thirty minutes to wash the SDS, and re-nature the protein. After washing with HPLC grade water two times, the gel was put into 100 mL of casein (2%) prepared in 50 mM (7.5 pH) Tris –HCl buffer, kept at 4 °C for 30 min to enable casein diffusion into the gel, and then further incubated for 3h at 37 °C. Finally, the gel was washed with HPLC-grade water, then staining and destaining was carried out as mentioned earlier. The visualization of the clear white band confirms the presence of proteolytic activity and thus the protease.

2.6.2 Effect of pH on Activity and Stability

The method mentioned by Gagaoua et al. (2017) with some modification, was applied to determine optimum pH of AGP. The protease assay was done as explained earlier within the pH range of 4-12; using citrate (4 - 5 pH), sodium phosphate (6 - 7 pH), and Tris–HCl (8 - 12 pH) buffers having 50 mM concentration. The stability at different pH was determined by estimating remaining activity after exposing the AGP for an hour to the given buffers of different pH (3 - 12).

2.6.3 Effect of Temperature on Activity and Stability

The profile of temperature-dependent activity of AGP was evaluated between 30°C and 100°C with some modifications (Gagaoua et al., 2017). The AGP activity accomplished at a different temperature was divided by the maximum activity achieved within the specified temperature to express the results as the percentage of relative activities. The temperature stability profile of AGP was assessed following the procedure outlined by Gagaoua et al. (2017). The AGP was pretreated to different temperatures (4 - 100°C) for 15 min, and then used to determine the remaining activity.

2.6.4 Kinetic Parameters

Kinetic parameter of AGP was studied following protease activity on casein at different concentrations (0 - 30 mg/mL) (Gagaoua et al., 2017). The data obtained were fitted to Michaelis–Menten plot and Lineweaver–Burk plot using Graphpad Prism 9.5.1 to estimate Vmax (maximum velocity) and K_m (Michaelis–Menten's constant).

2.6.5 Effect of Protease Inhibitors

Inhibitor's impacts on AGP performance were analyzed using the method explained by Hafid et al. (2020). Pre-incubation of AGP was carried out at 37°C for one hour with 5 mM of specific inhibitors; Iodoacetamide (IAA) for cysteine protease, phenyl methyl sulforyl fluoride (PMSF) for serine protease, and ethylene-diamine-tetraacetic acid (EDTA) for metalloprotease, and then used for assaying remaining activity. The results were presented in terms of relative residual activity, allowing for the enzyme activity without inhibitor as 100%.

2.6.6 Specificity to Natural Substrates

The substrate-specific activity of the AGP was evaluated on different substrates (casein, hemoglobin, whey protein, BSA, and gelatin) (Khadka et al., 2024). The protease assay was done at identical concentration (5 mg/mL) for each of the substrates. The outcomes were reported in relative activity, taking into account the activity on casein as 100%.

2.7 Enzymatic Hydrolysis of Whey Protein

Whey protein was hydrolyzed with TPP purified AGP and commercial porcine trypsin (TC245; 1000-1500 BAEE units/mg, HiMedia) under control conditions as mentioned by Ketnawa et al. (2014). Whey protein hydrolysis was conducted to compare the ability of protein hydrolysis and hydrolysis patterns, and antioxidant activities of generated hydrolysate by the enzymes. At first, trypsin solution 1 mg/mL was prepared and evaluated for protease activity by employing the protease assay as mentioned earlier which provides protease activity of 2.3 U/mL. Then, the AGP was also diluted to adjust the protein concentration 1.0 mg/mL (activity: 2.3 U/mL) before used for hydrolysis.

Whey protein concentrate (WPC 80%) was diluted to make 6 mg/mL (0.6%) with the TRIS-HCl (50 mM) buffers adjusted to pH 8 and 9, hydrolyzed at optimum temperature 40°C for trypsin and 60°C for AGP, respectively. For hydrolysis, the enzymes at different levels: 0.25, 0.50. 0.75, 1.00, 2.00, 4.00, 6.00 and 8.00 (%, v/v) were added. Hydrolysis was conducted in a shaking water bath for 2 h and halted by heating the hydrolysate at 95°C for ten minutes. Subsequently, the supernatant was recovered after centrifugation (5000 rpm for 10 min at 4°C) and filtration with whatman 41 filter paper, and kept at -20 °C until analysis.

2.7.1 Determination of degree of Hydrolysis and Hydrolysis patterns

The degree of hydrolysis (DH) of the un-hydrolyzed whey protein and hydrolyzed whey protein was estimated by determining the percentage of TCA soluble nitrogen (SN-TCA) methods with some modifications (Rutherfurd, 2010). Briefly 3 mL of hydrolysate were added with 3 mL of 20% (w/v) TCA, allowed to rest for 30 min, and then centrifuged at 3000g (at 4°C for five minutes) to separate the soluble protein and insoluble fractions (un-hydrolyzed protein). Then, the protein content of the supernatant and the hydrolysate samples were estimated following Lowry methods (Lowry et al., 1951). Briefly, 1 mL of the sample

was mixed with 5 mL of the reagent (50 mL of 2% w/v Na_2CO_3 in 0.1 M NaOH; 0.5 mL of 1% w/v $CuSO_4.5H_2O$; 0.5 mL of 2% w/v potassium sodium tartrate) and left to react at room temperature for 10 minutes. Then 0.5 mL of folin-ciocalteu reagent (diluted 2 times with distilled water) was added, and the mixture was left at room temperature for 30 minutes until absorbance at 750 nm was measured. A BSA (0 - 500 µg/mL) standard curve was used to measure the protein concentration. DH was estimated applying the formula as mentioned below:

$$DH\ (\%) = \frac{\text{Soluble protein in 10 \% TCA (mg)} \times 100}{\text{Total protein content (mg)}}$$

Patterns of whey protein degradation during hydrolysis were examined by using tricine SDS-PAGE applying 4% stacking gel and 15% separating gel (Schägger, 2006). The WPH samples after passing through a 0.22 microns nylon filter was combined with sample buffer (150mM Tris-Hcl buffer of pH 7 containing 12% SDS, 6% mercaptoethanol, 30% glycerol, 0.05%, CBBR-250) at 1:1 ratio, and 20 µL was loaded to well. At a constant 120 V, electrophoresis was run for four hours. Staining and destaining of the gel was executed as mentioned previously to visualize degraded proteins bands during the hydrolysis.

2.7.2 Determination of Antioxidant Activity

The antioxidant activity of the WPH and un-hydrolyzed whey protein was assessed by using two different methods: DPPH radical scavenging activity and ABTS radical scavenging activity.

The DPPH radical scavenging activity of hydrolysate was measured based on the method Corrons et al. (2012) after some modifications. Briefly, 300 µL of hydrolysate and un-hydrolysate samples (after filtration with 0.22 microns nylon filter) were mixed with 3 mL of DPPH (0.1 mM) solution, and kept in the dark for 1h. Following incubation, spectrophotometer was used to read the absorbance at 517nm. Control was performed by substituting the sample with distilled water while keeping all the other reagents the same. Sample blank and solvent blank were carried out by adding the 300 µL of hydrolysate and 300 µL distilled water, respectively, in the 3 mL methanol (80%) without adding the DPPH solution. The DPPH radical scavenging activity was calculated based on the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(1-(\frac{A_s-A_{sb}-A_b}{A_c-A_b})] \times 100$$

Where,

A_S: absorbance of sample
A_{Sb}: Absorbance of sample blank
A_c: Absorbance of control
A_b: Absorbance of solvent blank.

ABTS radical cat-ions scavenging activity of the WPH was determined by adopting the method of Hernández-Ledesma et al. (2005) with some modification. It is a spectrophotometric method that relies on antioxidant-induced reduction of the cat-ion radical ($ABTS^{\bullet+}$), which is produced by oxidation when potassium persulfate is added to ABTS. To make $ABTS^{\bullet+}$ stock solution, first 19.2 mg ABTS was dissolved in 5 mL distilled water, to which 88 µL of 140 mM potassium persulfate was added, and then kept in an amber vial in the dark for 12-16 h. $ABTS^{\bullet+}$ working solution was prepared by diluting (approx.1:70) stock solution with phosphate buffer saline (pH 7.4) until the optical density of 0.70 ± 0.02 was achieved. The un-hydrolyzed whey protein and WPH samples after syringe filtration (0.22 µm nylon filter) were diluted to 2 times (as some of WPH samples neutralize $ABTS^{\bullet+}$ within a few minutes), then 30 µL of diluted sample was added to 3 mL of working solution, and placed on dark condition for 10 min. After ten minutes, a decrease in absorbance at 734 nm was noted. To prepare the control, sample amount was replaced by same volume of buffer. The ABTS radical scavenging activity was determined and reported as percentage inhibition of absorbance.

$$\text{Inhibition (\%)} = (\frac{A_0-A_1}{A_0}) \times 100$$

Where,

A_0: absorbance of control
A_1: absorbance of the sample

2.8 Data Analysis

All experiments were performed three times with duplicates. The results were presented on mean ± standard deviation. Graphpad Prism 9.5.1 was used to estimate enzyme's kinetics parameter (Vmax and Km value). IBM SPSS Statistics (Version 20) was used for statistical analysis. To ascertain significant difference among the mean values and for the multiple comparison, analysis of variance (ANOVA) and Tukey's HSD approach were used, respectively, at the 5% level of significance. Microsoft Excel 2019 was used to create each and every graph.

3. RESULT AND DISCUSSION

3.1 Three Phase Partitioning

Several factors have been reported to be essential in the TPP process for effective partitioning of protease, which include $(NH_4)_2SO_4$, *t*-butanol, pH, and temperature (Gagaoua et al., 2017; Hafid et al., 2020; Patil & Rathod, 2021). Consequently, to accomplish partitioning of AGP in one-step, these parameters were first optimized**.** The ash gourd crude protease extract (AGP-CE), having protease activity of 2.44U/mL (Protein concentration: 5.57 mg/mL) was used as beginning material for the purification study.

3.1.1 Optimization of salt [$(NH_4)_2SO_4$]

$(NH_4)_2SO_4$ has been shown to be effective salt for partitioning protease in the TPP process (Gagaoua et al., 2017; Maskey & Karki, 2023). Salting out purifies as well as concentrate the protein in one of the phases depending on the protein's net charge and the concentration of the salt in the system (Gagaoua, 2018; Yan et al., 2018). So, optimization of the salt is an essential step in the effective separation of protease. In this study, $(NH_4)_2SO_4$ saturation level was varied from 30 - 80% to get the optimum salt concentration maintaining 1:1 proportion of AGP-CE to *t*-butanol, and result is depicted in Figure 1A. The highest purification fold (4.77) and recovery of AGP activity (112.43%) were achieved with IP fractions at $(NH_4)_2SO_4$ saturation level of 50%. The purification fold as well as the

recovery initially increased with salt saturation level from 30% to 50%. An additional increased in $(NH4)_2SO_4$ (> 50%) saturation level resulted a decrease in both the yield and the level of purification. This might be related to the permanent denaturation of the proteins (Gagaoua et al., 2017; Maskey & Karki, 2023). Previous studies also noted a similar impact of $(NH_4)_2SO_4$ in TPP separation, indicating 40% and 60% as the optimal concentrations for actinidin and cucumisin separation, respectively (Gagaoua et al., 2017; Maskey & Karki, 2023).

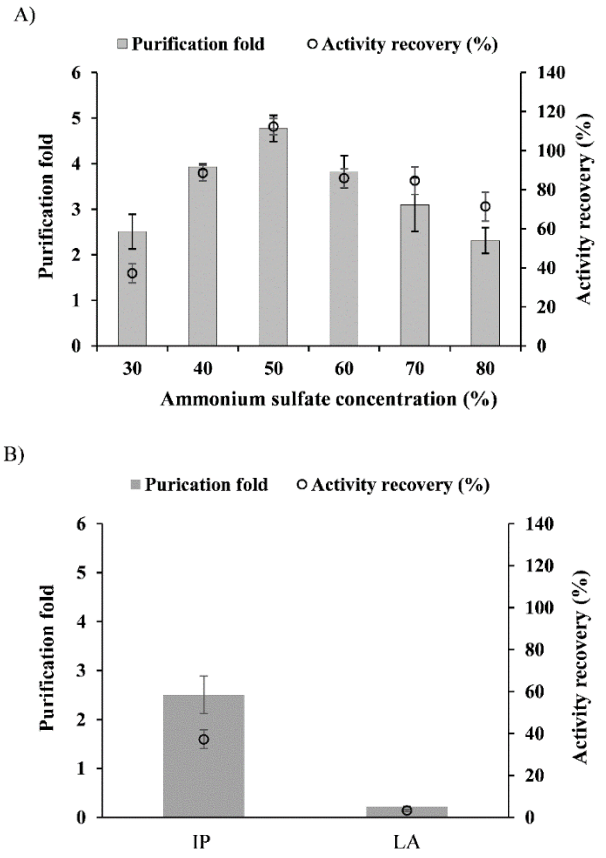


Figure1.
A) Effect of different salt concentration on percentage recovery and purification fold of IP fractions. B) Purification fold and activity recovery of IP (interfacial phase) and LA (aqueous phase) at 30% ammonium sulfate saturation level. Data represent mean ± standard deviation.

Salting out by sulfate ions in TPP is a mutual effect of the phenomenons such as ionic strength, kosmotrophy, osmotic dehydration, exclusion crowding and binding of salt ions to cat-ionic site of the proteins (Dennison & Lovrien, 1997; Gagaoua, 2018). Hence, 50% of ammonium salt concentration was concluded as optimum to concentrate and separate the AGP and was used for further optimization of the other factors.

3.1.2 Optimization of t-butanol

t-butanol; a miscible solvent has various benefits over the other solvents. In presence of *t*-butanol when an adequate amount of $(NH4)_2SO_4$ is added, the solution convert into the three phase layers and remove the lipids, phenolic pigments, and enzyme inhibitors into the upper phase (Dennison & Lovrien, 1997; Patil & Rathod, 2021; Yan et al., 2018). *t*-butanol, due to its size and branched structure, could not enter into the folded protein structure, hence could not cause the denaturation of proteins (Gagaoua, 2018). The impact of *t*-butanol ratios on AGP recovery and purification are presented in Figure 2.

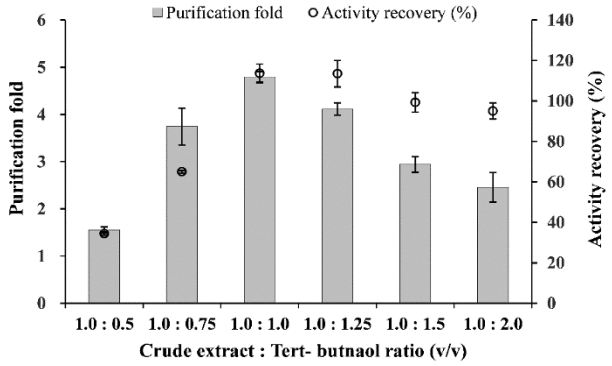


Figure 2
Effect of t-butanol ratio on percentage recovery and purification fold of IP fractions. Data represent mean ± standard deviation

The result disclosed that with the addition of the *t*-butanol, the protease concentrated in the IP with the maximum purification fold (4.79) and the highest recovery of activity (113.65%), at AGP-CE: *t*-butanol ratio of 1:1. But further increase in *t*-butanol ratio significantly reduced the purification fold and yield of the protease (Figure 2). Similar level of *t*-butanol with proportion to crude enzyme was reported efficient for the TPP concentration of serine protease obtained from *W. tinctoria* (Rajagopalan & Sukumaran, 2018). The result aligned with previous researcher's findings that the very high *t*-butanol ratio had high chance of causing protein denaturation, and could not perfectly synergize with the $(NH4)_2SO_4$ concentration, resulting in low recovery and purification (Gagaoua et al., 2014; Gagaoua et al., 2017; Maskey & Karki, 2023). Based on these results, the ratio 1.0:1.0 of AGP-CE to *t*-butanol was selected as an ideal condition for further evaluation of impacts of pH on TPP process.

3.1.3 Optimization of pH

The pH of the system in TPP purification can largely influence on the electrostatic interaction amongst the phases and charge retained by the protein to be separated (Dennison & Lovrien, 1997; Yan et al., 2018). The effect of the different pH from 5 to 10 on TPP recovery of the AGP activity and purification fold are highlighted in Figure 3.
The AGP was partitioned effectually in IP with a maximum 5.12 purification fold and with a corresponding maximum recovery of 118.39%, when the TPP system was maintained to pH 8. At pH 7, the recovery of activity and purification fold achieved were almost near to the values retrieved at pH 8. However, the purification fold and the recovery significantly decreased, when pH of the system was below 7 and above 8. These finding suggest that most of the target

protease transformed from aqueous phase to the IP at pH 7-8, most probably maintaining better conformational stability with the solvent (Gagaoua et al., 2017).

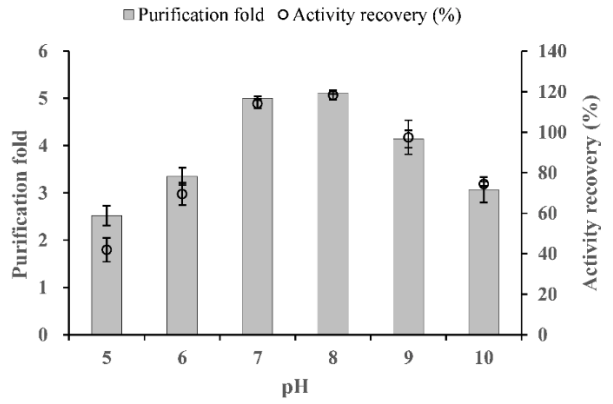


Figure 3
Effect of pH on percentage recovery and purification fold of IP fractions. Data represent mean ± standard deviation

The influence of the pH in the TPP separation is linked with the isoelectric point (*p*I) of targeted proteins (Gagaoua et al., 2017; Hafid et al., 2020; Maskey & Karki, 2023). pH below *p*I maintained positive charge on the proteins that fascinates the binding of salt to cat-ions, improve the electrostatic screening by minimizing repulsion between similarly charged protein in TPP process (Gagaoua, 2018). In most cases, the *p*I of cucumisin and cucumisin-like proteases has been mentioned to be between pH 8.7 to 9.5 (Gagaoua et al., 2017; Tripathi et al., 2011). Above the *p*I value, the net surface charge of the protein would be negative allowing their diffusion to lower aqueous phase; while below the *p*I value, surface charge would be mostly positive which enhance protein precipitation and enrichment in the IP (Hafid et al., 2020; Yan et al., 2018).

3.1.4 Effect of temperature

The influence of incubation temperature (10 - 40°C) on TPP partitioning of the AGP was assessed and the result obtained is presented in Figure 4.
When the incubation temperature was elevated from 10°C to 25°C, there was continuous increase in both the recovery of activity and purification of the AGP. At 25°C, the AGP was purified maximally by 5.15-fold with recovering the highest activity of 121.69%. The purification fold and recovery obtained at 25°C was closely same with the values obtained for 20°C but were reduced drastically at other incubation temperatures employed in the study. A temperature of 20°C has been reported to be ideal for the essential separation of cucumisin (Gagaoua et al., 2017). However, in many cases, the temperature between 20 - 40°C was suggested for optimum recovery of the proteases, and the optimum temperature found in this study was also within this range (Yan et al., 2018). The decrease in the recovery and purification at elevated temperatures could be a result of denaturation and inactivation of the enzymes (Chew et al., 2018; Gagaoua, 2018). Moreover, higher temperatures frequently resulted in volatilization of *t*-butanol; which lowered the solvent concentrations in the system, hindered the solvent's ability to properly synergize with the salt, and reduced the enzyme recovery (Gagaoua, 2018; Yan et al., 2018).

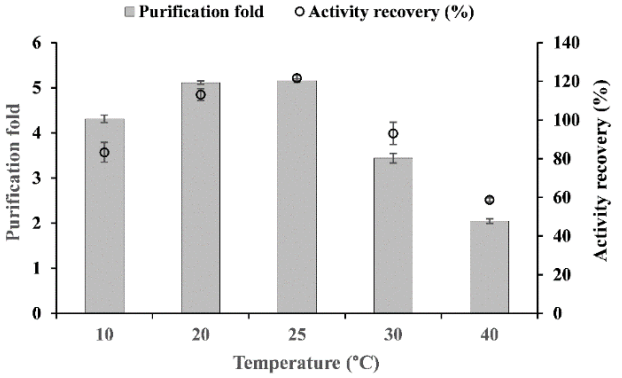


Figure 4
Effect of temperature on percentage recovery and purification fold of IP fractions. Data represent mean ± standard deviation

3.2 Overall Profile of TPP Purification

Table 1 summarizes the overall results of the single step TPP purification profile of AGP at optimal conditions - 50% $(NH_4)_2SO_4$ saturation, AGP-CE: *t*-butanol (1:1) ratio, pH 8, and 25°C temperature. At optimal purification conditions, a one-step TPP resulted the highest 5.17-fold purification and 122.67% activity recovery of AGP and the enzyme mainly concentrated in IP.

Table 1
Overall purification and recovery profile ash gourd protease by three phase partitioning process

Purification step	Protease activity (PA) (U/ml	Protein (mg/ml)	Total PA (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Crude extract (AGP-CE)	2.44	5.57	39.02	89.12	0.44	1.00	100.00
Interfacial phase (IP)	8.40	3.71	47.85	21.10	2.26	5.17	122.67
Lower aqueous phase	0.39	1.48	3.80	14.36	0.26	0.59	9.57

The purification fold and recovery accomplished in this study were on the same level or even higher than those found in the chromatographic purification methods for cucumisin from *C. trigonus* Roxburghi(Asif-Ullah et al., 2006) and other plant serine proteases like *Ficus carica* protease (Tokonami et al., 2023), strebilin (Tripathi et al., 2011), tamarillin (Li et al., 2018). The purification profiles are also comparable to the TPP purification profiles for cucumisin

from *C. melo* var. Reticulatus (Gagaoua et al., 2017) and better than stem protease from *W. tinctoria* (Rajagopalan & Sukumaran, 2018). TPP process causes the enzyme to activate concurrently, which supports the ostensibly higher activity recovery (>100%). This could be the outcome of the enzyme molecule's better adaptability (Gagaoua et al., 2017). Several researchers who had applied TPP process for separation and concentration of enzyme from different sources including microbial (Garg & Thorat, 2014), animals (Ketnawa et al., 2014) and plants (Gagaoua et al., 2017; Hafid et al., 2020; Maskey & Karki, 2023); have also noted considerably higher activity recovery. However, the purification fold despite of higher recovery achieved in this study was lower than that reported for *B. hispida* var Ryukyu protease (57-fold) and *B. cerifera* (18.8-fold) isolated from two steps chromatography (Kaneda & Tominaga, 1977; Uchikoba et al., 1998).

3.3 Characterization of TPP purified AGP

3.3.1 SDS-PAGE analysis

SDS-PAGE on reducing state showed only one thick band present in the TPP purified AGP in IP phase, corresponding to molecular weight (Mw) of 67.39 kDa (Figure. 5A). The corresponding casein zymogram also showed the intense white band in the similar region of electrophoretic mobility, and hence confirm the presence of active protease in the IP fraction of TPP (Figure 5B). Most of the bands appeared in the AGP-CE (Lane L_1 or $L_{1'}$) were disappeared in IP fractions (Lane L_2 and $L_{2'}$) showing AGP was concentrated on IP fractions. However, some minor bands below 67.39 kDa were also observed in the lanes of IPs, but their activities were not detected by zymography. It indicates that they were proteins not the enzyme which were not completely separated by the TPP process.

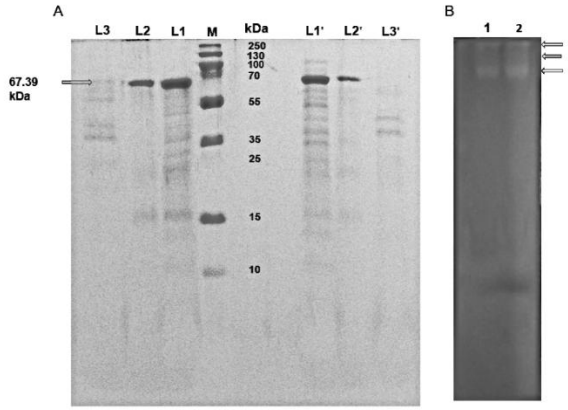


Figure 5
Electrophoresis analysis of TPP purified AGP: A) SDS-PAGE (M, protein marker; L_1, dialyzed crude AGP extract; L_2, TPP recovered IP fraction; L_3: AP fraction of TPP) on reducing condition. $L_{1'}$, $L_{2'}$ and $L_{3'}$ represent the same three samples from the replicate experiment; B) Casein zymogram of TPP Purified AGP (IP fraction at 2µg and 5µg protein).

The appearance of homogenous band corresponding to 67kDa were reported with cucumisin and cucumisin-like serine protease isolated from *C. melo* var Prince (Uchikoba et al., 1995), *C. trigonus* Roxb. (Asif-Ullah et al., 2006) and also from *B. hispida* var Ryukyu (Uchikoba et al., 1998). The Mw of the most active band of purified AGP obtained in this study was also very nearby to the Mw of serine protease from various plant sources such as *C. melo var*. Reticulatus (Mw:70kDa) (Gagaoua et al., 2017), and *Cyphomandra betacea* (Mw: 70kDa) (Li et al., 2018).

The two additional active bands were also observed in casein zyomogram of the IP fractions which were above 67.39kDa (Figure 5B) but were not observed in IP fractions (Lane L2) of SDS-PAGE. Although, two traces of bands (Approx. Mw 78 kDa and 91 kDa) which were above the 67.39kDa could be observed in crude extract (L_1') and IP fraction (L_2') of replicate sample of SDS-PAGE. They could most probably be the two active bands shown by casein zymograms. However, there was some difference in electrophoretic migrations. It indicated that the AGP obtained from the TPP process consist of three enzymes as represented by the zymography. The plant serine proteases had been stated to possess Mw from 19 to 110 kDa, while most of the protease obtained from the *Cucurbitaceae* family mentioned to be in the range of 60-80kDa (Antão & Malcata, 2005). In ash gourd (*B. hispida*), two serine proteases of Mw 67kDa from sarcocarp (Uchikoba et al., 1998) and 14 kDa gelatinolytic protease from seed (Das et al., 2018) have been reported up to now. Hence, the appearing of two active bands above 67.39kDa in zymography indicated some possibilities of novel protease (not reported before) present in *B. hispida* but further study is needed to confirm it.

The SDS-PAGE analysis and purification profiles obtained from TPP showed that TPP could be effective and feasible method for concentration and recovery of the AGP, though complete purification was not achieved. Many researchers have even used crude extract of the plant proteases for protein hydrolysis and bioactive peptide generation as cost-effective strategies (Corrons et al., 2012; Kheroufi et al., 2022; Mazarro-Manzano et al., 2020). Considering that, TPP-purified AGP could also be better option for the use in whey protein hydrolysis, based on the current purification profiles.

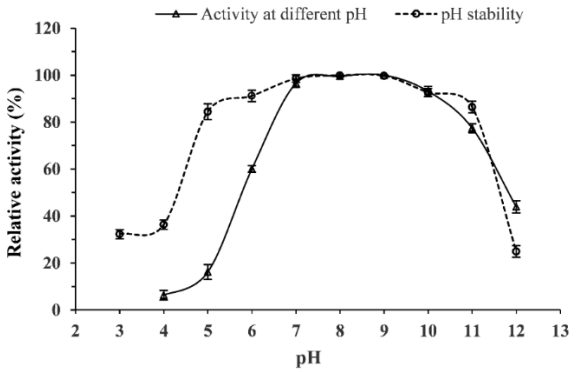


Figure 6
Influence of pH on activity and stability of TPP recovered protease. Data represent mean of triplicate ± standard deviation.

3.3.2 Effect of pH on activity and stability of AGP

The higher activity was displayed by the AGP in the neutral to alkaline pH range, with pH 9 exhibiting the peak activity **(**Figure 6**)**. The enzyme displayed more than 96% activity at 7 and 8 pH. At 10 pH, AGP showed relative activity of 93.12%. However, above pH of 11 and below pH of 7, a progressive decline in activity was noticed.

According to earlier research, the ideal pH range for plant-derived serine protease is between 7 and 11 (Table 2). As like finding of this study, the ideal pH of 9 was mentioned for the serine protease from *C. melo, B. cerifera*, and *Streblus asper* (Gagaoua et al., 2017; Kaneda & Tominaga, 1977; Tripathi et al., 2011). The optimum pH of 7.1 and 7.5 were reported for proteases from *C. melo* L. var. *prince* (Kaneda & Tominaga, 1975) and *C. dipsaceus* (Madhu & Sharada, 2019) respectively. In addition, the optimum pH of 11 has also been reported for the serine protease obtained from *C. trigonus* (Asif-Ullah et al., 2006).

Regarding pH stability, AGP was found to be stable retaining more than 80% of the residual activity from 5 to 11 pH (Figure 6). *B. hispida* protease has been demonstrated to be extremely resilient across the alkaline pH range, retaining more than 90% activity in the pH 7–11 range (Uchikoba et al., 1998). The pH stability range of AGP was within the ranges as mentioned by previous literatures related to plant serine proteases **(**Table 2**)**, and most closely resembles with the pH stability of cucumisin-like proteases (Asif-Ullah et al., 2006; Gagaoua et al., 2017; Kaneda & Tominaga, 1975, 1977; Uchikoba et al., 1998). Based on the pH activity and stability profiles, AGP could be beneficial to use in whey protein hydrolysis, as the pH greater than 7 increases the solubility of whey protein as well as transfolds β-lactoglobulin from dimeric to monomeric form, exposing more cleaving sites(Sakkas et al., 2022).

Table 2

Comparison of purification profiles and properties of plant serine protease purified from chromatographic and three phase partitioning methods

Plant Source (Part)	Purification steps/methods	Mw (kDa)	Opt. pH	Opt. Temp. (ºC)	pH Stability	Temp. Stability (ºC)	Purification fold	Recovery (%)	Reference
Cucumis trigonus Roxburghi (Fruit)	Cell extract, DEAE-Sepharose, $(NH4)_2SO_4$ precipitation (60%), CM-Sepharose	67	11	70	4-10	< 70	6.5	8.2	(Asif-Ullah et al., 2006)
Benincasa cerifera (Juice/pulp)	Juice, $(NH4)_2SO_4$ precipitation (60%), CM-cellulose, Sehadex G-75	50	9.2	70	4.5-9.5	Up to 65	18.8	21	(Kaneda & Tominaga, 1977)
Cucumis melo l.var. Prince (Sarcocarp)	Juice, $(NH4)_2SO_4$ precipitation (60%), CM sepharose, CM sepaharos	67	-	-	-	Up to 60 at pH 7.1	7.19	28	(Uchokoba et al.. 1995)
Benincasa hispida (Sarcocarp)	Juice, DEAE cellulose, CM sepharose	67	-	60	7-11	Up to 60	57	49	(Uchikoba et al., 1998)
Streblus asper (Latex)	Anion exchange (DEAE-Sepharose) chromatography	64	9	65	3-12.5,	15-85	4.6	75	(Tripathi et al., 2011)
Cyphomandra betacea (Tamarillo fruit)	$(NH4)_2SO_4$ precipitation (65%), DEAE-Sepharose chromatography	70	11	60	7-11	< 60	5.6	53	(Li et al., 2018)
Ficus Carica (Fruit)	$(NH4)_2SO_4$ precipitation (80%), Cation, Anion, Hydrophobic interaction, Gel filtration chromatography	70, 62, 60	7.5	50–60	8-11	Up to 70	54.6	2.60	(Tokonami et al., 2023)
Cucumis melo var. Reticulatus (Juice)	$NH4)_2SO_4$ precipitation (60%), 1:1.25 crude enzyme to *t*-butanol, pH 8, temperature 20ºC (TPP)	68.4	8-9	70	7-11	40-80	4.61	156	(Gagaoua et al., 2017)
Wrightia tinctoria (Stem)	$NH4)_2SO_4$ precipitation (60%), 1:1 crude enzyme to *t*-butanol, pH 7.5, temperature 50ºC (TPP)	95.62, 91.11, 83.23	7.5	50	-	-	2.34	9.24	(Rajagopalan & Sukumaran, 2018)
Benincasa hispida (Pulp)	Ammonium sulfate precipitation (60%), 1:1 crude enzyme to *t*-butanol, pH 8, temperature 25ºC (TPP)	67.39	7-9	60-70	5-11	Up to 70	5.17	122.67	Present study

3.3.3 Effect of temperature on activity and stability of AGP

The AGP remained active over a wide temperature range (40-80°C) but above 90°C the activity dropped rapidly. The highest activity was detected at 60-70°C, with notably equal level. Even at a high temperature of 80°C, AGP retained about 84.23% of the initial activity (Figure 7). These results were aligned with those of earlier research (Asif-Ullah et al., 2006; Gagaoua et al., 2017; Li et al., 2018; Uchikoba et al., 1998). For serine protease from pitaya peel, *C. trigonus*, *C. melo*, *B. cerifera*; the ideal temperature has been identified to be 70°C (Amid et al., 2014; Asif-Ullah et al., 2006; Gagaoua et al., 2017; Kaneda & Tominaga, 1977). Whereas, the optimum temperature of serine protease from *Cyphomandra betacea* (Tamarilin)*, F. carica, S. asper* (Strebilin) *and B. hispida,* has been reported to be within 60-70°C (Li et al., 2018; Tokonami et al., 2023; Tripathi et al., 2011; Uchikoba et al., 1998).

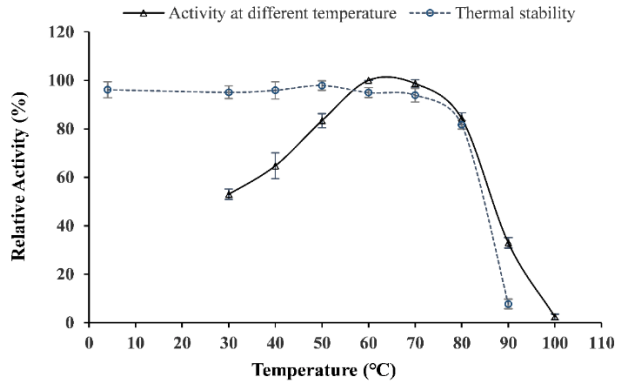


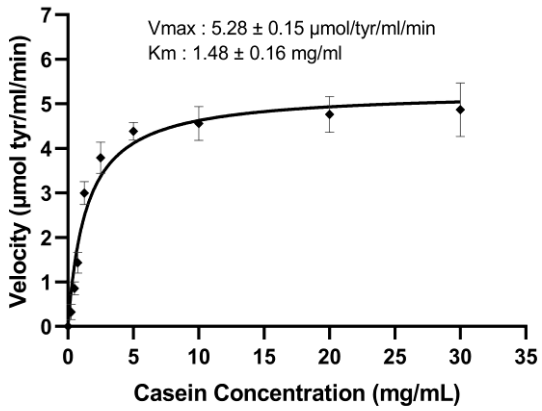
Figure 7
Influence of temperature on activity and stability of TPP recovered protease. Data represent mean of triplicate ± standard deviation.

The remaining activities of the enzyme after exposing to different temperatures for 15 min indicates that the AGP was stable at temperature between 4 -70°C, retaining > 90% activity (Figure 7). Even at 80°C, the AGP maintained around 81.67% of its activity, which was greater than that reported for serine proteases derived from many plant sources (Table 2). The enzymatic activity significantly decreased to 7.72% at 90°C indicating initiation of thermal denaturation. Most of the plant serine protease were thermostable in nature (Table 2). Therefore, the result revealed that AGP is a thermostable protease, with thermal stability comparable to various plant serine proteases including wrightin (75 - 80°C) (Tomar et al., 2008); AG_2 from seed of *B. hispida* (up to 70°C) (Das et al., 2018) and sterblin (15 – 85°C) (Tripathi et al., 2011), protease from *C. trigonus* (up to 70°C) (Asif-Ullah et al., 2006) and cucumisin (up to 70°C) (Gagaoua et al., 2017). Furthermore, Uchikoba et al. (1998) reported that the protease from *B. hispida* was inactivated at 90°C, at pH 7.5 after 15 min. Thermostable enzymes can reduces the chance of contamination and the expenses associated with cooling (Amid et al., 2014). The remarkable stability against the wide range of pH (neutral to basic) and high temperature indicates that the protease could be beneficial to use in many other industrial settings and biotechnological processes (Hafid et al., 2020; Khadka et al., 2024).

3.3.4 Kinetic parameters

The K_m value gives information concerning the enzyme-substrate affinity; lower K_m values indicate high substrate affinity (Khadka et al., 2024). V_{max} is commonly used to assess the catalytic activity, which is typically preferred to be maximum(Gagaoua et al., 2017). The kinetic parameters K_m and V_{max} of the AGP were determined employing casein as a substrate with concentrations varied from 0.25-30 mg/mL. By fitting data obtained from the kinetic experiment with the Michaelis-Menten model and Lineweaver-Burk plot, K_m and V_{max} of the AGP were ascertained (Figure 8 A-B).

A)



B)

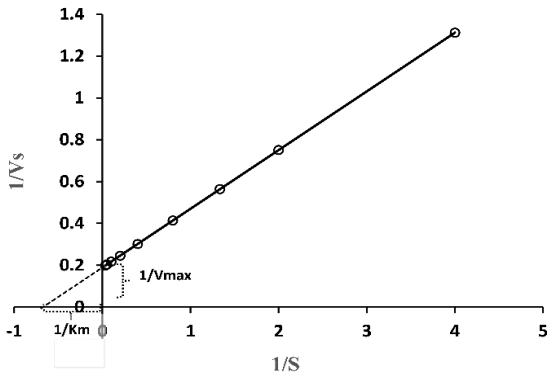


Figure 8
Michaelis –Menten plot (A) and Line-Weaver Burk plot (B) of TPP recovered protease

The K_m and V_{max} of AGP were obtained as 1.48 ± 0.16 mg/mL and 5.28 ± 0.15 µmol tyr /mL/min, respectively. Gagaoua et al. (2017) reported the K_m and V_{max} values for cucumisin as 2.24 mg/mL and 1048 µM/min respectively. The K_m value for wrightin was mentioned to be 50µM (Tomar et al., 2008). Similarly, at pH 8 and 70 °C, the K_m and V_{max} for *Hylocereus polyrhizus*-derived serine protease were 8 mg/mL and 31.20 U/min (Amid et al., 2014). The kinetic constants exhibit variability based on the enzyme

sources, purity level, reaction environment, assay methods, and substrate used (Bibi et al., 2015).

3.3.5 Effect of proteinase inhibitors

The inhibition studies provide information about nature of an enzyme including the properties binding site. A serine protease inhibitor (PMSF), exhibited significant inhibition (93.72%) compared to IAA (Cysteine inhibitor; 10 .28%). While EDTA (Metallo-protease inhibitor) had no significant inhibition (Figure 9). Higher inhibition by PMSF indicated that the AGP is most likely the serine protease. PMSF generally bind with serine molecule present in the active site of serine protease and cause irreversible inhibition (Antão & Malcata, 2005; Li et al., 2018). Previous studies also reported the inhibition of many plant serine proteases by PMSF (Asif-Ullah et al., 2006; Das et al., 2018; Gagaoua et al., 2017; Madhu & Sharada, 2019).

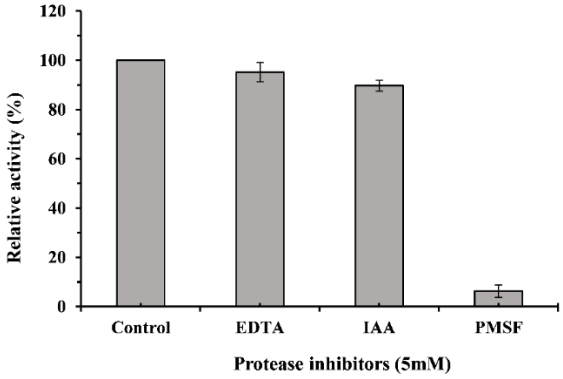


Figure 9
Influence of different inhibitors on activity of TPP recovered protease. Data represent mean of triplicate ± standard deviation.

3.3.6 Specificity towards the natural substrate

Among the tested substrate, AGP showed highest specificity to casein, followed by whey protein (52.94%), hemoglobin (49.50%), gelatin (34.74%) and BSA (24.59%), respectively (Figure 10).

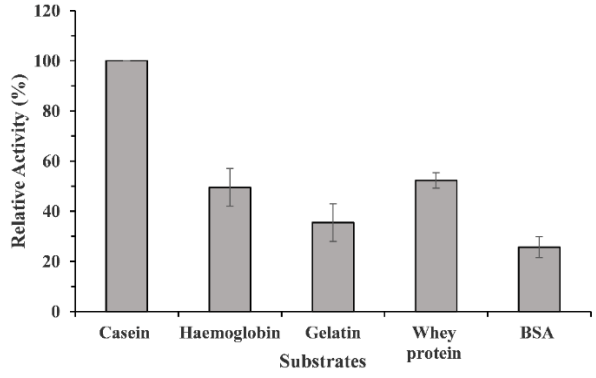


Figure 10
Activity of TPP recovered protease on different natural substrates. Data represent mean of triplicate ± standard deviation.

According to Khan et al. (2008), the serine protease from the seeds of *Holarrhena antidysenterica* showed greater activity to casein followed by hemoglobin, BSA, and gelatin. The higher activity to casein compared to hemoglobin, was also mentioned for other plant serine proteases (Tomar et al., 2008; Tripathi et al., 2011). While, Rawaliya et al. (2022) described that serine protease from *Cyamopsis tetragonoloba* act on substrate casein, gelatin, hemoglobin, and BSA with almost similar efficiencies. The differences in specificity of the enzyme is due to difference in shape and structure of the substrate and also the active site of enzyme, which again rely upon the position, sequence and structure of the amino acids (Hrmova & Fincher, 2001). The specificity of AGP to various natural proteins highlighted its possibilities to use in different animal and food proteins hydrolysis, and most probably beneficial for protein hydrolysate preparation, bioactive peptide generation, milk clotting, meat tenderization, and food waste treatment (Patel et al., 2019).

3.4 Application of AGP in whey protein hydrolysis

3.4.1 Degree of hydrolysis and hydrolysis patterns

Whey protein (0.6%) was hydrolyzed with the different concentration (0-8% v/v) of AGP and trypsin, the obtained DH is presented in Figure 11A. Trypsin was selected as control as it was belong to serine family and one of the mostly used animal protease in whey protein hydrolysis (Irazoqui et al., 2024). The DH was greater for AGP than for trypsin at every concentration of the added enzyme. The DH continually increased with increased enzyme concentration, attaining the highest value of 48.79% at 6% of AGP and 43.34% at 8% of trypsin. As observed in Figure 11A, a significant increase in DH was observed with AGP hydrolysis up to 2% added enzyme level and with further increase in enzyme level to 4%, there was some increase in DH but not significant. This could be attributed to availability of sufficient substrate (intact peptide bonds) for the enzymes. But at 4 %, enzyme level has been increased and there could be some competition between the enzymes to bind the limited substrate which could probably responsible for insignificant increase in the DH. But at 4-5% enzyme level, there was a significant sharp increase in the DH and then the DH started to drop but not significantly at 6% added enzyme level. Some released peptides during hydrolysis could have possibility to become the substrate to free enzymes that could enhance the activity causing a plateau and convert the peptide to inhibitory products which could cause drop in the DH (Edison et al., 2020**)**. The drop in DH after optimum enzyme level could also reported to be due to enzymatic aggregation, inhibition of substrate diffusion and then substrate saturation(Noman et al., 2018). However, no such plateau and dropped in the DH were noticed in trypsin hydrolysis, most probably due to highly specific nature of the enzyme and also not reaching the substrate level to complete saturation. An increase in DH of whey protein with various plant serine proteases were well reported before (Babij et al., 2015; Corrons et al., 2012). A dose-dependent increase in DH was reported for whey protein hydrolysis by papain, bromelain and chymotrypsin (Valchkov et al., 2023). Similarly, a high DH at a higher

ficin concentration (1%) compared to a lower concentration (0.5%) at different hydrolysis time was also mentioned (Kheroufi et al., 2022).

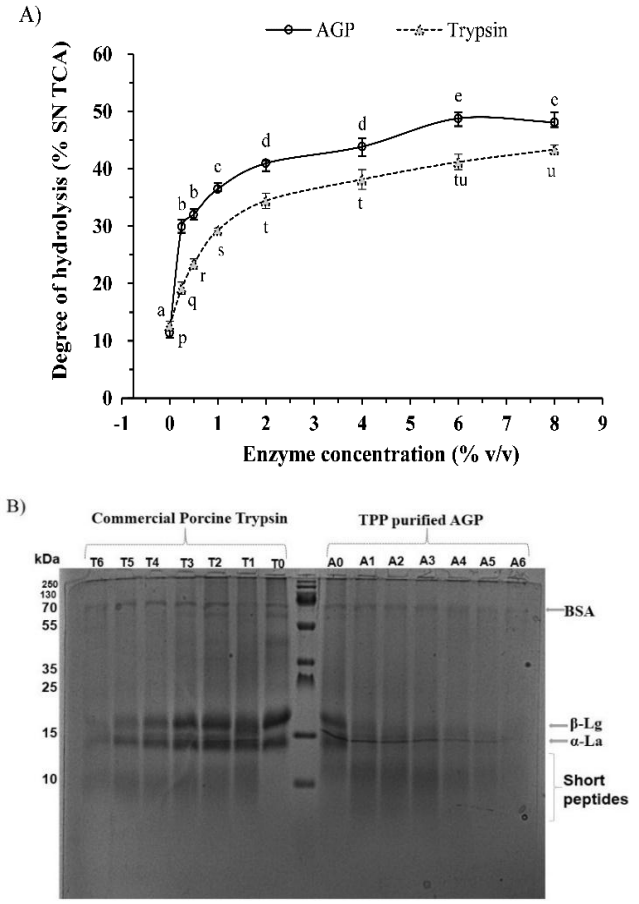


Figure 11
Degree of hydrolysis of WPC (0.6%) obtained from TPP recovered protease (AGP) and trypsin at different added concentration of enzyme (0-8%, v/v), values are mean of triplicate ± standard deviation and different lower-case letter across the same line indicate significantly different ($p<0.05$); B) Electrophoresis (tricine SDS-PAGE) results of whey protein degradation patterns by commercial trypsin and TPP purified AGP. M; protein standard ladder, t; Trypsin hydrolysate, A; AGP hydrolysate, Lane T0 –T6 and Lane A0 – A6 represent the enzyme concentrations (0, 0.5, 1, 2, 4, 6 and 8% v/v) of trypsin and AGP, respectively used for hydrolysis of whey protein (0.6%) for 2h.

Regarding the hydrolysis patterns, AGP degraded both the whey proteins but more efficiently than trypsin; and β-lactoglobulin band was observed to decrease more intensely as compared to α-lactoalbumin with increased concentration of the enzyme (Figure 11B). Similar intensive degradation β-lactoglobulin compared to α-lactoalbumin were also found with the crude serine protease extract from trompillo berries and melon as compared to *C. aurantium* flowers proteinase extract (Mazarro-Manzano et al., 2020). As alike *C. aurantium* flowers proteinase and cynarase (Plant aspartic proteases) which act poorly on β-lactoglobulin (Mazarro-Manzano et al., 2020; Tavares et al., 2012), AGP was found to cleave β-lactoglobulin efficiently, and this properties could be beneficial for reducing the whey protein allergenicity too (Mazarro-Manzano et al., 2020).

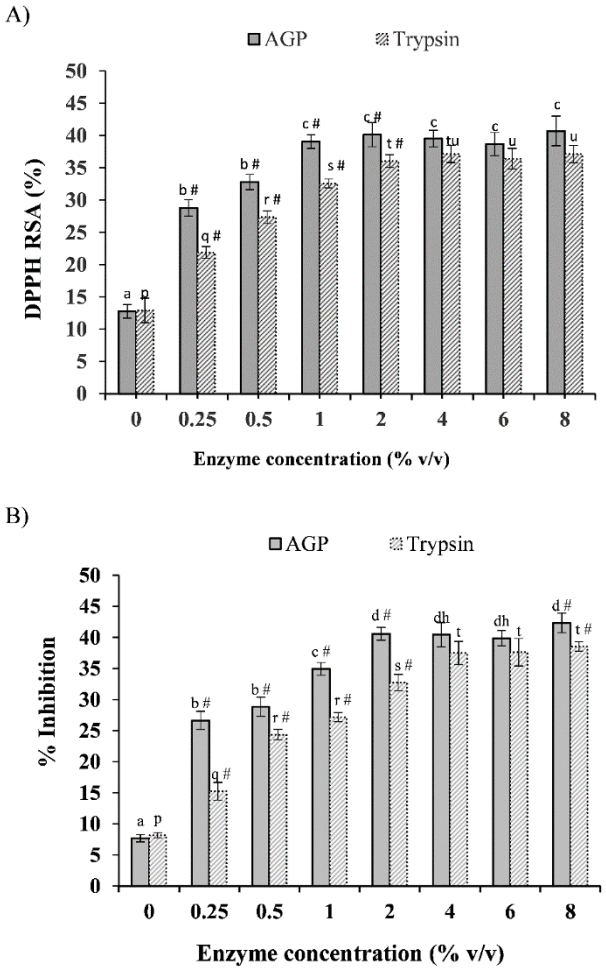


Figure 12
Antioxidant activities of why protein (0.6%) hydrolyzed by TPP recovered protease (AGP) and trypsin as a function of enzyme concentration (0-8% v/v); A) DPPH radical scavenging activity; B) ABTS inhibition activity. Values are mean of triplicate ± standard deviation, Different lowercase letters (a,b,c…., or p,q,r,s…) among the bars of different added concentration of same enzyme represent significantly different value ($p<0.05$), and the same symbol (#) on the bars at same level of added concentrations between the enzyme represent significantly different values ($p<0.05$).

3.4.2 Antioxidant activities

The antioxidant activities of the generated hydrolysate with increased volume of AGP and trypsin with 2h of hydrolysis were estimated using two approaches; DPPH radical scavenging activity and ABTS inhibition activity (Figure 12). As seen in Figure 12, at each concentration; basically, at lower concentration range of enzyme, AGP compared to trypsin exhibited comparatively higher antioxidant activities as observed from both methods. Trypsin has highly specific enzyme especially prefer to cleave bond at lysine or arginine of the substrate, while protease with broad specificity (e.g.,

cucumisin like protease) has ability to cleave many peptide bond resulting higher extent of hydrolysis which could be responsible for efficient fast production of bioactive peptides at initial lower concentration (Kleekayai & FitzGerald, 2022).

The DPPH radical scavenging activity significantly ($p < 0.05$) rose from 12.76 % to 39.06% as the concentration of AGP increased from 0 to 1% and 12.91% to 36.04% as the concentration of trypsin went up from 0 to 2% (Figure 12A). But, no notable changes in activity were detected with both enzyme on further increment of concentrations. The highest DPPH radical scavenging activity obtained from AGP (1%) and trypsin (2%) hydrolysate was, respectively 3.06 and 2.79 times greater than that of un-hydrolyzed whey protein. Concerned to ABTS inhibition activity, WPH produced from AGP had higher inhibition activity at every concentration of enzymes (0-8% v/v) as compared to trypsin. The highest inhibition of 40.59% and 37.60%, which were 5.27- and 4.59-fold increments as compared to un-hydrolyzed whey protein, were obtained at an enzyme concentration of 2% AGP and 4% trypsin, respectively (Figure 12B). But, beyond these concentrations, no significant changes in inhibition were observed with both the enzymes (Figure 12B).

Higher DPPH radical scavenging activity of plant serine protease extract from melon as compared to commercial alcalase in fish protein hydrolysate was well reported earlier (Alavi et al., 2019). Similarly, WPH obtained from plant proteases (Papain and bromelain) as compared to commercial chymotrypsin, has been shown to be better in DPPH radical scavenging activity (Valchkov et al., 2023). The author also reported an increase in activity with an increased enzyme concentration from 0.1 to 0.5 mg/mL, but a slight reduction in activity was mentioned at higher enzyme concentration (1 mg/mL) (Valchkov et al., 2023). The significant higher antioxidant activity with ABTS assay at higher enzyme concentration (1%) compared to the lower concentration (0.5%) and un-hydrolyzed whey protein has been shown earlier in ficin hydrolyzed whey protein (Kheroufi et al., 2022). Thus, our findings are in agreement with those findings. Insignificant increases in DPPH scavenging and ABTS inhibition activities at higher concentrations could be related to substrate dilution effect which resulted in limited generation of bioactive peptides.

The significant linear increase in antioxidant activity, at lower range enzyme concentration indicated that peptides generated during the hydrolysis by the proteases are responsible for these activities. The differences found in antioxidant activities by the two methods mainly depend upon the natures of the peptides produced at the time of hydrolysis. The features of the bioactive peptides, including size, composition, structure, and position of amino acid residue, greatly attributed to particular antioxidant properties of the hydrolysate (Karami & Akbari-adergani, 2019). In ABTS scavenging activity, cysteine followed by the other hydrophobic amino acids; tryptophan, tyrosine, and histidine are mentioned to be mainly responsible (Coscueta et al., 2016). Whereas, the hydrophilic fraction of peptides has shown to be correlated with DPPH radical scavenging activity (Martin-del-Campo et al., 2019). Collectively, the study reveals that AGP possesses the efficient ability to hydrolyze whey protein and to generate bioactive peptides for enhancing the antioxidant activities.

4. CONCLUSION

TPP can be used as an affordable, straightforward, and effective way to separate and concentrate the protease from ash gourd crude extract. By using an optimized one-step TPP system with 50% $(NH_4)_2SO_4$, crude extract: *t*-butanol ratio of 1:1 at pH 8 and 25°C, protease from ash gourd crude extract can be effectively concentrated and recovered in the IP fraction with 5.17-fold purification and 122.67% recovery of activity. The recovered protease characterization reveals the presence most active serine protease of 67.39 kDa, and is stable at a wider pH (5-11) and temperature up to 70°C. The recovered enzyme also hydrolyzed whey protein more effectively than the commercial trypsin with improved antioxidant activity of hydrolysate by 3.06 - 5.27 times higher than the un-hydrolyzed whey protein. Overall, the enzyme demonstrated its potential to use in whey protein hydrolysis to improve the biological functional properties such as the antioxidant activity. Further in-depth study regarding optimization of hydrolysis condition, characterization of hydrolysate with respect to generated peptides, establishing relation between peptide structure and biological functionality are recommended to explore application of ash gourd protease in whey protein hydrolysis for their effective utilization in food and pharmaceutical applications.

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CONFLICTS OF INTEREST

No potential conflict of interest was reported by author(s).

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