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Purification and Characterization of Protease from Chickpeas

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Abstract

The main aim of the present study was to isolate, purify, and characterize a protease from chickpeas. A 0.1 M phosphate buffer at pH 8.0 was used for protease extraction. Following the rinsing of unattached proteins with a (pH 8.0) 20 mM Tris-2 mM CaCl2 solution and subsequent size exclusion filtering using Sephadex G-50 in the same buffer, the required protein was subsequently eluted using 0.5 M NaCl. The protease was subjected to further optimization by precipitating ammonium sulphate at a 75% concentration. Protease exhibited activity throughout the pH range of 6.0 to 8.0, it was entirely active at 35°C. Subsequently, DPPH, FTIR, and HPLC analysis was applied.

Keywords: chickpea, protease, purification, characterization, HPLC, DPPH, FTIR.

Introduction

The enzymes that carry out proteolysis, or the digestion of proteins, are known as proteases or proteinases [1]. These enzymes play a crucial role in the continued existence and functioning of the organism as they participate in numerous biological processes [2; 3]. Proteases are excellent for complex organisms since they have evolved to cope with a wide range of situations (such as pH fluctuations and reductive environments). They are categorized according to the various catalytic processes used for the hydrolysis of substrates [4]. According to their mode of action, they can either be aspartic, metallo, or glutamic proteases or serine, cysteine, or threonine proteases. Except at the ends of the molecule, proteases specifically cleave proteins. Endopeptidases can originate from any place on the molecule, while aminopeptidases and carboxypeptidases might originate from the N or C terminal. Numerous vital functions need proteases as a fundamental component for sustaining the life cycle, cellular development and apoptosis, reconstruction of tissues, homeostasis, wound healing, and immunological response [4]. Proteases work by cleaving proteins. The third-ranked pulse is the chickpea (*Cicer arietinum*), which is a significant source of dietary protein and carbohydrates [5]. Chickpeas have been shown to reduce the risk of several illnesses, including cancer, diabetes, obesity, dermatological and hematological problems, as well as hepatic and splenic ailments.

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Studies [6; 7; 8; 9] have shown that protease can be isolated and purified from a wide range of plants and animals. Thus, we set out to purify and investigate the physical and chemical properties of the protease. Its stability in different conditions. Finding new protease sources and learning more about the functions of proteases could both benefit from this research. In certain respects, these studies can also aid in our understanding of the significant function that proteases serve in industry.

Materials and Procedures

We bought chickpea seeds from the local market in Baghdad. Chemicals for measuring protease activity: All additional compounds were of the analytical variety.

Protease Extraction and Purification

Chickpea seeds were blended in a blender with a pH 8.0 buffer (20 mM Tris HCl) and 2 mM $CaCl_2$. The coarse remaining particles were eliminated from the crude extract by filtering it through muslin cloth. After that, it was centrifuged for 45 minutes at 4°C at 8000 rpm. Ammonium sulfate was added to the supernatant to increase saturation to 75%. The mixture underwent centrifugation for an extra 45 minutes at 8000 rpm, followed by constant mixing overnight at 4°C, to precipitate the proteins that had been pre-salted with ammonium sulfate. The protein pellet was collected and dialyzed numerous times against the same buffer (20 mM Tris, 2 mM CaCl2, pH 8.0) to remove ammonium sulfate. The dialyzed material was placed on a 2 x 30 cm DEAE-cellulose column for further purification as required. Protein was extracted using 0.5 M NaCl in the same buffer after free proteins were washed utilizing (pH 8.0) 20 mM Tris–2 mM CaCl2 buffer. Next, the proteins were placed onto a Sephadex G-50 column (2 x 100 cm) in 20 mM Tris, 2 mM CaCl2, and pH 8.0 for size exclusion filtering [10].

Protease Activity Determination

The method described in [11] was slightly modified and was applied to evaluate protease activity using azo-casein as a substrate. 50µl of buffer containing 20 mM Tris-HCl and 2 mM CaCl2 (pH 8) was added to 50µl of sample for each purification stage. The reaction was terminated by the addition of 100 µl of (30% (v/v)) triacetic acid following a 10-minute incubation at 25 °C. The absorbance of the reaction mixture was recorded at a wavelength of 410 nm.

Estimation of Protein

The protein content of the crude extract was assessed using the biuret method [12] with bovine serum albumin (Sigma Chemical Co.) serving as the reference. The mg/ml unit of measurement for protein was used.

Optimum pH

The optimal pH of the protease enzyme was determined by using buffer solutions with different pH values (citrate buffer, phosphate buffer, glycin buffer 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 for 30 minutes).

Optimum Temperature

The effect of the optimal temperature on the activity of protease was examined by adjusting the temperature of the reaction mixture (15, 25, 35, 45, 55, 65, and 75 ◦C). The reaction mixture was incubated at the same temperatures in order to look into the ideal condition of the enzyme.

The Antioxidant Assay uses DPPH (α,α-Diphenyl-β-picrylhydrazyl).

Following the methodology outlined in [13], a mixture was prepared by combining 1.5 mL of the

sample solution with 1.5 mL of DPPH, along with 0.1 mM of DPPH dissolved in 95% ethanol. The weight of the sample was measured at 0.0039 g in a volume of 100 mL of 95% ethanol. The solution was allowed to rest for 30 minutes at ambient temperature in the absence of light following rigorous agitation. The absorbance of the resulting solution was measured at 517 nm. Declined absorbance readings suggest improved DPPH scavenging activity and can be expressed as:

Scavenging activity (%) = [(blank A517—sample A517) / blank A517] × 100%.

High Performance Liquid Chromatography (HPLC)

For chromatographic separation, the Shimadzu HPLC system operating at a flow rate of 0.8 ml/min and using (300Å, 3 µm, 4.6 x 300 mm) HPLC column was utilized. An isocratic gradient utilizing a (pH 7.0) 150 mM phosphate buffer mobile phase was employed for chromatographic separations. The column was kept at ambient temperature. The injection valve and auto-sampler syringe were systematically cleansed with a methanol/water (70/30; v/v) solution for minimizing residual contamination. A sample of five microliters was added to the system. Using a diode array detector, collagen was found at a wavelength of 214 nm.

Sample Preparation

The chickpea extract was vigorously agitated to achieve a consistent viscous syrup. A highly concentrated sample of 10 µL was diluted 1000 times with distilled water after the homogenization process to achieve a final concentration of 5 µg/ml. After that, five µL of this combination were added to the HPLC-SEC-UV apparatus, and it was measured at 214 nm [14].

FT-IR Analysis

Potassium bromide KBr pellets were prepared from discs containing two milligram samples of collagen in around 200 milligrams of potassium bromide (KBr) in dry conditions, and the FTIR analysis was performed using this equipment in the SHIMADZU (Japan) model apparatus [15].

Results and Discussions:

Protease was successively purified from crude extracts prepared in Tris-buffer. The protease purification was conducted using standard methods. The purification of the crude extract involved a series of steps, including 75% ammonium sulfate precipitation, followed by dialysis and DEAE cellulose chromatography, as detailed in Table (1) for the purification process flow chart.

Crude enzyme has a specific activity of 40.11 (U/ml). The protease was effectively precipitated at 75% ammonium sulphate saturation. The precipitate protease-specific activity was 146.08 (U/ml), and the yield and fold of purification were 3.64 and 48.18%, respectively. The protease was first put onto a DEAE-cellulose column following dialysis. Combining purification techniques can increase protein folding and recovery. The solution with the concentration of 0.02-0.5M NaCl was utilized for protein elution, and the absorbance was measured at 280 nm (Figure 1 A). Ion exchange chromatography yielded a protease fold of purification of 10.61 times. Other plant species,

such as chickpeas, also showed similar outcomes [16].

Afterwards, the gel filtration process was performed utilizing a Sephadex G-50 column (2 × 100 cm), which was equilibrated with (pH 8.0) 0.1 M Tris HCl buffer, comprising 0.2 M NaCl (Figure 2A). The column was eluted at 4 °C with a flow rate of 30 mL/h, and 4.0 mL fractions were retrieved using the same solution. Following the three stages of purification, the fractions displaying protease activity surfaced. Gel filtration was used to load the pooled peak fractions. Protease-specific activity peaked at 393.94 (U/ml), had a 9.82-fold purification factor, and had a 20.66% recovery rate.

Optimum pH

Chickpea protease exhibited relative stability across the pH range of neutral to slightly alkaline, with an optimal pH of 8.0 (Figure 2). A correlation frequently occurs between net charge and protein stability at a certain pH. Chickpea protease demonstrated optimal activity at a pH of approximately 8, with a significant decline to about 50% at pH levels of 5-6. In comparison, sardine protease showed stability within the pH range of 7-8 [6; 7; 8]. This chickpea protease pattern is comparable to that of fish protease, which is unstable at pH values that are either highly alkaline or acidic [17]. Regarding thermostability, chickpea protease resembles fish sources that have been purified, meaning it remains extremely stable at lower temperatures, and over 55°C becomes more and more inhibited [8; 17; 18]. Protein stability and net charge at a specific pH are generally correlated.

Table (1): Specifics about the protein at each stage of purification, including its concentration, activity, and purity.

Purification steps	Volume (ml)	Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/ml)	Total activity	Purification Fold	Yield (%)
Crude enzyme	230	65.66	1.637	40.11	15101.8	1	100
Precipitate by ammonium sulphate 75%	55	132.06	0.904	146.08	7263.3	3.64	48.18
Dialysis	60	143.26	0.568	252.21	8595.6	6.28	56.92
Ion exchange DEAE - Cellulose	15	202.11	0.475	425.49	3031.65	10.61	49.51
Gel filtration G-50	20	156	0.396	393.94	3120	9.82	20.66

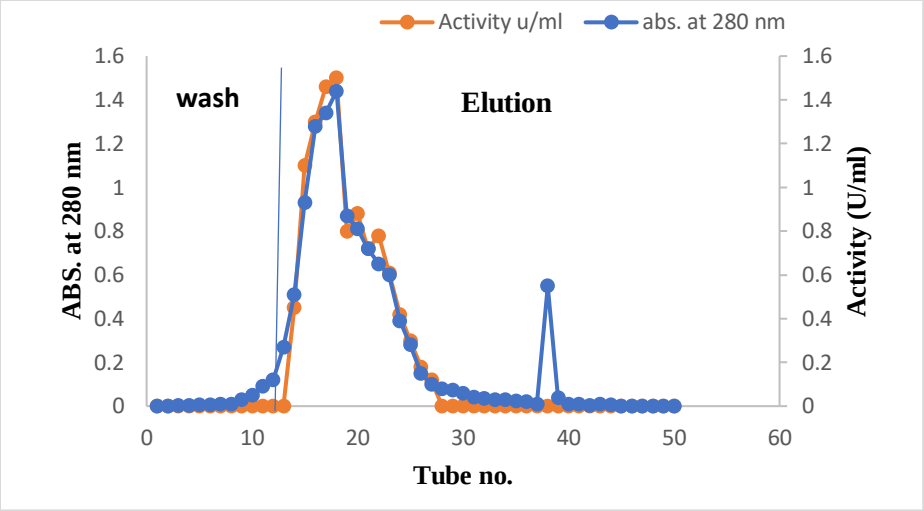


Figure (1A): An ion exchange chromatography assay was conducted utilizing a DEAE-cellulose protease column (2 × 30 cm) with a flow rate set at 40 ml/h. A total of 4 ml of unbound proteins was obtained and subsequently rinsed with a 20 mM Tris-2 mM CaCl2 solution at pH 8.0. A linear gradient of 0.02 to 0.5 M NaCl was implemented in a 2 mM Tris solution at pH 8.0 for the extraction process.

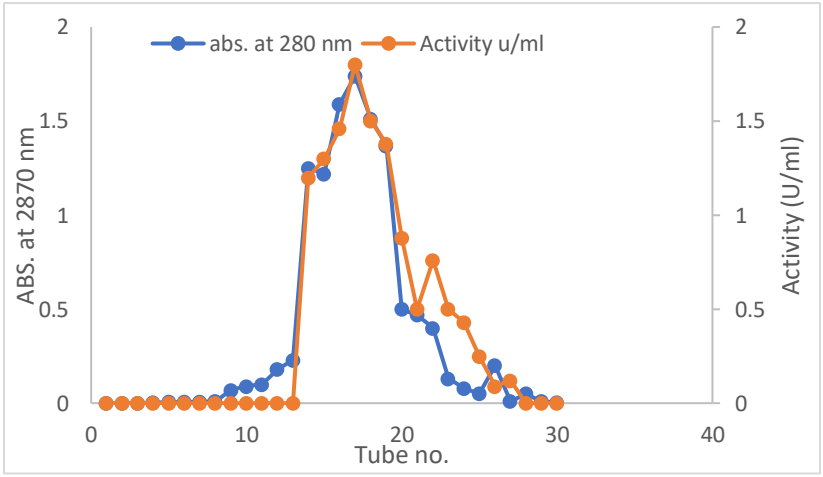


Figure (2A): Gel filtration chromatography procedure using a Sephadex G-50 column. A G-50 column (2 × 100 cm) was equilibrated with a 0.1 M Tris-HCl buffer (pH 8.0) supplemented with 0.2 M NaCl. The column was eluted at 4 °C using the same solution at a flow rate of 30 mL/hour, and 4.0 mL fractions were collected.

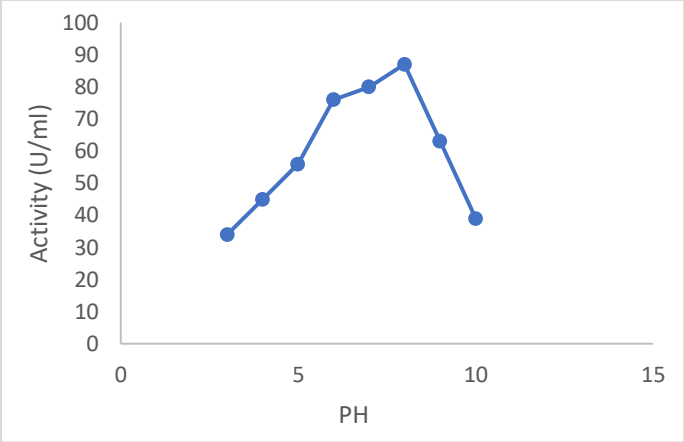


Figure (2): Effect of pH on protease activity

Optimum Temperature

The findings displayed in Figure 3 confirm that pure protease remained active at temperatures between 30 and 40 ◦C, peaking at 35 ◦C, as shown in Figure 3 [10]. Shows that the protein denatures at 60℃ and that activity drastically decreases at 50°C.

Antioxidant Assay α,α-diphenyl-β-picrylhydrazyl (DPPH)

The method relies on the reduction of an alcoholic DPPH solution when hydrogen-donating antioxidants exist, resulting in the formation of the non-radical form, DPPH-H. The DPPH was determined to exhibit a 43.82% scavenging activity initiated by pure protease. Hydroxyl radicals are extremely active free radicals that can be generated from superoxide anions and hydrogen peroxide when metal ions, like copper or iron, are present. Hydroxyl radicals are characterized by their high chemical activity, allowing for easy interactions with biomolecules, including amino acids, lipids, DNA, and proteins [19]. The elimination of hydroxyl radicals is likely one of the most significant defenses of an organism against several illnesses. Assessing hydroxyl radical scavenging activity presents valuable insights into antioxidant capabilities. Consequently, eliminating hydroxyl radicals is likely among the most potent protective mechanisms of a living organism against a range of diseases. The assessment of hydroxyl radical scavenging activity yields valuable insights into antioxidant functions.

High Performance Liquid Chromatography (HPLC) test

Figure 4 (a and b) displays the retention time and area of the standard protease and chickpea protease. The findings from the HPLC UV-VIS analysis are described in Table 2. The concentration of chickpea protease amounted to 704.4 µg/ml, as shown by the retention time peak, whereas the standard protease was 10 µg/ml.

FT-IR:

The FTIR spectra were recorded between 4000 and 500 cm−1 (Figure 5a, Figure 5b) on an FTIR spectrometer (Schimadzu, Japan). The spectra of protease and standard protease are similar, indicating that tyrosine is one of the major amino acids released by the action of protease from chickpeas.

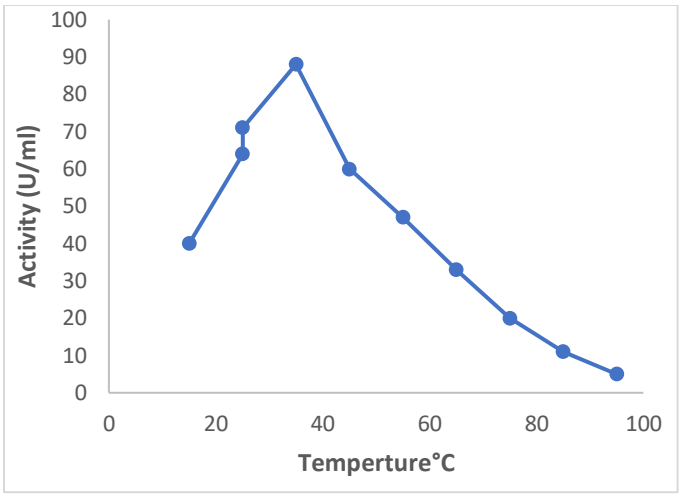
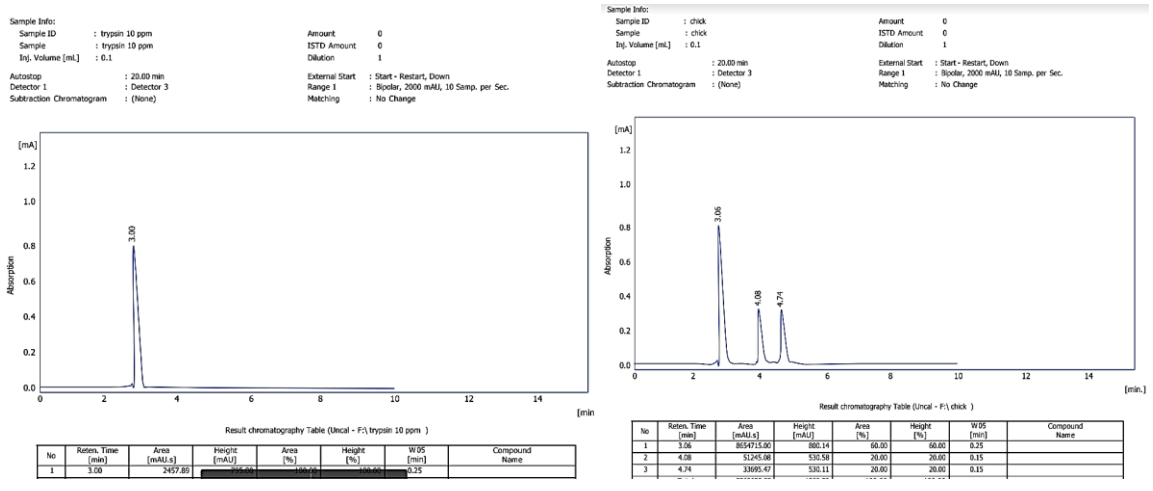


Figure (3): Influence of temperature on protease activity

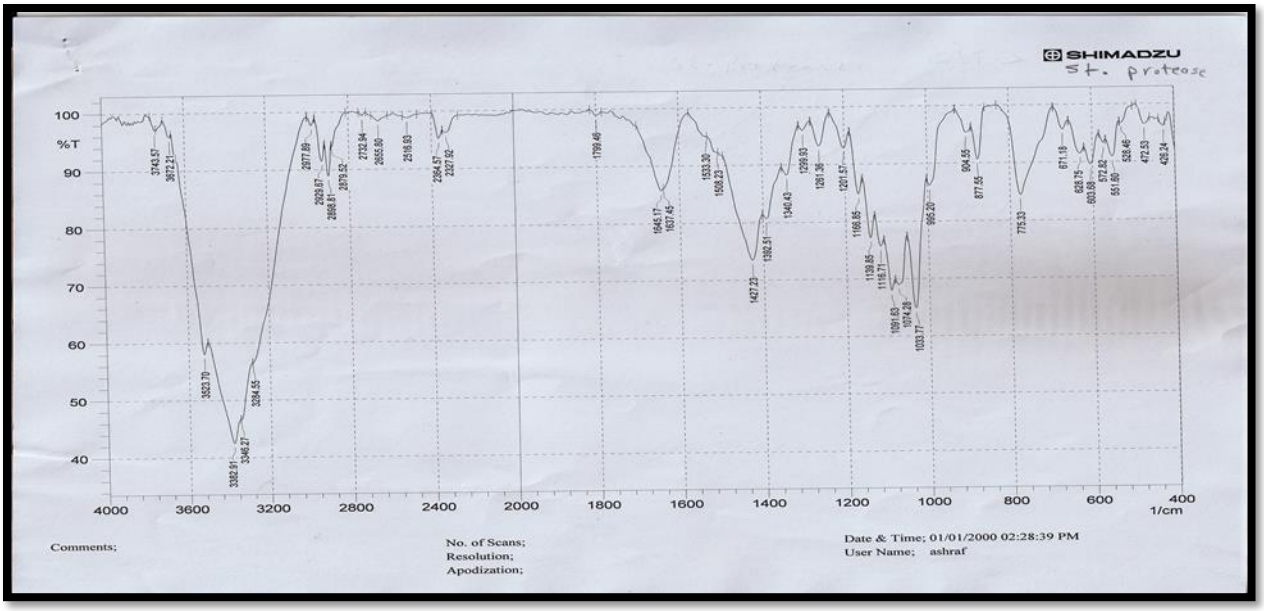
Table (2): Concentration of chickpea protease according to standard protease by HPLC.

Sample	Retention Time (min.)	Concentration (ppm)
Standard protease	**3**	**10**
Chickpea	**3.06**	**704.4**

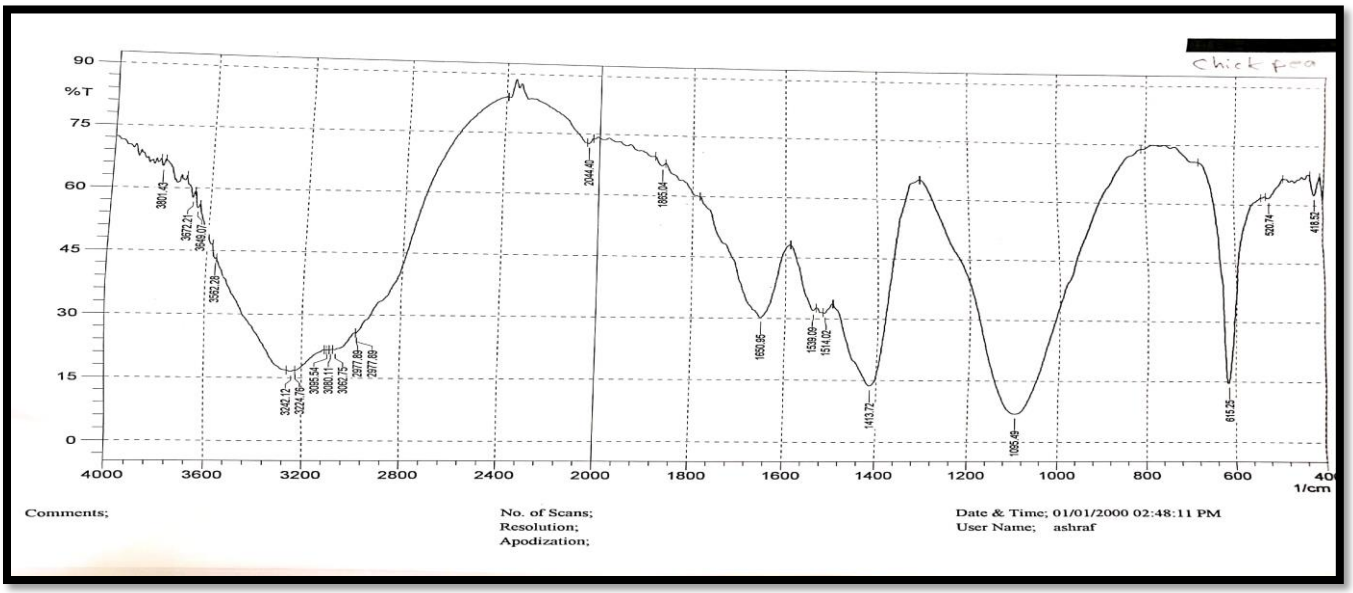


(a) **(b)**

Figure (4): High-performance liquid chromatography of (a) standard protease and (b) chickpea protease.



(a) Standard protease



(b) Chickpea protease

Figure (5): FTIR of (a) standard protease and (b) chickpea

Conclusion

We have effectively isolated protease with a high yield in a limited number of stages from chickpea seeds. After soaking the seeds for the entire night in Tris buffer (20 mM Tris–2 mM CaCl2; pH 8.0), the seeds were homogenized and centrifuged. A 75% fraction of ammonium sulfate precipitation and dialysis was used to precipitate the protein. For Ion-exchange chromatography, the dialyzed sample was placed onto a DEAE-cellulose column, and the protease was eluted using gel filtration and an elution buffer containing 0.02M–0.5M NaCl. We discovered that our purification process yields highly pure protease and is quick, effective, and simple and requires less time. The evaluation of the isolated protease revealed significant technical potential, particularly as its peak activity occurred at an alkaline pH of 8.0 and an optimal temperature of 35°C. These characteristics indicate that the enzyme has protease qualities, making it potentially valuable as a biotechnological tool in the food, tissue culture, proteomics, and cancer research fields.

Conflict of interest: NIL

Funding: NIL

References

1. Habib H, Khalid MF (2007) Plant protease inhibitors: a defense strategy in plants. Biotechnology and Mol Biol Rev 2: 68-85.
2. Joanitti GA, Freitas SM, Silva LP (2006) Proteinaceous Protease Inhibitors: Structural Features and Multiple Functional Faces. Curr Enz Inhib 2: 199-217.
3. Neurath H (1989) Proteolytic processing and physiological regulation. Trends Biochem Sci 14: 268-271.
4. Turk B (2006) Targeting proteases: successes, failures and future prospects. Nat Rev 5: 785-799.
5. Duke JA (1981) Handbook of legumes of world economic importance. Starch 34: 52-57. 6. Panlasigui LN, Panlilio LM, Madrid JC (1995) Glycaemic response in normal subjects to five different legumes commonly used in the Philippines. Int J Food Sci Nutr 46: 155-160.
6. Castillo-Yanez FJ, Pacheco-Aguilar R, Garcia-Carreno FL, Navarrete-Del, Toro MA. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey sardine Sardinops sagax caerulea. Comp Biochem Physiol B Biochem Mol Biol 140: 91-98.
7. Singh SJ, Singh LR, Devi SK, Singh SS, Devi CB, et al. (2015) Purification and Characterization of a Thermostable Caseinolytic Serine Protease from the Latex of Euphorbia heterophylla L. Protein Pept Lett 22: 828-835.
8. Ishwarya S, Sangeetha R. (2013) A new serine protease from the leaves of Thespesia populnea. Prep Biochem Biotechnol 43: 95-107.
9. Ranjbar M, Zibaee A, Sendi JJ (2014) A trypsin-like proteinase in the midgut of Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae) purification, characterization, and host plant inhibitors. Arch Insect Biochem Physiol 85: 1-12.
10. Tooba Naz Shamsi, Priyankar Sen, and Sadaf Fatima. (2016). Purification and Characterization of a Protease from Green Seeded Chickpea (Cicer arientum). J. Res Development 2016, vol. 4:issu 2, P2-5. DOI: 10.4172/2311-3278.1000146.
11. Erlanger BF, Kokowsky N, Cpenhen W (1961) The preparation and properties of two new

chromogenic substrates of trypsin. Arch Biochem Biophys 95: 271-278.

12. Gornall, A. G., Bardawill, C. L. and David, M. M. (1949). Determination of Serum Protein by means of the biuret reaction. J.Biol. Chem. 177: 751-753.
13. Salman Z. O.,B. M. J. Alwash E.and Kadhim J. (2019). Effect Of Essential Oil of Cestrum nocturnum FlowersL Cultivated In IRAQ As Antioxidant And Elonation Cold Storage Period Of Minced Meat. Iraqi Journal of Agricultural Sciences:50(2):601-607.
14. Bilgin, Vatansever, Burcu (2015). A quantitative method for the measurement of hydrolyzed type-I collagen protein in dietary supplement syrup using HPLC-SEC-UV technique. J. Chem. Metrol. 9:1,1-15.
15. Muyonga, J.; Cole, C. G. And Duodu, K. (2004). Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult nile perch (Lates Niloticus)‖, Food Chem., 86: 325–332.
16. Kansal, R, Gupta, RN, Koundal, KR, Kuhar, K, Gupta, VK (2008) Purification, characterization and evaluation of insecticidal potential of trypsin inhibitor from mungbean (Vigna radiata L. Wilczek) seeds. Acta Physiol. Plant. 30:761-768.
17. Hag SK, Khan RH. (2003). Characterization of a proteinase inhibitor from Cajanus cajan (L.). J Protein Chem 22: 543-554.
18. Heu MS, Kim HR, Pyeun JH. (1995). Comparison of trypsin and chymotrypsin from the viscera of anchovy (Engraulis japonica). Comp Biochem Physiol B Biochem Mol Biol 112: 557-567.
19. J.Y. Je, Z.J. Qian, H.G. Byun, S.K. Kim. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis Process Biochemistry, 42, pp. 840-846.