International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

Isolation and Characterization of Chemostable Protease from Senegalia rugata

Latha B V[1] and Likhitha R[2]

[1]*Assistant professor, Postgraduate Department of Biochemistry, JSS college of Arts, Commerce and Science, Mysuru, Karnataka*

[2]*Senior Research Fellow, Postgraduate Department of Biochemistry, JSS college of Arts, Commerce and Science, Mysuru, Karnataka*

Corresponding Author: Latha B V

Date of Submission: 01-08-2021 Date of Acceptance: 14-08-2021

ABSTRACT: The present study included isolation, purification and partial characterization of protease from Senegalia rugata seeds. Extraction of protease was done by ammonium sulphate precipitation. Ion exchange chromatography and gel permeation chromatography was employed for protease purification. Proteolytic activity of the purified protease was determined in the gel by Gelatin Zymogram. The molecular weight of the isolated protease was found to be 33.94kDa with optimum proteolytic activity at pH 9.5 with incubation for 60 minutes at 40°C. The purified protease enzyme revealed high stability and compatibility towards oxidizing, reducing, and bleaching agents. In addition, enzyme also showed stability towards organic solvents and commercial detergents. Thus the obtained protease has fascinating properties which can be exploited in various important bio-formulations and industrial applications.

KEYWORDS: Protease, Senegalia rugata, zymogram, chemostable.

I. INTRODUCTION

Senegalia rugata of the family Leguminosae is native to Asia, and commonly referred as "fruit for the hair". Washing hair with their fruit can promote hair growth and reduce dandruff. The infusion of the leaves and pod decoction are used in the treatment of malarial fever and biliousness, also acts as a strong laxative.

[1].Fatty acids and esters like palmitic and linoleic acid, furfural, 5-methyl-2-furfural, methyl salicylate, methyl palmitate, isopropyl palmitate and linalool oxide are found to be main constituents of volatiles which contribute to the aroma. [2].The extracts of bark and pod showed antimicrobial property. [3].Saponins named kinmoonosides AC extracted from pods exhibit cytotoxicity against human HT-1080 fibrosarcoma cells. [4]. The methanolic fraction of pods has exhibited strong immunological adjuvant activity.

[5].Proteases play an important role in physiological functions of plants and animals. They are reported to be involved in germination, senescence, apoptosis, complement activation, inflammation, microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation and many more. [6].They constitutes one of the largest groups of enzymes having industrial importance. Among 60% of enzymes produced, proteases have more importance in enzyme market. [7].Proteases have been widely used in leather processing for soaking, dehairing and baiting to remove protein and bloodstain from the skin. Proteases are used in food industry for tenderizing meat, brewing, cheese elaboration and in bread making. Proteases, because of their high substrate specificity and stability over a wide range of pH and temperature have gained importance in textile, detergent, pharmaceutical, medicinal and biotechnology industries.

The diverse properties of proteases and their wide applications attract the interest of current day researchers. Current work revealed Senegalia rugata as medicinally important the piezoelectric control based pilot allows for direct regulation of other engine valve parameters including variable lift and seating velocity.

II. MATERIALS AND METHODS

Senegalia rugata seeds were procured from the local market at Mysuru, Karnataka. All chemicals used were of analytical grade and obtained from SIGMA (Merck), SRL and HiMedia. Commercial detergents were purchased from local market.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

III. EXTRACTION OF PROTEASE

Senegalia rugata seeds (50g) were ground into fine powder and soaked overnight in 0.1M NaCl solution. The slurry is then centrifuged at 10000rpm for 15 minutes. The protein concentration and protease activity in the supernatant is estimated. Ammonium sulphate was added at two different concentrations (40% and 80%) to precipitate protein. The protein separated by salting out was separated by centrifugation at 10000rpm for 15 minutes. The pellet was dissolved in 0.1M phosphate buffer and dialysed overnight. The protein concentration and protease activity of the dialysed fraction was analysed.

IV. PURIFICATION OF PROTEASE

The dialysed protein fraction was further purified by DEAE cellulose ion exchange chromatography. The sample was loaded on to DEAE cellulose column previously equiliberated with 0.1M NaCl and eluted with linear gradient of 0-1.0M NaCl solution at a flow rate of 2mL/4 minutes. The fractions are collected and tested for protease activity, the protease active fraction obtained from ion exchange chromatography was passed through Sephadex G-100 column, pre-equiliberated with 0.1M phosphate buffer and eluted with the same buffer at a flow rate of 2mL/5 minutes. Protein and protease content of each fractions were determined by their absorbance at 280nm. Fractions with high protease activity were pooled. Protein concentrations of crude and purified fraction are determined by Lowry's method [8] using bovine serum albumin (BSA) as standard.

V. CASEINOLYTIC ACTIVITY

Caseinolytic activity was analysed according to [9] using casein as substrate. Casein (0.4mL) buffered with 0.5M Tris-HCl buffer of pH 8.5 was incubated with protein fractions in a final volume of 1mL for 1 hour at 37°C. The reaction was arrested by adding 1mL of 7% TCA and undigested casein was allowed to precipitate for 30 minutes. The reaction mixture was centrifuged at 2500rpm for 15 minutes. The supernatant (1mL) was treated with 2.5mL of 0.4M sodium carbonate and 0.5mL of FC reagent. The mixture was allowed to stand at room temperature for 30 minutes. The blue colour developed was measured at 660nm. Activity was expressed as units/hour. One unit of enzyme activity was defined as the amount of protease required to increase an absorbance of 0.001units per minute at 660nm [10].

VI. OPTIMIZATION OF INCUBATION CONDITIONS

Casein buffered with 0.5M Tris-HCl of pH 8.5 was incubated at different interval of time (0, 15, 30, 45, 60, 75 and 90 minutes) with purified protease enzyme at 37°C. Protease activity was estimated through the method as described by Chimbekujwo et al. with slight modifications [11]. Optimum incubation time was determined. Caseinolytic activity was analysed according to the method described above [9] using casein as substrate buffered with 0.5M Tris-HCl of different pH (7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5) and was incubated with purified protease for 1 hour at 37°C. Optimum pH for protease was determined. Activity of purified protease enzyme was determined by incubating casein buffered with 0.5M Tris HCl (pH 9.5) with protease for 1 hour at different temperature (4°C, 15°C, 25°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C) to determine optimum incubation temperature.

VII. EFFECT OF INHIBITORS AND ACTIVATORS ON PROTEASE ACTIVITY

The effect β-mercaptoethanol, iodine and EDTA were estimated by pre-incubating the purified protease with 1mM of β-mercaptoethanol, iodine and 5mM EDTA in different volumes at 40°C for 10 minutes [12]. The protease activity was performed by the method mentioned above. The residual activity was measured relative to control. The activity of control was taken as 100%.

VIII. EFFECT OF METAL IONS ON PROTEASE ACTIVITY

Effect of monovalent (Na^+ and K^+) and divalent (Ca^{2+}, Zn^{2+}, Cu^{2+} and Ba^{2+}) metal ions on enzyme activity at a concentration of 5mM was investigated by using casein as substrate [12]. The reaction mixture was pre-incubated with purified protease enzyme with metal ions for 10 minutesat 40°C and the proteolytic activity was determined. Enzyme activity without metal ions was considered as 100%.

IX. EFFECT OF SURFACTANTS AND OXIDIZING AGENTS ON PROTEASE ACTIVITY

The suitability of the purified protease as a detergent additive was determined by testing its stability to surfactants (Tween 20, Triton X-100 and SDS) and oxidizing agents (H_2O_2 and bleach). The reaction mixture was prepared by pre-incubating the purified enzyme with surfactant and oxidizing

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

agents at different concentrations for 10 minutes at 40°C and protease activity assay was carried out. Enzyme activity without any surfactant and oxidizing agent was considered as 100%.

X. EFFECT OF ORGANIC SOLVENTS ON PROTEASE ACTIVITY

The effect of various organic solvents such as propanol, DMSO, toluene, ethanol, butanol and hexane on purified protease activity was tested. The organic solvents were pre-incubated with the purified enzyme for 10 minutes at 40°C. Protease activity assay was carried out. Enzyme activity without any organic solvent was considered as 100%.

XI. STABILITY OF PROTEASE TOWARDS COMMERCIAL DETERGENTS

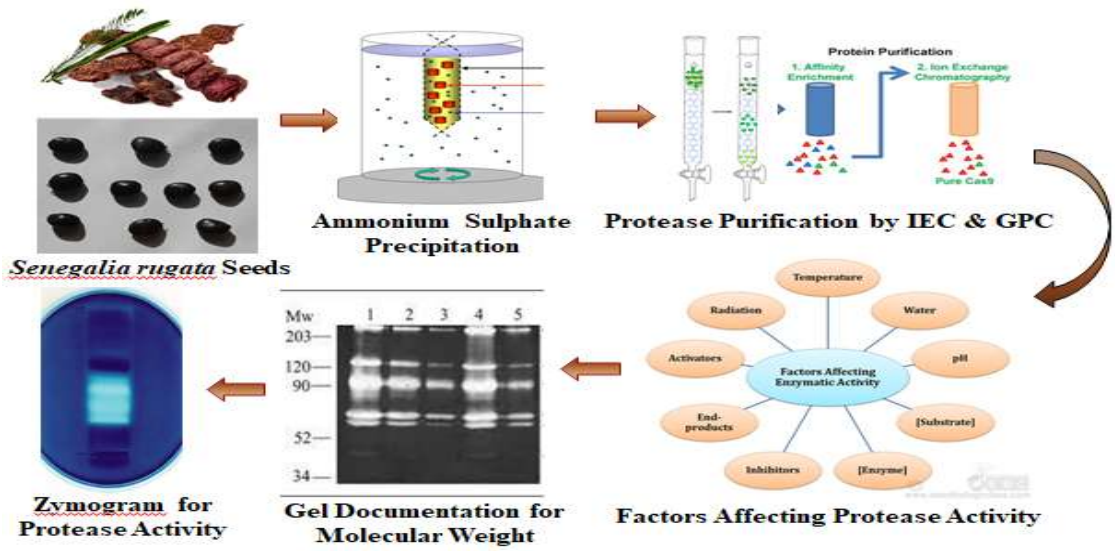
The stability of purified protease was checked against several commercial detergent powders such as Ariel, Tide, Surf, Wheel, Kite and Rin. Detergent solutions were prepared in 0.1M Tris-HCl buffer (pH 8.0) to get final concentration of 1, 10 and 100mg/mL of each detergent. Prior to assay detergent solutions were heated at 100°C for 1 hour to deactivate the endogenous proteases present in the commercial detergents. The reaction mixture was prepared by pre-incubating the purified enzyme with each detergent with different concentrations for 10 minutes at 40°C and protease activity was determined. The activity of enzyme without detergent was considered as 100%.

XII. DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE

Purified rotease enzyme was loaded on 15% SDS-PAGE with standard protein markers and electrophoresis was carried out. Gel was stained with 0.25M Coomassie Brilliant Blue R-250 and destained to visualize protein bands. The molecular weight of the protease is determined by gel documentation.

XIII. ZYMOGRAM SHOWING PROTEASE ACTIVITY

Gel was prepared similar to SDS-PAGE along with gelatin substrate copolymerized within the polyacrylamide gel atrix (7.5% gelatin gel). Sample with equal volume of 2x non reducing sample buffer (2.8mL distilled H_2O; 1mL 0.5M Tris-HCl; pH 6.8; 0.8mL glycerol; 3.2mL 10% (w/v) SDS; 0.2mL 0.2% bromophenol blue) was loaded. After electrophoresis, gel was washed for 1 hour with wash buffer (2.5% TritonX100 in 50mM tris, pH 7.4, 5mM $CaCl_2$, 1µM $ZnCl_2$). The gel was incubated overnight at 37°C with incubation buffer (50mM tris, pH 7.4, 5mM $CaCl_2$, 1µM $ZnCl_2$). The gel is then stained by 0.5% Coomassie G-250 in 30% ethanol with 10% acetic acid for 30 minutes and destained by 30% ethanol with 10% acetic acid for clear band.



GRAPHICAL REPRESENTATION OF THE WORK DONE

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

XIV. RESULTS AND DISCUSSION

The Senegalia rugata seed protein extracted at 80% ammonium sulphate saturation showed high protein content and protease activity, hence considered for further purification and characterization of protease. The fraction eluted with 0.2M NaCl in DEAE cellulose ion exchange chromatography (IEC) showed high protease activity with high protein concentration (Fig.1A). The fractions of this peak were pooled for further purification through gel permeation chromatography (GPC). Three distinct peaks were obtained in GPC (Fig.1B), third peak showed highest protease activity among the three, hence pooled and used for characterization. The protein concentration of crude sample, 80% ammonium sulphate precipitated sample, IEC fraction and GPC fraction were 9.9mg/mL, 8.1mg/mL, 3.0mg/mL and 1.95mg/mL respectively (Fig.2A). The protease activity of crude, 80% ammonium sulphate precipitated sample, IEC fraction and GPC fraction were found to be 1220U, 1140U, 620U and 440U respectively (Fig.2B).

Protease activity increased with increase in incubation time upto 60 minutes after which the activity remained constant (Fig.3A) suggesting 60 minutes as optimum incubation time. The protease activity was maximum at 40°C, below and beyond this temperature activity decreased (Fig.3B). Maximum protease activity was observed with Tris-HCl of pH 9.5 (Fig.3C).

Protease activity of purified sample was inhibited by EDTA and iodine while β-mercaptoethanol acted as activator. The 5mM EDTA, 1mM iodine and 1mM β-mercaptoethanol was added in different volumes (200µL-800µL) among which highest inhibition of 28% and 40% was observed at the addition of 800µL of 5mM EDTA and 1mM iodine which decreased with decreasing volume of EDTA and iodine (Fig.7A). The protease activity of the purified protease increased by 27% by the addition of 800µL of 1mM β-mercaptoethanol (Fig.4).

The effect of metal ions on protease activity is shown in Fig.5A. The enzyme retained 100% of its activity in the presence of 5mM NaCl and 5mM $BaCl_2$, 96% in the presence of 5mM KCl and $CuSO_4$. The activity increased by 5% in the presence of 5mM $CaCl_2$ and 5mM $ZnSO_4$. Enzyme activity of purified protease increased by 55, 1%, 12%, 17%, 6% and 17% with toluene, butanol, propanol, ethanol, DMSO and hexane respectively (Fig.5B).

The non-ionic surfactants increased enzyme activity of purified protease while 1% of anionic surfactant (SDS) decreased the activity by 6% (Fig.6A & 6B). The enzyme activity increased by 46%, 70% and 96% in presence of 1%, 5% and 10% Tween-20 respectively. The enzyme activity increased by 57%, 51% and 50% with 1%, 5% and 10% TritonX100 respectively. The purified protease enzyme retained its activity upto 75% in the presence of commercial detergents Kite, Ariel, Rin and wheel whereas enzyme showed 80% activity in the presence of Surf and Tide (Fig.7B).

Molecular weight of purified Senegalia rugata seed protease was found to be 33.94kDa which was determined by SDS-PAGE and Gel documentation (Fig.16 & 17). The protease activity of the purified sample was confirmed by the clear band/zone obtained against the blue background indicating the protease activity on gelatin substrate (Fig.18).

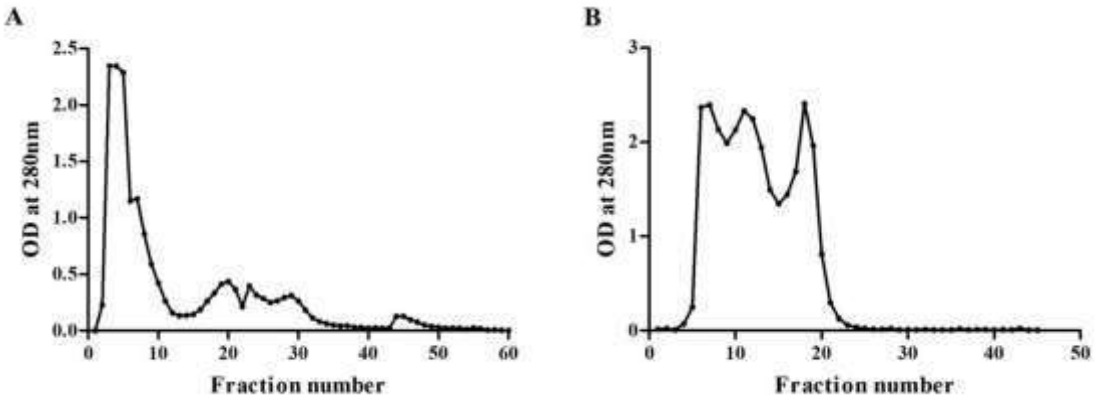


Figure 1. A. Ion Exchange Chromatographic separation of Senegalia rugata seed Protease
B. Gel Permeation Chromatographic separation of Acacia concinna seed Protease

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

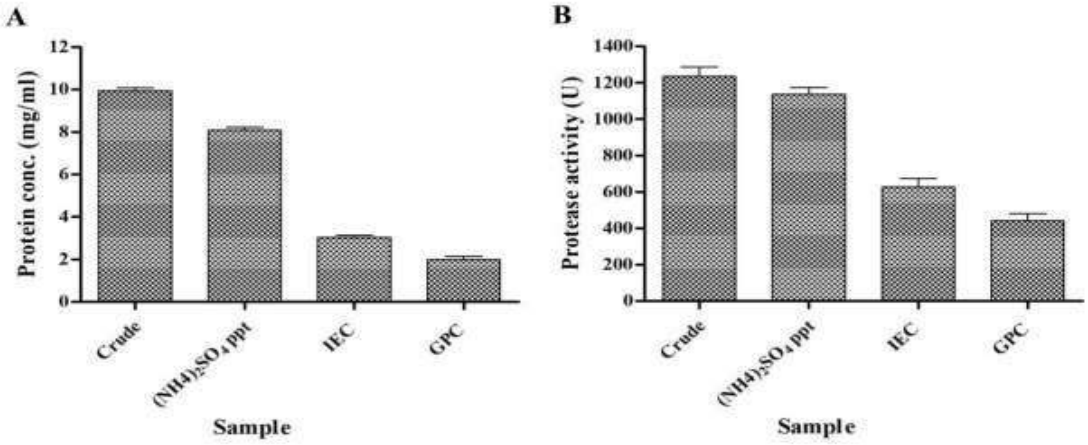


Figure 2. A. Graph showing protein concentration and B. Caseinolytic activity of Senegalia rugata seed protease at different stages of protease purification

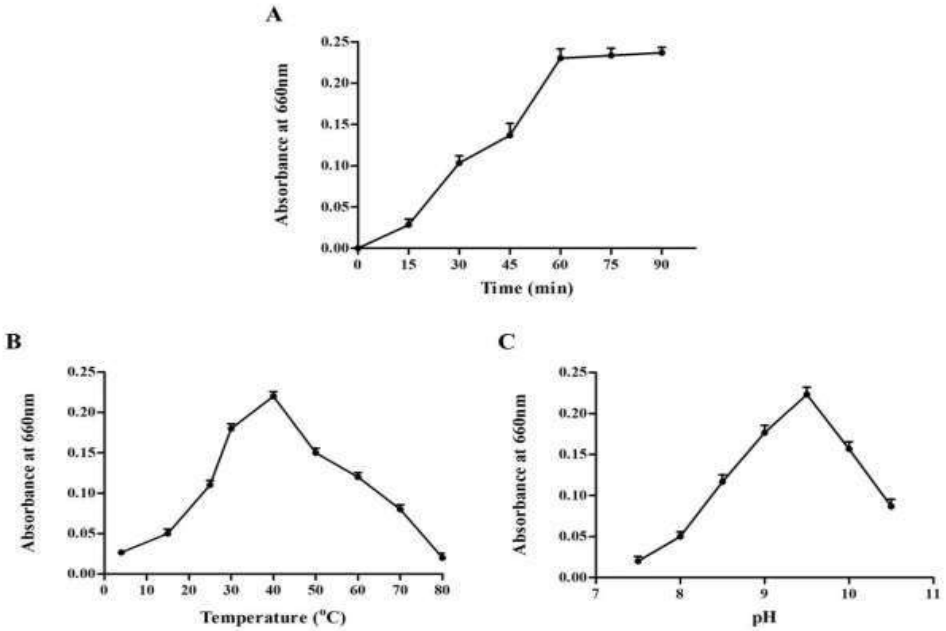


Figure 3. Optimization of Incubation A. Time, B. Temperature and C. pH of purified Senegalia rugata seed protease

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

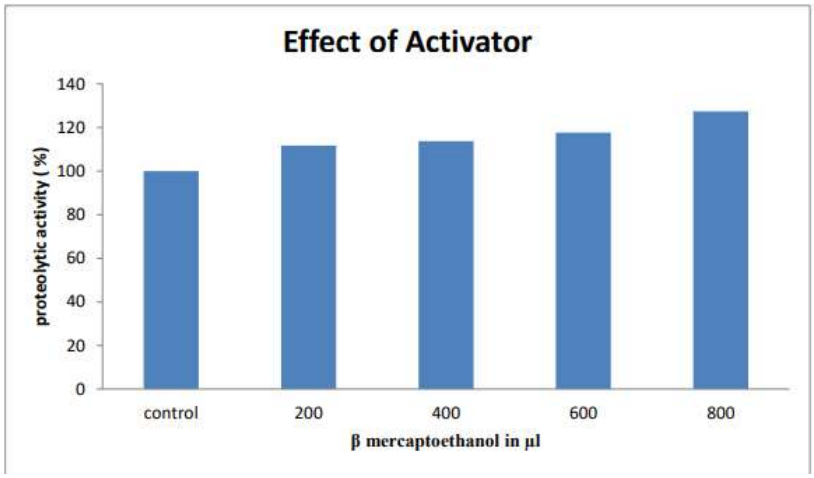


Figure 4. Effect of β-mercaptoethanol on protease activity of purified Senegalia rugata seed protease

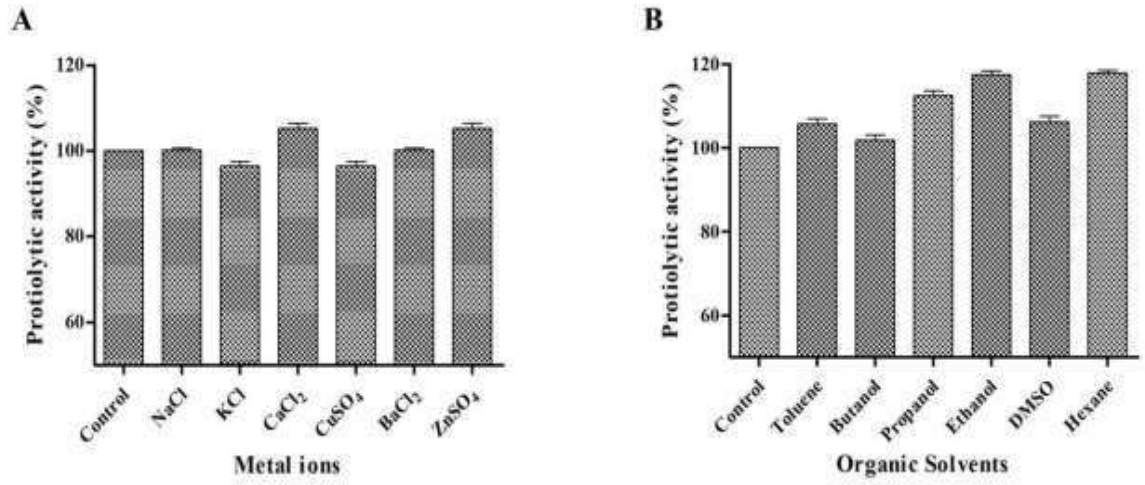


Figure 5. Effect of A. metal ions and B. organic solvents on protease activity of purified Senegalia rugata seed protease

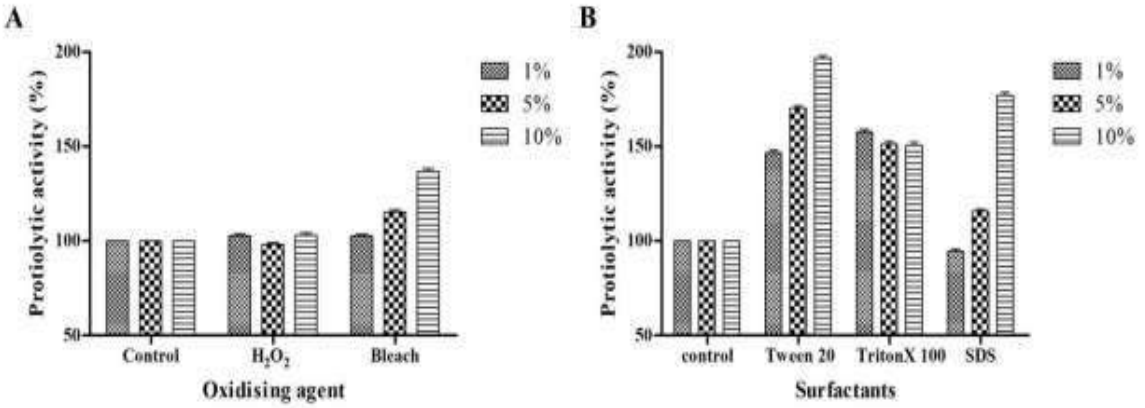


Figure 6. Effect of A. oxidizing agents and B. surfactants on protease activity of purified Senegalia rugata seed protease

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

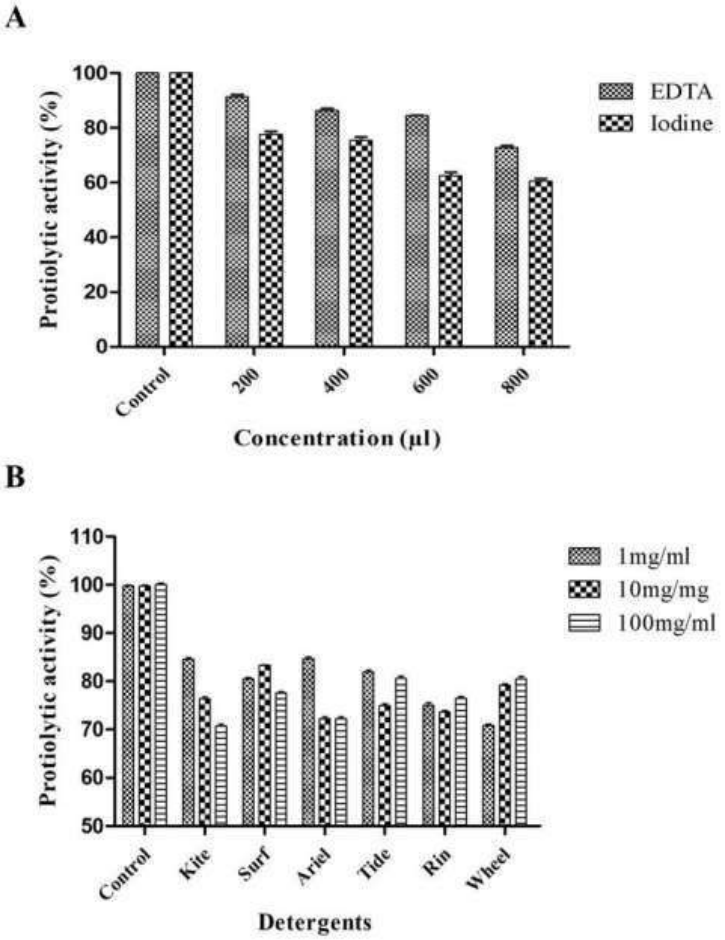
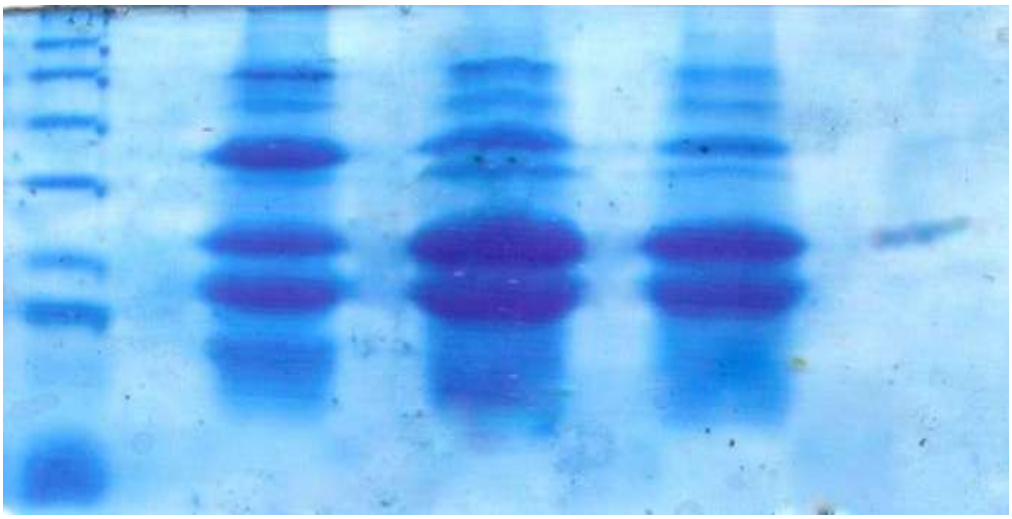


Figure 7. Effect of A. Inhibitors (EDTA & Iodine) and B. Detergents on protease activity of purified Senegalia rugata seed protease



International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

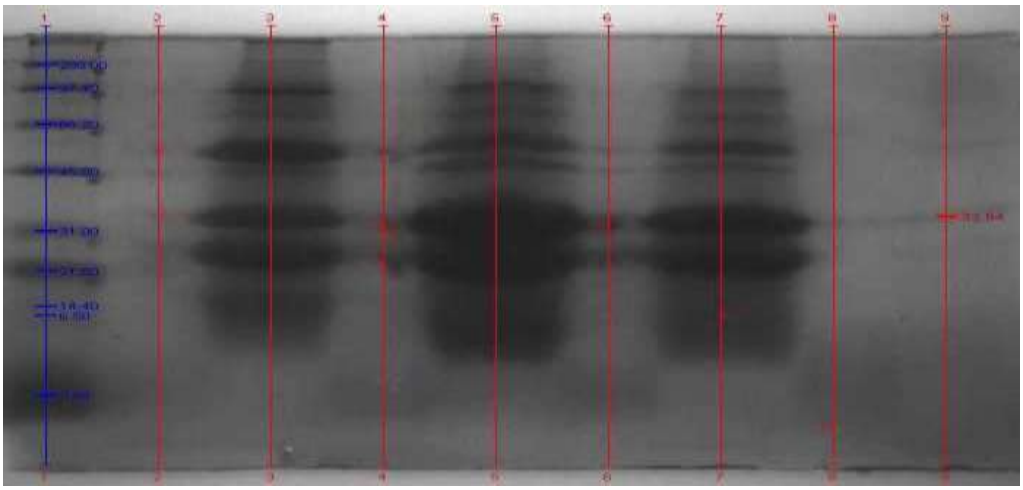


Figure 8. A. Stained SDS-PAGE gel B. Stained SDS-PAGE gel under gel documentation (lane 1- prestained protein marker, lane 2- crude protease extract, lane 3- 80% ASP, lane 4- IEC purified fraction and lane 5- GPC purified protease fraction)

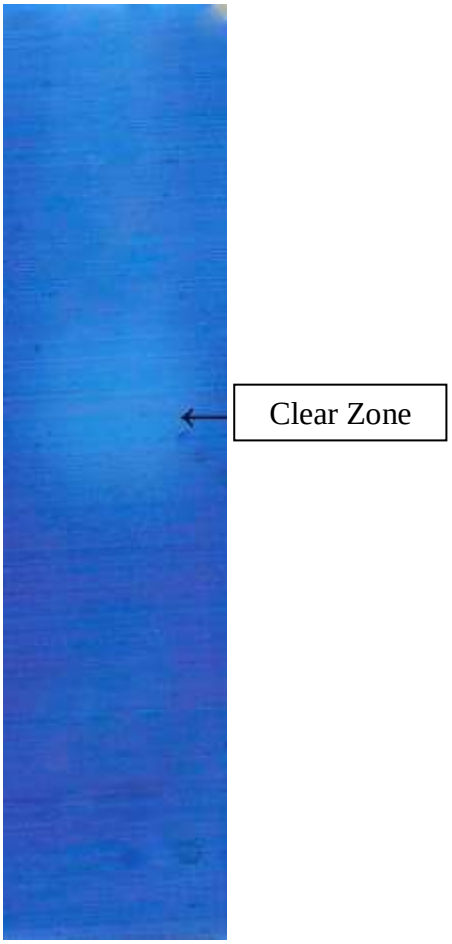


Figure 9. Zymogram of purified Senegalia rugata seed protease showing clear zone

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1146-1154 www.ijprajournal.com ISSN: 2249-7781

XV. CONCLUSION

Looking back on this project, the overall outcome of results showed that the purified protease isolated from Senegalia rugata seeds have very high potential to be used as an alternate for commercially available proteases. As very few work has been done on proteins of Senegalia rugata seed, this work can bring in a new insight into the applications of these seeds. These natural proteases can be used as tenderizers for their ability to breakdown protein in meat. This protease can be of great importance in food and leather industries and also can be exploited in various bio-formulations.

REFERENCES

[1] Sombatsiri, P; and Chairote, G., 2005, "Volatile Compounds from 'Som Poy' (Acacia concinna DC.)" 5:189-194; https://doi.org/10.17660/ACTAHORTIC.2005.679.23

[2] Win, T.T; Thandar, S; and Nu, T.T., 2020, "Investigation of the Physicochemical Properties and Antimicrobial Activity of the Crude Extracts of Acacia concinna DC. (Kin-mun-gyin) Leaves," University Journal of Creativity and Innovative Research 2020; 01:350-353

[3] Zhuoyue, Z; Ruangaram, W; and Kato, E., 2021, "Saponins are responsible for the anti-obesogenic activity of Acacia concinna." Journal of Natural Medicines 2021; https://doi.org/10.1007/s11418-021-01530-0

[4] Boonmee, S; and Kato-Noguchi, H., 2017, "ALLELOPATHIC ACTIVITY OF ACACIA CONCINNA POD EXTRACTS," Emirates Journal of Food and Agriculture 2017, 29-4:250-5, doi:https://doi.org/10.9755/ejfa.2016-07-964

[5] Chew, L. Y; Toh, G. T; and Ismail, A., 2019, "Chapter 15 - Application of Proteases for the Production of Bioactive Peptides," Enzymes in Food Biotechnology 2019, pp. 247-261, https://doi.org/10.1016/B978-0-12-813280-7.00015-3.

[6] Jabalia, N; Mishra, P. C; and Chaudhary, N., 2014, " Applications, Challenges and Future Prospects of Proteases: An overview," Journal of Agroecology and Natural Resource Management 2014, 1:pp 179-183

[7] Naveed, M; Nadeem, F; Mehmood, T; Bilal, M; Anwar, Z; and Amjad, F., 2021, "Protease-A Versatile and Ecofriendly Biocatalyst with Multi-Industrial Applications: An Updated Review," Catalysis Letter 2021, 151(2): 307-323

[8] Sarkar, S; Mondal, M; Ghosh, P; Saha, M; and Chatterjee, S., 2020, "Quantification of Total Protein Content from Some Traditionally Used Edible Plant Leaves: A comparative Study," Journal of Medicinal Plants Studies 2020, 8(4): 166-170

[9] Hou, J; Han, D; Zhou, Y; Li, Y; and Cui, H. L., 2020, "Identification and Characterization of the Gene Encoding an Extracellular Protease from Haloarchaeon Halococcus salifodinae," Microbiological Research 2020, SAE Paper No. 126468, https://doi.org/10.1016/j.micres.2020.126468

[10] Tito, F. R; Pepe, A; Tonon, C. V; Daleo, G. R; and Guevara, M. G., 2020, "Determination and Characterisation of Milk-Clotting Activity of Two Solanum tuberosum Aspartic Proteases (StAPs)," International Dairy Journal 2020, SAE Paper No. 104645, https://doi.org/10.1016/j.idairyj.2020.104645

[11] Chimbekujwo, K. I; Jaafaru, M. I; and Adeyemo, O. M., 2020, "Purification, Characterization and Optimization Conditions of Protease Produced by Aspergillus brasiliensis Strain BCW2," Scientific African, 2020, 8:e00398, https://doi.org/10.1016/j.sciaf.2020.e00398

[12] Badoei-dalfard, A; Khankari, S; and Karami, Z., 2020, " One-pot Synthesis and Biochemical Characterization of Protease Metal Organic Framework (protease@MOF) and its Application on the Hydrolysis of Fish Protein-Waste," Colloids and Surfaces B: Biointerfaces 2020, 196:111318, https://doi.org/10.1016/j.colsurfb.2020.111318