Asian Journal of Biochemistry, Genetics and Molecular Biology

8(1): 30-36, 2021; Article no.AJBGMB.66390
ISSN: 2582-3698

Extraction, Purification and Characterisation of Plant Enzyme (Calotropain) from the Unripe Pods and Bark of Sodom Apple (*Calotropis procera* (AIT))

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2021/v8i130186
Editor(s):
(1) Prof. Gul Ozcan, University of Istanbul, Turkey.
Reviewers:
(1) Lee Fu Haw, Universiti Putra Malaysia, Malaysia.
(2) Gengjun Chen, Kansas State University, USA.
(3) Ranjana Das, Jadavpur University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/66390

Original Research Article

Received 06 February 2021
Accepted 16 April 2021
Published 11 June 2021

ABSTRACT

Aims: This study was conducted to determine the potential Extraction, Purification and Characterisation of Calotropain from the unripe pods and stem bark of *Calotropis procera* for establishing its applications in food industry.
Methodology: Crude extracts were purified by ammonium sulphate precipitation and two-step salt precipitation. A One-way ANOVA analysis was used to determine the significant difference between the two methods at 5% significance level.
Results: The results obtained from the study inferred that proteolytic activity was present in both plant part extracts. The protein concentrations of the sample crude extracts were 0.86 mg/ml and 0.92 mg/ml for stem bark and pods respectively. The protein concentrations after ammonium sulphate precipitation were 0.57 mg/ml and 0.69 mg/ml for stem bark and pods respectively. The protein concentrations after two-step salt precipitation were 0.51 mg/ml and 0.61 mg/ml for stem bark and pods respectively. Proteolytic activities of the crude enzyme extracts were 0.038

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µmol/min/ml for stem bark and 0.02 µmol/min/ml for pods. Higher values of 0.075 µmol/min/ml and 0.05 µmol/min/ml were calculated for the enzyme extracts for stem bark and unripe pods respectively after ammonium sulphate precipitation and after two-step salt precipitation. The enzyme extracts for stem bark and pods respectively recorded proteolytic activities of 0.099 µmol/min/ml and 0.07 µmol/min/ml.

Conclusion: The outcome of this study showed how the proteolytic activities of the plant parts could be enhanced for protein hydrolytic processes in the production of food.

Keywords: Calotropis procera; calotropain; proteolytic activity; ammonium sulphate precipitation; two-step salt precipitation.

1. INTRODUCTION

Enzymes are tools that have been used in medicine and industries for decades, especially proteolytic enzymes. Proteolytic enzymes also called Proteases are collective enzymes utilized industrially to breakdown protein molecules into simpler molecules [1]. Though proteases play significant role in humans and animals as protein digesters and invading microbes destroyers, they are also exploited in for *in vitro* studies and industrial processes in food industry [2,3]. Although enzymes are essential for food production in Ghana and Africa, they are expensive since they are imported, which in turn makes their food products costly [4]. Interestingly, proteolytic enzymes from plant sources are gradually becoming part of our everyday life and play major roles in food processes and industrial processes. Hence the proteolytic enzymes have been detected in some plants locally and international through several research. The well-known plant proteolytic enzymes of commercial value are papain from *Carica papaya*, ficin from *Ficus* species and bromelin from pineapple [5]. These have been called biological scalpels because of their specific action on chronic and acute suppurating lesions and in debridement following burns without causing any deleterious action on healthy tissues. One of such plants that has been given much attention due to its potential of harbouring an important proteolytic enzyme is Sodom apple (*Calotropis procera*). Calotropain extracted from *Calotropis procera* can completely substitute animal and microbial rennet used for commercial cheese production [6].

Calotropis procera (Sodom Apple) is a member of *Asclepiadaceae* family that is widely used in Ghana. It is commonly called '*mpatu asa'* in Akan in Ghana and '*bomubomu*' in South Western Nigeria. *Calotropis procera* have been reported to contain Calotropain, a protease isolated from the root/root bark, latex, stem and fruits of the plant. And due to the presence of Calotropain, the extract of the plant is utilised in the traditional production of soft unripened cheese variety known as '*Wara*' cheese in Nigeria. In Ghana also, the latex from the leaves and stems of *Calotropis procera* is used to prepare '*warankasi'* a local cheese; the extract however causes bitterness in cheese due to its high proteolytic activity [7,8]. Hence, *Calotropis procera* have been indicated to completely substitute animal and microbial rennet used for commercial cheese production due to high level of protease [6].

However, since the various parts of the plant contain Calotropain, other part like the unripe pods and stem bark of the plant have not vigorously been reported in literature. This could offer an alternative source of Calotropain that would be highly efficient as compared to the other parts. Hence the purpose of this study which is to extract Calotropain from the pods and stem bark of *Calotropis procera* and determine the protein concentration as well as the proteolytic activity. Moreover, compare the effectiveness of two purification processes; ammonium sulfate salt precipitation and two step salt precipitation.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant Sodom apple was obtained from a farm in Ayeduase, a community located near Kwame Nkrumah University of Science and Technology (KNUST), Kumasi-Ghana. The unripe pods were plugged from the plant whiles the stem bark was obtained by up-rooting the plant. The unripe pods and stem bark were washed with distilled water and cut into pieces. The collected samples were placed in Ziploc bags on ice and transported to the Department of Biochemistry and Biotechnology laboratory in KNUST for further analysis.

2.2 Crude Enzyme Extract Preparation

100 g of each part (unripe pods and stem bark) were homogenised in a kitchen blender (Binatone, BLG) containing 500 ml of 0.05M sodium acetate buffer with a pH of 5.5. The blended mixture of each sample was sieved through a clean cheese cloth to remove suspended coarse materials into a sterile beaker. 230 ml of each of the filtrate was separately divided into four parts in a 50 ml centrifuge tubes and the remainder (30 ml) stored in refrigerator at 4^{o}C. The filtrate was then centrifuged at 4000 rpm for 15 minutes at 4^{o}C. Afterward, the supernatant (crude extract) was poured into a sterile beaker and stored in the refrigerator at 4^{o}C for further analysis [9].

2.3 Purification of Crude Extracts

2.3.1 Ammonium sulfate salt precipitation

A volume of 10 ml each of the crude enzyme extracts was pipetted separately into twelve sterile beakers; six each for crude enzyme extract from unripe pods and stem bark respectively. Afterwards, the percentage saturation which is the volume of saturated ammonium sulfate required to give the desired ammonium sulfate concentration in the crude enzyme extract was calculated at 70%.The purification factor corresponding to raising the ammonium sulfate from 0 to 70% was 2.33. The initial 10ml of crude enzyme extract was then multiplied by the purification factor (2.333) to give the volume of the saturated ammonium sulfate solution (23.3 ml). The 23.3 ml saturated ammonium sulfate was added to 10 ml of the crude enzyme extract with gentle stirring followed by incubation at 4^{o}C for 30 min for precipitation to take place. The precipitate was centrifuged at 4000 rpm for 15 minutes at 4°C. Both, the supernatant and pellet were collected after each centrifugation and the pellet was resuspended in sodium acetate buffer (pH 5.5). The alliquots of precipitated fractions were analyzed for its protein concentration and enzyme activity [10].

2.3.2 Two step salt precipitation

A modification of the two-step precipitation method proposed by Baines and Brocklehurst [11] was employed for the purification of *calotropain* from the crude enzyme extracts. The first precipitation was done using ammonium sulphate while sodium chloride was used for the second precipitation. Crude enzyme extracts prepared from unripe pods and stem bark were pipetted separately into different sterile beakers and mixed with 40 mM cysteine and the pH of the suspension was adjusted to 5.6 by using 6 M HCL and then stirred slowly for 15min at 4^{o}C. The resulting mixture was filtered through a cotton wool and its pH was adjusted to 9.0 using 6 M NaOH. The filtrate was centrifuged to remove insoluble materials and this was carried out at 9000 x g for 30 min at 4^{o}C. The supernatants were precipitated with ammonium sulphate at 45% saturation. At this step, the percentage saturation which is the volume of saturated ammonium sulfate required to give the desired ammonium sulfate concentration in the crude enzyme extract was calculated at 45%. 10ml of the supernatant was multiplied by the purification factor (0.82) to give the volume of the saturated ammonium sulfate solution (8.2mm). The 8.2 ml saturated ammonium sulfate was added to 10 ml of the crude enzyme extract with gentle stirring. The salt-enriched solutions were incubated with at 4^{o}C for 30 min. The precipitate, after being collected by centrifugation as above (9000 x g for 30 min at 4^{o}C) was dissolved in 20 mM Cysteine. The solution was kept at a temperature of 4^{o}C before sodium chloride (10% w/v) was added and the resulting mixture was slowly stirred followed by centrifugation to separate the enzyme. Water was used to dissolve the enzyme and stored at 4^{o}C [11].

The protein concentrations and proteolytic activity in subsequent analysis was determined for stem bark and unripe pods of crude extracts, purified extracts by ammonium sulphate and purified extracts by two-step salt precipitation.

2.4 Characterisation of Calotropain

2.4.1 Determination of protein concentration (Bradford assay)

The concentration of proteins was determined following the method of Bradford [12] using bovine serum albumin (BSA) as standard. The bovine serum albumin solution was prepared by the dissolution of 0.1 g bovine serum albumin in 50 ml of distilled water. After which it was topped up to the 100ml mark. In details, volumes (i.e., 10 µl, 20 µl. 40 µl, 60 µl, 80 µl, 100 µl and blank) of bovine serum albumin solution were pipetted into sterile centrifuge tubes and was adjusted to 100 ul with distilled water. A volume of 100 µl of each of crude extracts and purified extracts was

pipetted into the tube followed by 5 ml of the Bradford reagent. The solution was mixed by inversion and absorbance measured at 595 nm after 2min and before 1 hour. The weight of the protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in the unknown samples.

2.4.2 Determination of enzyme activity (Casein hydrolysis method)

The extracts were assayed for protease activity by a casein degradation method using the method of Ladd and Butler [13]. To prepare 1% casein solution, 0.5 g casein from bovine milk was dissolved in 50 m1 of distilled water. The casein solution was heated gradually to 80^0C for ten (10) minutes at a constant stirring speed on a Stuart stirrer hotplate (Aggrey-Korsah, 2011).The casein solution (1%) was prepared in 0.05 M citrate phosphate buffer pH 7.5 and heat treated at 100°C for 15 minutes in water bath, cooled and used as substrate. The substrate (1ml) was thoroughly mixed with the enzyme extract (1.0 ml), however the control consists of 1.0 ml each of the substrate and citrate buffer. This was incubated for 1 h at 35°C and the reaction was terminated by adding 3.0 ml of cold (2°C) 10% Trichloroacetic Acid (TCA). 10% TCA was prepared by dissolving 5 g (w/v) of trichloroacetic acid in distilled water to the 50 ml mark in a 50 ml falcon tube (Aggrey-Korsah, 2011). The tubes were allowed to stand for a minimum of 1 h at 2°C in a refrigerator (Midea, HD-113F(N)) to allow the undigested protein to precipitate. Thereafter, the mixtures were centrifuged at 3000 rpm for 30 minutes under room temperature and the absorbance of the clear supernatant was measured at 275 nm, the control was used as blank. One unit of proteolytic activity was defined as the amount of enzyme that liberated 1µmol of tyrosine per minute under the standard assay conditions. The absorbance of different concentration (20-220 µl) of tyrosine solution (1.1 mM) was used to generate a standard tyrosine curve. A Bio base spectrophotometer (double beam, BK-D560) was used to measure the absorbance of the different tyrosine solution and blank at 275 nm [13].

2.5 Statistical Analysis

The results from the study were determined from the means of triplicates values and expressed as mean ±SD. Collected data were analysed using SPSS statistical software package (version 25). A One-way ANOVA was used to analyse the significant differences of mean values of the proteolytic activity and protein concentration of each sample and graphical representation of data was done using Microsoft Office Excel 2019 package. All test procedures were made at 5% significant level.

3. RESULTS AND DISCUSSION

The protein concentrations of the various samples for stem bark and unripe pods (i.e. crude extracts, purified extracts by ammonium sulphate and purified extracts by two-step salt precipitation) are presented in Table 1. The presence of proteins from the whole latex of *Calotropis procera* from a previous study is in line with the results of our study which indicates that, all parts of Sodom plant are rich sources of protein [14]. From the results of the study (Table 1), there was a significant difference (P <0.05) in terms of protein concentration between the crude and purified enzyme extract in the two plant parts. Unripe pods showed higher protein concentration as compared to the stem bark. In terms of the plant part, crude enzyme extract from the stem bark showed the highest protein concentration (0.86 ± 0.02), followed by ammonium sulfate purified extract (0.57±0.03) and two step salt purified extract (0.51±0.02).

Similarly, in the unripe pods, the crude enzyme extracted recorded the highest protein concentration (0.92 ± 0.03), followed by ammonium salt purified extract (0.69±0.03) and two step purification extract (0.61±0.03). Also, there was a significant difference (P<0.05) between the methods of purification. Respective samples purified by ammonium sulphate precipitation showed higher protein concentration (0.057±0.03 mg/ml for stem bark and 0.69±0.03 mg/ml for unripe pods) than the samples purified by two-step salt precipitation (0.051±0.02 mg/ml for stem bark and 0.61±0.03 mg/ml for unripe pods).

Furthermore, there was an observed significant difference (P<0.05) in the protein concentrations of the crude extracts and other crude extracts purified by ammonium sulphate precipitation and two-step salt precipitation of both stem bark and unripe pods of the plant. This suggest that, the concentration of protein is always dependent on the purification step as a significant decrease in the protein concentration was observed after crude enzymes extract were purified. The reason being that, the purification processes ensure the retrieval of wanted proteins from a solution of proteins hence the observed decrease in the

protein concentrations [15]. In an experiment conducted by Vernet et al. [16], after an ultra-purification method was employed, there was a decrease in protein concentration from 165 mg/ml to 64 mg/ml. This might be due to the purification step used as different purification steps may result in several degrees of purification. However, between the two purification methods in this present study, ammonium salt purification had a higher protein concentration as compared to two step purification method for both plant parts and was also significantly different. This is obvious because, ammonium sulfate precipitation is a useful initial step for protein purification due to its quickness and bulk precipitation. This method results in the precipitation of other substances along with the protein [17], hence justifying the results of our study. Similarly, Aggrey-Korsah, [18] used different precipitation methods in the purification of papain extracts which influenced the degree of purification of the enzyme. In her study, two phase aqueous extraction was employed in purification of papain from latex. This produced an enzyme with a higher activity compared to the use of ammonium sulphate precipitation. Therefore, two step salt precipitation which employed sodium chloride for subsequent purification after ammonium sulfate resulted in a lower protein concentration with no contaminants and increased proteolytic activity. In contrast, a study that compared aqueous two phase and two step salt precipitation in the purification of papain noticed a protein increase in each step. However, two salt precipitation was not efficient in the purification of papain as compared to the aqueous two phase since the proteins were contaminated with other proteins [19].

In Table 1, the proteolytic activities of the various samples for stem bark and unripe pods (i.e., crude extracts, purified extracts by ammonium sulphate and purified extracts by two-step salt precipitation) are presented. Both of the plant parts showed proteolytic activity showing the wide distribution of calotropain in various plants parts. There was a significant difference (P<0.05) between the proteolytic activities of the crude extracts and the activities after purification by ammonium sulphate precipitation and two-step salt precipitation for both the plant parts. Comparing proteolytic activity for stem bark and unripe pods, it was observed that the various samples for stem bark showed higher activity (0.075 ± 0.002 and 0.099 ± 0.001 µmol/min/ml) than the samples for unripe pods (0.05 ± 0.003 and 0.07 ± 0.002 µmol/min/ml). This result, with stem bark having a higher proteolytic activity, corroborate with earlier reports that the *Calotropis procera* contained calotropain and some other cardiac glycosides and several proteinases [20], as supported by the findings of Atal & Sethi [21], that the proteolytic activity of calotropain from *Calotropis procera* is greater than that of papain, ficin and bromelin and also free from toxins. This agrees with Awoyinka & Shokunbi, [22] who indicated a higher proteolytic activity in *Calotropis procera* than *Carica papaya* and *Musa* paradisaca.

The proteolytic activities of the plant parts revealed the ability of the plant parts to hydrolyse casein suggesting that proteolytic enzyme is distributed in both parts of the plant. This validates with that reported by Oseni & Ekperigin [9], who screened various parts of *Calotropis procera* for protease activity and found that the latex of the plant exhibits the highest activity, followed by the root, stem, leaf and pods. However, the recorded proteolytic activity in our study was also higher in both plant parts as compared to that which was indicated by Oseni & Ekperigin, [9], (0.052) and Awoyinka & Shokunbi, [22] (0.0563) in the latex of the leaves.

Table 1. Summary of protein concentration and proteolytic activity of various purification steps of plant parts (mean ± SD, n = 3)

Plant Part	Purification step	Protein concentration (mg/ml)	Proteolytic activity (mg/ml)
Stem bark	Crude extract	0.86 ± 0.02^{a}	0.038 ± 0.002^{a}
	Ammonium salt precipitation	0.57 ± 0.03^{b}	0.075 ± 0.002^{b}
	Two step salt precipitation	0.51 ± 0.02^{c}	0.099 ± 0.001^{c}
Unripe pods	Crude extract	0.92 ± 0.03^{a}	0.02 ± 0.002^{a}
	Ammonium salt precipitation	0.69 ± 0.03^{b}	0.05 ± 0.003^{b}
	Two step salt Precipitation	0.61 ± 0.03^{c}	0.07 ± 0.002^{c}

**Different superscript (a, b, c) indicates significant differences at P < 0.05.*
**Mean ± S.D = Mean ± Standard deviation of triplicates*

Further it was observed that, there was a significant increase in the activities after application of the purification processes for both of the plant parts. Two step salt precipitation proved to be the most effective purification method. This might be because the solubility of proteins varies according to the ionic strength of the solutions, two-step salt precipitation provides an array of system where different proteins could be precipitated out easily and effective in a process called “salting-out” [17].

4. CONCLUSION

This current study conducted revealed high protein concentration and proteolytic activity of Calotropain from the unripe pods and stem bark of *Calotropis procera*. The study revealed that the newly isolated enzyme Calotropain is superior in its proteolytic activity to some of the commercially available proteolytic enzymes of plant origin. Crude extracts from different parts of *Calotropis procera* contain proteolytic enzyme activity that can be applied in proteolytic assays. More so, two step salt precipitation have been showed to be every efficient in the purification of the enzyme as compared to ammonium sulfate precipitation. Further studies on characterisation and properties of the purified enzyme and possibly the mechanism of milk coagulation from this plant would be interesting and this should be reported to fully ascertain its food applications.

ACKNOWLEDGEMENTS

I would like to thank the Almighty God who endowed me with grace, strength and enthusiasm to pursue and complete this study. My sincere appreciation goes to my parents and siblings for their undying love, support and motivation. To my supervisor, Prof. Hilary Domakyaara Zakpaa, I say God bless you for your criticisms and suggestions which made this work a success. To all the Laboratory Technicians of the Biochemistry Department especially Mr. Agyemang Duah, I say thank you, for your technical advice and support. I am also indebted to all lecturers of the Department of Biochemistry and Biotechnology for their inputs at seminar presentations, encouragement and role played in seeing this work through. God bless you all.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/66390