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Research Paper

Purification of a Protease from the Seeds of *Mangifera indica* cv. Bangalora

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A B S T R A C T

A protease was purified from the seeds of *Mangifera indica* cv. Bangalora using ammonium sulphate fractionation, ion exchange and gel filtration chromatography. The protease was fractionated at 80% ammonium sulphate saturation. The chromatographic purification steps resulted in a 4.7-fold purification of the enzyme with a yield of 75%. After the chromatographic purification steps, the purity and molecular weight of the protease was checked by SDS PAGE. The protease was visualized as a single band on the PAGE gel showing that it was purified to homogeneity and the molecular weight was determined to be approximately 20 kDa. The protease from the seed of *Mangifera indica* cv. Bangalora is therefore a low molecular weight protease. The presence of the approximately 20 kDa protease was further confirmed by the presence of clear zone of casein hydrolysis in the area of the purified protease band after casein zymography. Since mango seeds are generally thrown as waste, an attempt was made in this study to isolate protease from the seeds to investigate if they could be used for industrial processes or therapeutic applications.

***Key words*:** Protease, Purification, Seeds, Low molecular weight, Chromatography

Many plants possess proteases with good activity and unique properties. This makes them very useful for industrial purposes such as food processing, textile processing and detergent production as well as in the medical field as therapeutic agents for a variety of disorders such as digestive and inflammatory disorders, cancer, etc. [1-2]. Several researchers have isolated, purified and characterized plant proteases of industrial and medical importance. Such proteases have been isolated from many different parts of the plant such as the roots, stem, leaves, fruit, fruit peel and fruit seed [3]. In most cases, researchers have used ammonium sulphate fractionation followed by ion exchange and gel filtration chromatography to purify the desired protease [4-6]. Some have reported high molecular weight proteases and some have reported low molecular weight plant proteases. In many cases more than one protease each having its own substrate specificity and affinity have also been isolated. Zymography has also been used by many of the researchers as a confirmation of the activity of the proteases purified through chromatographic separation. Zymography is easy to perform involving the incorporation of the substrate, for which the enzyme shows good affinity, into the polyacrylamide gel and then electrophoresing the enzyme sample. The formation of clear zone of substrate hydrolysis indicates the presences of the enzyme at that zone on the gel [7-8]. Many plant materials, especially the seeds, are often thrown as waste after the use of the desired part of the plant as food. Use of these waste plant materials for isolating enzymes would be a cost-effective method of enzyme production for industry. Moreover, there is an increasing demand for plant-derived proteases in the food industry over bacterial enzymes [9]. Investigations of enzymes, especially protease enzymes in such plant material would aid the discovery of novel enzymes with unique properties.

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MATERIALS AND METHODS

Preparation of the crude seed extract

For preparing the crude extract of the mango seed, 25 g of fresh seed sample was homogenized in 100 ml of 0.05 M Tris HCl buffer (pH 7.5). The homogenate was centrifuged at 8000 rpm for 15 min and the supernatant was collected.

Partial purification of the protease

The protease was partially purified from the crude extract by ammonium sulphate fractionation at 80% ammonium sulphate saturation. The white precipitate obtained was collected by centrifugation at 8000 rpm for 15 min, dissolved in 10 ml of 0.05 M Tris HCl buffer (pH 7.5) and dialyzed overnight against the same buffer.

Purification of the protease by ion exchange chromatography

The dialyzed protease sample was purified first by ion-exchange chromatography. A DEAE-Cellulose column which was equilibrated with 0.1 M Tris-HCl buffer, pH 7.2 (equilibrating buffer) was used. The column was washed with 100 ml of the equilibrating buffer to remove unbound proteins. The bound proteins were then eluted using 500 ml of a salt gradient ranging between 0.1 – 0.5 M sodium chloride in the equilibrating buffer. The flow rate was maintained at 1ml/min. The protease activity of the eluted fractions (5 ml fractions)

were determined by the modified method of Tsuchida *et al*. 1986 and the fractions showing protease activity were pooled.

Purification of the protease by gel filtration chromatography

The pooled sample obtained after ion exchange chromatography were subjected to gel filtration chromatography. The sample was loaded onto a SephadexG-100 column and eluted with 0.1 M Tris-HCl buffer, pH 7.2 with a flow rate of 1ml/min. The protease activity of the eluted fractions (2 ml fractions) were determined by the modified method of [10] and the fractions showing protease activity were pooled.

Determination of the protein concentrations of the protease samples at various stages of purification

The protein concentration of the crude and dialyzed samples and the protease samples after ion exchange and gel filtration chromatography was determined by the Bradford method using bovine serum albumin (BSA) as the standard [11.

Determination of the protease activities of the protease samples at various stages of purification

The activity of the protease was determined according to the modified procedure of [10]. 1% casein in Tris HCl buffer (pH 7.5) was used as the substrate for the protease. 0.5 ml of the casein solution was incubated with 0.5 ml enzyme extract at 37°C for 10 min after which the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The reaction mixture was subjected to centrifugation at 8000 rpm for 15 min and the supernatant was mixed with 5 ml of 0.44 M sodium carbonate and 1 ml of two-fold diluted Folin Ciocalteau reagent. After 30 min of incubation at room temperature, the blue colour that developed was read at 660 nm against a reagent blank. Tyrosine was used as the reference standard. One unit (U) of protease activity is defined as the amount of enzyme required to catalyze the formation of 1 µmol of tyrosine per minute. The enzyme activity and specific activity were determined. The enzyme activity was calculated as follows:

$$\text{Enzyme activity} = \frac{\text{µmols of Tyrosine released} \times \text{Reaction volume}}{\text{Sample volume} \times \text{Reaction time} \times \text{Volume assayed}}$$

Determination of the fold purification and % yield

The fold purification and percent (%) yield at various stages of purification were calculated as follows:

$$\text{Fold purification} = \frac{\text{Specific activity in the fraction}}{\text{Specific activity in the crude sample}}$$

$$\text{\% yield} = \frac{\text{Enzyme activity in the fraction}}{\text{Enzyme activity in the crude}} \times 100$$

Determination of the purity and molecular weight of the purified protease by SDS PAGE

The crude, ammonium sulphate fractionated sample, dialyzed sample, the protease sample after ion exchange chromatographic separation and the protease sample after gel filtration chromatographic separation were subjected to SDS polyacrylamide gel electrophoresis (PAGE) according to the method of [12]. A 12% separating gel was used for separation of proteins and the protein bands were stained with Coomassie Brilliant blue R -250.

Native PAGE of the purified protease sample

The crude, ammonium sulphate fractionated sample, dialyzed sample, the protease sample after ion exchange chromatographic separation and the protease sample after gel filtration chromatographic separation were subjected to Native PAGE (without SDS) according to the method of [12]. The samples were not heated before loading. A 12% separating gel was used for separation of proteins and the protein bands were stained with Coomassie Brilliant blue R -250.

Casein zymography

Casein zymography was performed according to the method of [13] with slight modifications by adding 0.2% w/v casein with a 12.5% separating gel and performing the SDS PAGE as per the method of [12]. The gel was then incubated for 30 minutes at room temperature on a gel rocker in a buffer of 50 mM Tris-HCl (pH 7.4) containing 2.5% Triton X-100 to remove SDS. The gel was then incubated in a buffer of 30 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 10 mM $CaCl_2$. The gel was left in the buffer at 37°C for 12 h on the gel rocker. The gel was stained with 0.5% w/v Coomassie brilliant blue for 30 min after which it was detained in 10% methanol and 5% acetic acid until a clear zone of casein hydrolysis appeared against the blue background of the gel.

RESULTS AND DISCUSSION

After ion exchange chromatography, the fractions 32 to 50 showed protease activity and were pooled and subjected to gel filtration chromatography. After gel filtration chromatography, the fractions 12 to 25 showed protease activity and were pooled and used as the purified protease sample for further investigations. Ion exchange has been used in industrial and pilot scale purified preparations of many proteins, especially enzymes and therapeutic proteins. Ion exchangers such as DEAE cellulose that contain cross-linked cellulose provide are spherical with high porosity. Such ion exchange matrices provide increased flow properties and improved separation of biological macromolecules [14]. The advantage of using gel filtration chromatography for purification is that the stability and activity of the molecules are maintained without compromising on resolution. Because of its simplicity, reliability, versatility, and ease of scale-up, gel filtration chromatography has been successfully used for the purification of several industrially important proteins [15]. Ion exchange and gel filtration chromatography have been used as the purification methods of choice for several plant proteases with good yields of enzyme [16].

The protein concentrations of the purified protease fractions of ion exchange and gel filtration chromatography were also determined and used for the calculation of the specific activities of the samples and therefrom the purification fold was calculated as shown in (Table 1). The protein concentration in the final purified protease sample after gel filtration decreased by nearly 84% showing that the unwanted proteins were effectively eliminated during the two chromatography steps. This is also confirmed by the increase in the specific activity of the protease as the sample is passed through the various steps of the purification process. As shown in Table 1, the specific activity of the purified protease after gel filtration has increased nearly four times. Thus, a 4.7-fold purification of the protease was obtained at the end of the

purification process with an enzyme yield of 75% relative to the enzyme in the crude extract. The percent yield of enzyme is bound to reduce with every purification step due to the natural loss of enzyme during the processing steps.

Table 1 Purification of the protease

Purification step	Enzyme activity (U/ml)	Percent yield	Specific activity (U/mg)	Total protein (mg/ml)	Fold purification
Crude	34.44	100	8.2	4.2	1.0
Dialyzed	32.0	92.9	16.0	2.0	1.97
Ion exchange	28.14	81.7	33.1	0.85	4.03
Gel filtration	25.83	75.0	38.55	0.67	4.7

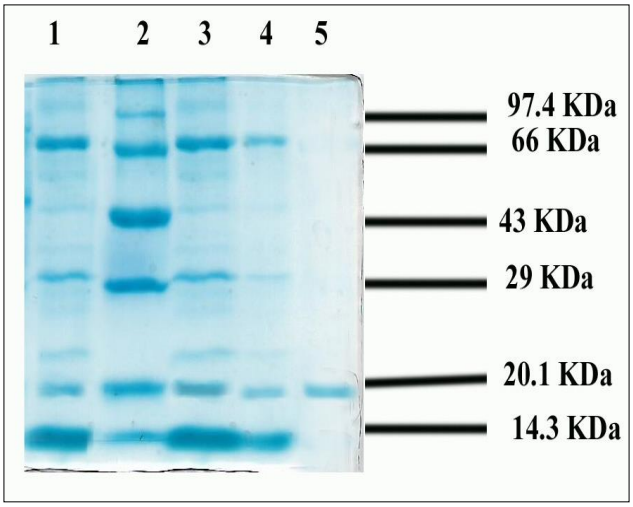

Fig 1 SDS PAGE of the protease sample at each stage of purification (Lane 1 – crude sample, Lane 2 – molecular weight marker, Lane 3 – dialyzed sample, Lane 4 – protease sample after ion exchange chromatography, Lane 5 – protease sample after gel filtration chromatography)

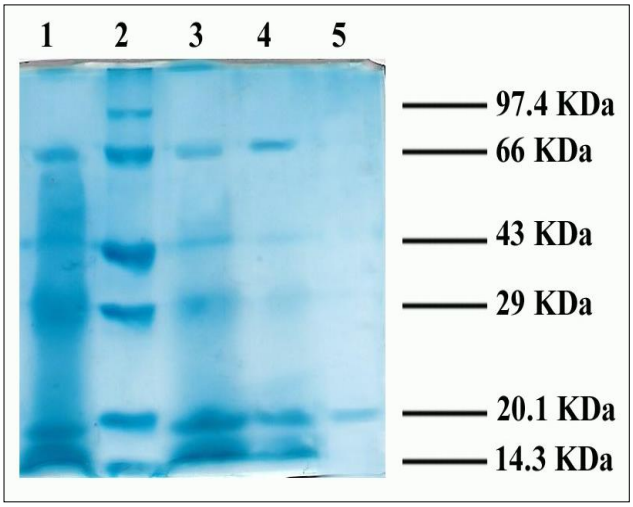

Fig 2 Native PAGE of the protease sample at each stage of purification (Lane 1 – crude sample, Lane 2 – molecular weight marker, Lane 3 – dialyzed sample, Lane 4 – protease sample after ion exchange chromatography, Lane 5 – protease sample after gel filtration chromatography)

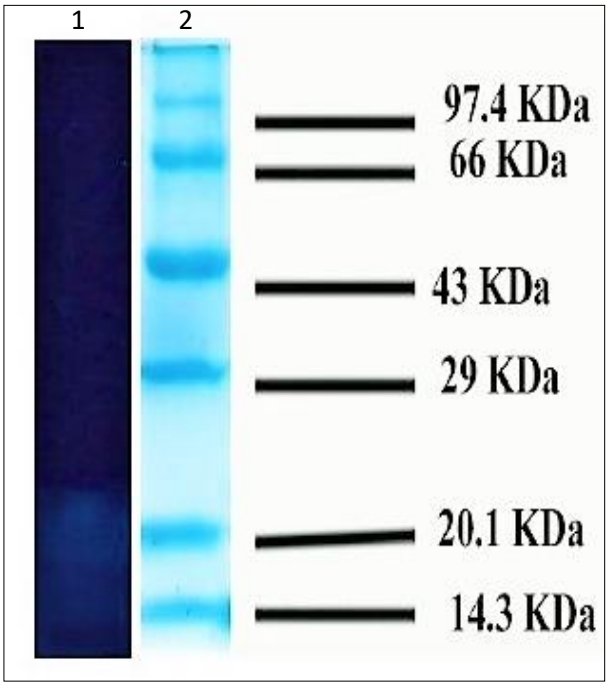

Fig 3 Casein zymography of the purified protease (Lane 1 – zymogram; Lane 2 molecular weight markers)

SDS PAGE of the crude, dialyzed and purified samples after ion exchange and gel filtration are shown in (Fig 1). It can be clearly seen from (Fig 1), that the final step of the purification process, i.e., gel filtration chromatography yielded a single protein band of pure protease sample with a molecular weight of approximately 20 kDa. The protein bands obtained when the crude, dialyzed and purified samples after ion exchange and gel filtration samples were subjected to native PAGE are shown in (Fig 2), which again reveals a single band of purified protease after gel filtration with a molecular weight of approximately 20 kDa. The confirmation that this band of 20 kDa was the purified protease was obtained with the casein zymogram (Fig 3). The casein zymogram showed a clear zone of clearance on the gel corresponding to approximately 20 kDa. Similar low molecular weight proteases have been obtained from various plant sources. Low molecular weight serine proteases of 25 kDa has been obtained from the seeds of *Holarrhena antidysentrica* [17], seeds of *Citrullus colocynthis* [18] and seeds of *Nelumbo nucifera* [19].

CONCLUSIONS

Proteases from plant sources are now being used in a variety of industries due to their unique properties. Discovery of such plant-derived proteases would be beneficial to industry. Many such plant-derived proteases show promise as therapeutic agents. This work has thrown light on the fact that mango seeds that are generally thrown away as waste may be good bioreactors for the production of proteases. Further investigation of the properties of this protease is required to determine its use for industrial purposes or for therapeutics.

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