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Purification of Protease from *Coriandrum sativum* using Ion-exchange Chromatography and Gel Filtration Method

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ABSTRACT

In the past decades, the interest towards plant proteases has increased significantly. Plant proteases are widely used in environment field, food and medicine industries. Proteases such as bromelain, papain and ficin are used in various areas such as in biosensors for detection of heavy metals, meat tenderization, brewing, cancer treatment, milk-clotting, viral disorders and digestion. In this study, protease from coriander leaf (*Coriandrum sativum*) was evaluated for protease activity using a Bradford-protease-casein assay system. This enzyme was purified through anion exchanger using DEAE-Cellulose column and gel filtration using Agilent ZORBAX column. Its molecular weight was around 55 kDa. The specific activity of the purified protein was 45.0 units/mg protein, total activity was 2745.0 units, yield 33.2% and fold purification was 2.8. Further investigation on gene sequence should be performed in order to identify the type of protease involved.

INTRODUCTION

Over the decades, the use of natural product has grown tremendously. However, industries using enzymes of plant origin is still less but growing rapidly. Enzyme isolations from plant-based sources have been utilized in industry from ancient times, even before the properties and the nature of the enzymes were identified [1]. Among all, protease group of enzyme is the most potential enzyme in commercialization due to its multiple applications in the detergent, pharmaceutical as well as food industries [2]. Proteases are well known in diary processing, meat tenderization and brewing. Among the proteases that are widely used currently are bromelain from pineapple, papain from papaya and ficin from fig tree [3].

Papain are currently introduced in the food industry for brewing, cheese, and beverage industries for the preparation of soluble hydrolysates proteins (papain-like proteases), as a food additives [4,5] to tenderize meats and soften the eggs [6,7] and for the emulsifiers production [8]. In other industries, proteases like keratinases are widely used in leather industry for dehairing [9,10] and in the essential amino acids production of such as lysine and to overcome wastewater clogging issues [11].

Proteases also have been widely used in pharmaceutical industries. Plant-based proteases with high levels of proteolytic enzymes have been traditionally used for a long time for the treatment of anti-tumorals [12,13], cancer [14,15], digestion disorders [16,17] and immune-modulation problems. Bromelain protease has been reported to prevent platelet aggregation, prevent edema and metasis due to its capacity in modifying cell structure through peptide cleavage [14]. Salas *et al.* [18] reported that the pharmacological activity of plant cysteine proteases able to immunomodulation, heal wound, adjust the digestive conditions and altering the neoplastics. Thus in this study, several plants have been identified to screen against protease activity. The plant with the highest protease activity was further with protease purification steps using affinity chromatography and gel filtration. This research aims to identify and purify protease from new plant resource, which can be a fundamental study before application in industries.

MATERIALS AND METHODS

Sample Preparation

Several fresh fruits and vegetables (star fruit, sweet potato, brinjal, mango, cucumber, coriander, lime and curry leaf) were purchased from wet market (Pasar Borong Selangor) and were brought to the laboratory for sample preparation. Samples were immediately washed, air dried and packed in ventilated polyethylene and refrigerated prior to analysis.

Preparation of crude plant extracts for screening towards proteases

Samples were screened to identify the most potential sample to show highest protease activity. 500 g of samples were used for extraction purpose. The leaves were cut and were submerged in sodium phosphate buffer (50 mM; pH 7) in the ratio of 1:2 (w/v). The samples were centrifuged at 10,000 X g for 15 min at 4 ºC and the supernatant was used in the purification procedures. All the steps were performed at 4ºC to avoid denaturation of the extracted enzyme.

Enzyme assay

The procedure described by Ellman *et al*. (19) with a modification for microassay [20] was used for measuring protease activity, which is expressed as the amount of casein degraded per minute under the specified conditions. Casein is broken down by protease to amino acids and reacted with Bradford reagent to produce brownish color solution (19), which is measured at 595 nm. The amount of solute present in brownish solution was measured using the extinction coefficient for Bradford [21]. The reaction mixture consisted of potassium phosphate buffer (50 mM; pH 8.0; 110 µl), casein (50 mM; 50 µl) and crude enzyme (40 µl) of at final volume of 200 µl. Experiments were carried out in triplicates.

The assay was carried out by mixing potassium phosphate buffer (50 mM; pH8.0; 110 µl) with of casein (50 mM; 50 µl). To start the reaction, protease (40 µl) was added into the mixture, mixed and incubated at room temperature for 10 min. The absorbance of the sample was measured at 595 nm. Crude (40 µl) was substituted with of potassium phosphate buffer (40 mM; pH 8.0) for control. The enzymatic reaction rate was quantified against blank for each activity measurement. Protease specific activity was calculated as described by Silver [22].

Enzyme activity = $\triangle$ Absorbance / 6.67 x 10^4 min^{-1} (1)
where;

$\triangle$ Absorbance = Wavelength at 595nm after 10 min of incubation (final- initial)

$0.667 mM^{-1}.cm^{-1}$ = Extinction coefficient of bovine serum albumin
Specific Enzyme Activity = Enzyme activity (µmol/min) / Amount protein (mg) (2)

Total enzyme activity = Specific activity of fraction x total protein content (mg) (3)

Purification of protease using ion-exchange chromatography

Partial purification was done on selected coriander extract that showed highest enzyme activity. Ion exchange chromatography is commonly used for protein purification [23-25], which is based on the isoelectric point (pI). In accordance with the method described by Jiang *et al.* [26], DEAE-Cellulose was used as the matrix in this study since the pH of buffer selected was considered as a factor that influence the selection matrix in any purification steps.

The column was equilibrated with potassium phosphate buffer (50 mM; pH 8.0; 250 ml), crude enzyme (200 ml) was then loaded into the column. The column was rinsed with potassium phosphate buffer (50 mM; pH 8.0; 100 ml) and a 3-ml fraction was collected until the protein content of the eluted solution was below the detection limit of the Bradford protein assay. The adsorbed enzyme was then eluted with 50 ml of the same buffer containing sodium chloride (1.0 M) and 3 ml of fractions were collected continuously using fraction collector.

Purification of protease using gel filtration chromatography

Further purification process was carried out using gel filtration involving Agilent ZORBAX column (GF250) (Agilent ® technologies, Germany). A column (4.6 mm in diameter and 250 mm in height) packed with spherical silica with pore diameter of 150 Å was used. Flow rate was set at 1.0 ml/min by gravity filtration. The column was equilibrated with 5 batch volumes of potassium phosphate buffer (50 mM; pH 8.0) and then crude enzyme (100 µl) was loaded into the column. 1 ml fractions of effluents were collected continuously and the protein content were analyzed using the Bradford protein assay.

Protein separation using gel electrophoresis

In this study, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate purified protein. The molecular weight of protease was visualized in SDS-PAGE under denaturing conditions [27] using the PROTEAN® 3 CELL apparatus (BioRad). Protein separation is carried out through a stacking and separating gels systems.

The resolving gel (10%) consisted of Tris-HCl (1.5 M; pH 8.8; 2.5 ml), deionized water (4.17 ml), acrylamide:bis (3.3 ml; 29.2:0.8) SDS (10%; 0.1 ml) ammonium persulphate (APS) (10%; 50 µl) and N,N,N',N'-tetramethylethylenediamine (TEMED) (20 µl) added in this sequence. The stacking gel consisted of of Tris-HCl (1.5 M; pH 8.8; 2.5 ml), deionized water (6.1 ml), acrylamide:bis (1.3 ml; 29.2:0.8), SDS (10%; 0.1 ml), APS (10%; 50 µl) and TEMED (20 µl) added in this sequence.Stock sample buffer was prepared by mixing deionized water (6.8 ml), Tris-HCl (0.5 M; pH 6.8; 1.2 ml), glycerol (1.0 ml), SDS (10%; 2.0 ml) and bromophenol blue (0.5%; 0.5 ml) and mercaptoethanol. The crude and partially purified protease was mixed with sample buffer in the ratio of 5:1 in microcentrifuge tube before heated in water bath for 10 min at 90 ^oC. Samples were allowed to cool to room temperature before being loaded onto the polyacrylamide gradient gel. After the run, the gels were stained using Coomassie brilliant blue R-250. If the concentration of the sample is too low, then the staining was changed to silver staining.

Protein content determination

Enzyme sample (20 µl) was loaded into a microplate well containing of Bradford reagent (200 µl) which was prepared in a 10-fold dilution from the commercialized stock solution. The mixture was incubated for 10 min before being measured spectrophotometrically at 595 nm. For blank, the sample was

replaced with deionized water (20 µl). Experiments were carried out in triplicates.

Calculation of fold purification and yield

The purification fold was calculated to determine the number of purification times compared with the crude extract. Percentage yield was calculated to display the highest enzyme activity that can be achieved.

$$\text{Fold purification} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of crude fraction}} \quad (4)$$

$$\text{Yield (\%)} = \frac{\text{Total enzyme activity of fraction}}{\text{Total enzyme activity of crude fraction}} \times 100\% \quad (5)$$

RESULTS AND DISCUSSIONS

Screening towards plant proteases

Identification of plant source that gives the highest enzyme activity is very crucial in this project, since bioassay is only practical for enzyme that shows high enzymatic activity. This is because, the significant color changes of the Bradford reagent is only observable if the protease have high activity. Table 1 shows the percentage activity between plant sources. Among all plant samples, coriander gave the highest enzyme activity. Although high enzyme activity does not correlate with increase or decrease enzyme sensitivity towards heavy metals, this plant source was used for further investigations since the activity is sufficiently high for inhibitive assay studies. For a good protease inhibitive assay for heavy metals, the difference in absorbance should be of more than 0.2 after a maximum of one hour of incubation for practical purposes [28]. Coriander have senescence related serinyl protease which is involved in the physiological role and metabolic activities of the plant [26]. The crude fraction may possibly consisted of one or more proteases. Proteases from coriander is feasible for large scale uses since it is cheap (about RM3 per kg) and it is easily available in markets. Therefore, coriander was chosen to be studied for subsequent investigations due to its high proteolytic activity.

Table 1. Proteolytic activity of different crude plant extracts.

Samples	Percentage of activity (%)
Control	0
Star fruit	10
Sweet potato	12
Brinjal	15
Mango	14
Cucumber	49
Coriander	95
Lime	21
Curry leaf	32

Purification of protease from coriander

Ion exchange chromatography

In this study, anionic exchanger (DEAE-Cellullose) was used for purifying protease from coriander. After crude sample were applied to the column, those proteins which have no affinity for the matrix were removed during the wash of the column by channeling sodium phosphate buffer. Then, the adsorbed protein was removed in the elution step by elution with high strength buffer (50 mM sodium carbonate containing 1M NaCl) at a flow rate of 0.3 ml/ml using gradient mixer in place of step elution. Since different proteins generally bind to an ion exchange matrix with different affinities, it is possible to separate them by gradually increasing the salt concentration during the elution step. All these steps were done in the cold room in order to avoid the enzyme to get denatured. Samples eluting out were collected using fraction collector every three minutes and all were assayed after undergone dialysis process. Dialysis is a very important step to discard unwanted salt from the sample in order to prevent denaturation of the enzyme. In this case, fraction 26 and 27 were identified to gave highest enzyme activity (**Figs.** 1, 2 and 3). The results obtained were summarized in the Table 2. Based on table 2, the crude extract from coriander contained 512 mg protein with specific activities of 16 Units/mg protein. The concentrated eluent from DEAE- Cellulose anion exchanger column gave specific activity of 33.2 Units/mg protein and the purification fold was 2.1. The yield obtained was 72.5%. Yield was reduced as more fractionation methods were employed to get pure enzyme. Fold purification denotes how pure is the enzyme preparation compared to crude. An increase in fold purification suggests that most of the impurities have been removed.

Gel filtration

After the ion exchange chromatography step, the results obtained from the SDS-PAGE analysis (Fig. 5) showed the existence of multiple bands. Thus for better purification, gel filtration using ZORBAX was done. Fraction 26 and 27 were pooled together before being loaded in the ZORBAX gel iltration column. The eluents were collected using a fraction collector at 1.0 ml/min.

Table 2: Purification table.

Step	Total Protein (mg)	Specific Activity (Units/mg protein)	Total Activity (units)	Yield (%)	Fold Purification
Crude	516.2	16.0	8259.2	100.0	1.0
Anion Exchanger	180.3	33.2	5985.9	72.5	2.1
Gel Filtration	61.0	45.0	2745.0	33.2	2.8

Based on the fig. 4 the highest peak of protein was determined at tube 15 (the 15th minute). The highest peak indicates the highest protein content. Enzyme assay and assessment towards the purity of the enzyme by performing SDS-PAGE were done. The results obtained were summarized in the Table 2. Based on Table 2, the concentrated eluent from ZORBAX column gave higher specific activity compared to the eluent from anion exchanger. The specific activity was 45.0 Units/mg protein. Meanwhile, the total protein content was 61.0 mg compared to 180.3 mg of eluent from anion exchanger. The total protein content was reduced due to the removal of the unwanted proteins. Fold purification of gel filtration step was 2.8 and this value was higher compared to the fold purification of the anion exchanger which was 2.1. An increase in fold purification denotes that many impurities have been removed.

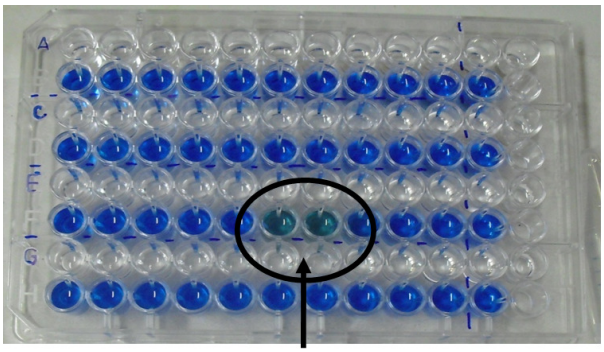

Fig. 1. Fractions that were assayed in the optimized condition. Fraction 26 and 27 show the highest enzyme activity.

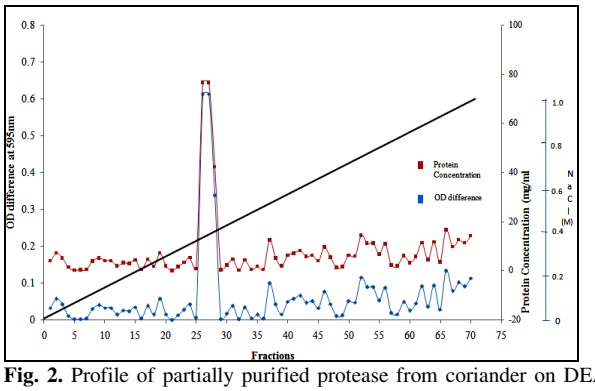

Fig. 2. Profile of partially purified protease from coriander on DEAE-Cellulose anion exchange column.

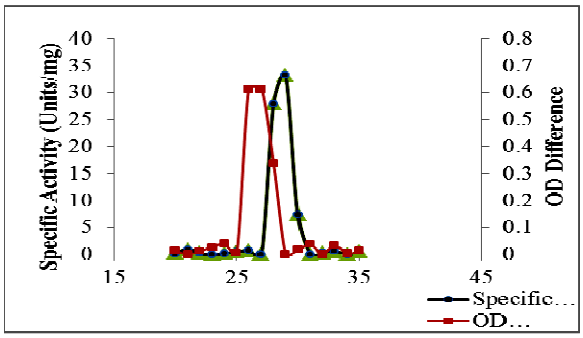

Fig. 3. The elution of protease from coriander on anion exchange chromatography. The specific activity were 27.9 units/mg protein and 33.2 units/mg protein for fraction 26 and 27 respectively.

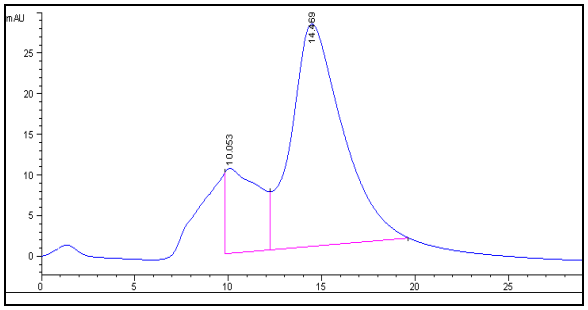

Fig. 4. The elution profile of protease from coriander on ZORBAX gel filtration using HPLC Agilent software.

SDS-PAGE is an inexpensive, reproducible and rapid procedure for quantifying, comparing and characterizing proteins. This method separates protein based on their molecular weights and size of the protein molecules [27]. The hydrophobic portion of protein binds with SDS to trigger its folded structure, therefore allowing it to exist stably in an opened conformation in solution. Thus, the length of the SDS-protein complex is directly proportional to its molecular weight and size.

Fig. 5 shows the comparison of bands formed for protein marker, partially purified and purified protease from coriander. In general, smaller molecular weight protein travels faster and thus the distance from wells is shorter. This explains the formation of first band, which represent the smallest protein molecular weight (15 kDa), and vice versa. There were four bands in partially purified protease after the anion exchanger and only one band after the second purification step (gel filtration). The four bands show that the desired protease was mixed with other proteins in the sample. Meanwhile, the presence of single band indicates that the target protease had been successfully isolated and other impurities or unwanted proteins had been removed. Since the Coomasie staining could not stain the protein, silver staining method was used instead. Silver staining is much more sensitive staining compared to the Coomasie staining since it can detect up to nanograms quantity of protein.

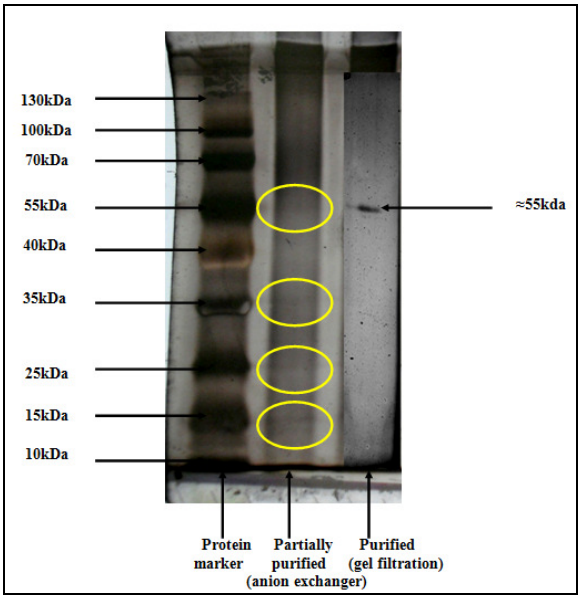

Fig. 5. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of purified protease from coriander in a 10% polyacrylamide gel.

CONCLUSION

Protease from the coriander leaves (*Coriandrum sativum*) had been fully purified through anion exchanger chromatography method by using DEAE-Celluose column and gel filtration using Agilent ZORBAX (GF250) column. The purified enzyme gave highest enzyme and total activity, highest enzyme yield and fold purification compared to the crude and partially purified enzyme. For further studies, it is highly recommended to explore the sequences of the protease and optimization study involving

enzyme concentration, substrate concentration, pH, temperature and incubation time to increase the efficiency of the protease. More research on the characterization of the protease should be carried out in order to understand the role of the enzyme especially in food and environmental industries.

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