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Properties of proteolytic enzyme from ginger (*Zingiber officinale* Roscoe)

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Abstract

Proteases in ginger rhizome have the potentials in industrial applications. This study was conducted to extract and characterize the proteolytic enzyme from ginger (*Zingiber officinale* Roscoe). Ginger protease (GP) was extracted from ginger rhizome by homogenization with 100 mM potassium phosphate buffer pH 7.0 containing 10 mM cysteine and 5 mM EDTA which were found to be the most efficient extraction buffer and stabilizers. After centrifugation at 10,500 x g, protein in the crude extract was precipitated using 60% ammonium sulfate following which the precipitate was redissolved in 50 mM potassium phosphate buffer pH 7.0, dialyzed and then lyophilized. The extraction method yielded 0.94% (w/w of fresh weight) of GP with a specific activity of 27.6 ± 0.1 Unit/mg protein where 1 Unit is defined as the amount of protease causing an increase in absorbance by 1 unit per minute using azocasein as the substrate. Results show that the GP was completely inhibited by heavy metal cations i.e. Cu^{2+} and Hg^{2+}, and a thiol blocking agent or inhibitor, n-ethyl maleimide (NEM), indicating that GP is most probably a cysteine protease. The enzyme has an optimum temperature at 60°C and the optimum pH ranged between pH 6 to 8. Monovalent cations (K^+ and Na^+) have no significant effect on activity of GP, but divalent and trivalent cations showed moderate inhibitory effect. Detergents such as sodium dodecyl sulfate increased the activity of GP while Tween 80 and Tween 20 slightly reduced the activity.

Introduction

Proteases are a highly demanded group of enzymes in various industries with a market share of 60% of the total enzyme market (Gaur *et al*., 2010). Global market for industrial enzymes was $2.3 billion in 2007, and is projected to grow to $ 2.7 billion in 2012 with a compound annual growth rate of 4% (BCC, 2008). Proteases available in the market are not sufficient to meet most industrial needs. Two major plant proteases that are important in the food industry, papain and bromelain, meet only 8% of market demand (Adulyatham and Owusu-Apenten, 2005). Therefore, the search for new plant proteases from different sources is crucial.

Since its discovery as a new protease source (Thompson *et al*., 1973), ginger (*Zingiber officinale* Roscoe) has been drawing a huge interest among researchers to extract, purify, characterize and study its application in various food products. Ginger powder has been used to improve meat tenderness and flavor in chicken kabab, an Indian traditional food (Bhaskar *et al*., 2006), while ginger rhizome extract (GRE) was reported to improve the properties of patties made from goat meat (chevon) (Pawar *et al*., 2007). The collagenase activity of ginger proteases (GP) is better than other plant cysteine proteases such as papain and bromelain based on its ability to hydrolyze native collagen (Kim *et al*., 2007). In addition, ginger with milk clotting activity has been traditionally used in the preparation of ginger milk curd in southern China (Su *et al*., 2009).

As one of the major ginger growing country, Malaysia produces ginger rhizome at 2,500 metric tons per year on 1,000 hectares land area (Ravindran and Babu, 2005). True ginger or halia (*Zingiber officinale* Roscoe) is the major ginger rhizome available in the marketplace and is usually used as a spicy condiment in cooking or even consumed directly as beverage. To enhance the application of ginger proteases (GP) in food or other industries, extraction and characterization of GP should be conducted.

Previous studies indicated that GP usually exhibit

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high proteolytic activity (Choi and Laursen, 2000; Adulyatham and Owusu-Apenten, 2005; Bhaskar *et al*., 2006; Kim *et al*., 2007; Su *et al*., 2009). However, it has poor stability when stored in solution. Cysteine could protect the GP from autolysis as well as improve its thermostability (Adulyatham and Owusu-Apenten, 2005). Similarly, ascorbic acid was also reported to help in maintaining the stability of fresh GP against oxidation which may occur during extraction (Adulyatham and Owusu-Apenten, 2005; Su *et al*., 2009). Ethylenediaminetetraacetic acid (EDTA) has also been used as an additive in ginger protease extraction (Adulyatham and Owusu-Apenten, 2005) but it gives unremarkable effect on proteolytic activity. However, a combination of these additives has not been investigated yet. Therefore, this study was also aimed at investigating the effect of stabilizers, such as cysteine and ascorbic acid in combination with EDTA, to enhance the stability of the ginger proteases. The proteolytic activity of most industrial proteases was reported to be unstable against organic solvents, surfactants and oxidants (Rao et al., 2009). Hence, by using different surfactants such as EDTA, Triton X-100, sodium dodecyl sulfate (SDS), Tween 20 and Tween 80, the stability of ginger protease could be investigated.

In order to encourage the commercial use of ginger protease on food and other industry it is important to extract and characterize the enzyme from different varieties of ginger. It has been shown that different varieties of ginger could promote different proteolytic activities, providing different potential applications. In India, Bangalore, a local ginger variety showed the higher proteolytic activity than another Indian local variety called Coorg (Bhaskar *et al*., 2006). Therefore, to enhance the usage of ginger, an extraction and characterization of protease from common Malaysian ginger should be conducted to deliver a proper application either in food or non-food industries.

Materials and Methods

Materials

Ginger rhizomes of commercial maturity were purchased from wet markets in Serdang, Malaysia. Usually, ginger is harvested between 7-9 months after planting. Azocasein, cysteine, ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were purchased from Sigma-Aldrich, (St Louis, MO, USA). Other chemicals used in this experiment were of analytical grade. Three batches of commercial ginger were used in the study.

Extraction of ginger protease (GP)

Ginger rhizome was washed and cut into fine pieces, and 100 g were homogenized in a Waring blender (Connecticut, USA) with 200 mL of 100 mM potassium phosphate buffer (pH 7.0). The homogenate was filtered through a piece of cheesecloth and the filtrate was centrifuged at 10,500 x g (Sartorius Model 3-18 k, Sartorius AG, Weender Land Strasse, Gottingen, Germany) and 4^{o}C for 30 min. The proteolytic activity of the supernatant was measured and compared with control (without any stabilizers) to determine the most effective stabilizer as described below.

Effect of stabilizers on extraction of ginger protease

To test the effect of stabilizer, three additives were examined for their ability to protect the activity of ginger protease during extraction. In the extraction procedure described above, 100 g of washed and cut ginger rhizome were homogenized with 200 ml of 100 mM potassium phosphate buffer (pH 7.0) containing one of the following groups of stabilizer(s): 0.2% ascorbic acid, 0.2% ascorbic acid and 5 mM EDTA, and 10 mM cysteine and 5 mM EDTA (Adulyatham and Owusu-Apenten, 2005; Su *et al*., 2009). Each homogenate was filtered through a piece of cheesecloth and the filtrate was centrifuged at 10,500 x g and 4^{o}C for 30 min. as previously described. The proteolytic activity of the supernatant was measured and compared with the control (without any stabilizer) to determine the most effective stabilizer. The extracts were also stored at 4^{o}C for four days where proteolytic activity was measured during each day of storage after bringing the extract to room temperature for approximately 20 min.

Assay of proteolytic activity

The proteolytic activity of GP was assayed using the colorimetric method as described by Adulyatham and Owusu-Apenten (2005) with a slight modification. The substrate, 1.0 mg/mL azocasein, was prepared in 100 mM potassium phosphate buffer (pH 7.0). One milliliter of azocasein solution was pipetted into a 2 mL Eppendorf tube containing 0.6 mL of the buffer solution and 0.1 mL of crude extract. The mixture was incubated at 60^{o}C for 20 min. The reaction was terminated with the addition of 0.3 mL 10% (w/v) trichloroacetic acid solution, followed by centrifugation at 9,000 x g for 10 min at room temperature. The absorbance of the supernatant was then measured at 410 nm. A blank was prepared as above except that the enzyme was first inactivated in a boiling water bath for 5 min. One Unit of enzyme

activity was defined as the amount of protease causing 1 unit increase in absorbance per mL extract per minute.

Protein determination

The protein content of the crude extract and dried GP were determined by the modified Lowry method (Lowry *et al*., 1951; Peterson, 1977) using bovine serum albumin (Sigma Chemical, USA) as the protein standard. The protein content is expressed as mg/mL for crude extract.

Preparation of GP powder

To produce dried GP, a total of 100 g of ginger rhizome was used. The enzyme was first extracted from the rhizome as described above using 200 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM cysteine and 5 mM EDTA. After centrifugation at 10,500 x g and 4°C for 30 min, the supernatant was filtered through Celite (diatomaceous earth) to remove any suspended materials and then mixed with 60% ammonium sulfate to partially concentrate and purify the enzyme. The precipitate that was obtained was collected by centrifugation at 10,500 x g and 4°C for 30 min and then resolubilized in minimal volume of 50 mM potassium phosphate buffer (pH 7.0). The enzyme solution was dialyzed against two changes of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA for 16 h at 4°C. The dialysate was centrifuged at 10,500 x g and 4°C for 20 min to remove any insoluble substances and then lyophilized using a freeze dryer (Model-7753032, Freeze Dry System, Labconco, Kansas City, MO). The yield of dried GP was determined gravimetrically while the proteolytic activity was measured as described above using a 1.0 mg dried GP/mL solution. The GP was then stored at -20°C prior to further analysis. The production of GP powder was conducted in triplicate.

Characterization of ginger protease (GP)

Determination of optimum temperature

To determine the optimum temperature of the enzyme, the activity of enzyme solution (1.0 mg/mL dried GP solubilized in 100 mM potassium phosphate buffer, pH 7.0) was assayed at various temperatures ranging from 20°C to 90°C for 20 min using 1.0 mg/mL azocasein as the substrate according to method as described above.

Determination of optimum pH

Different pH values ranging from 3 to 10 were used to determine the optimum pH of GP. Buffers used were 100 mM citrate (pH 3.0 - 5.0), 100 mM potassium phosphate (pH 6.0 and 7.0), 100 mM tris–HCl (pH 8.0 and 9.0) and 100 mM sodium carbonate (pH 10). Azocasein was dissolved in each buffer to obtain 1.0 mg/mL solutions and then used to assay the activity of a solution of 1.0 mg/mL dried GP.

Effect of cations and detergents on enzyme activity

The effect of cations which were prepared from their chloride salt (K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Al^{3+}) on GP activity was measured by adding 0.1 mL of 10 mM (to give a final concentration of 0.5 mM) of each cation into separate proteolytic assay reaction mixture. GP solution containing 1.0 mg/mL of dried GP was prepared prior to the proteolytic activity analysis as described above.

To determine the effect of detergents on GP activity, 0.1 mL of 5 mM (to give a final concentration of 0.25 mM) of EDTA, Triton X-100, sodium dodecyl sulfate (SDS), Tween 20 and Tween 80 were added into separate reaction mixture. The activity of GP was expressed as the relative activity based on the percentage of proteolytic activity of enzyme treated with either cation or detergent against the control (without added cation/detergent).

Determination of protease type

Four types of protease inhibitor were used to classify the type of GP. Aqueous solutions of 10 mM o-phenanthroline, phenyl methyl sulfonyl fluoride (PMSF), EDTA and n-ethyl maleimide (NEM) were prepared prior to analysis. The activity of 1.0 mL GP solution (1.0 mg/mL dried GP) after pre-incubation with 1.0 ml of each inhibitor solution for 1 h at 25°C was determined using the standard proteolytic method as described above. The activity obtained is expressed as the relative activity based on the percentage of proteolytic activity of inhibitor-treated enzyme against the control (100%). The protease type of GP was determined based on the rate of inhibitory effect.

Statistical analysis

The experiments were conducted in triplicate. The data which were presented as mean ± standard deviation were analyzed using analysis of variance (ANOVA) with Minitab 14 (Minitab Inc., 2003).

Results and Discussion

Extraction of ginger protease (GP) and effect of stabilizers

Proteases from ginger rhizome have been found to be instable and require an effective stabilizer to retain its activity during extraction (Adulyatham and

Table 1. Effect of extraction stabilizers on activity of ginger protease

Stabilizer	Enzyme activity (Unit/ml) at different day of storage at 4°C				
	0	1	2	3	4
Control	12.5 ± 0.6^{Aa}	5.6 ± 0.5^{Ba}	2.1 ± 0.2^{Ca}	1.6 ± 0.0^{Ca}	1.3 ± 0.3^{Ca}
0.2% Ascorbic acid	27.8 ± 0.5^{Ab}	24.7 ± 0.1^{Bb}	21.8 ± 0.6^{Cb}	19.7 ± 0.5^{Db}	18.6 ± 0.4^{Eb}
A, 0.2% Ascorbic acid + 5 mM EDTA	31.5 ± 0.7^{Ac}	29.4 ± 0.2^{Bc}	29.1 ± 0.4^{Bc}	28.7 ± 0.2^{Bc}	28.2 ± 0.5^{Bc}
a,b 10 mM Cysteine + 5 mM EDTA	34.6 ± 0.9^{Ad}	34.0 ± 0.3^{Ad}	$33.2 \pm 0.1^{A,Bd}$	32.8 ± 0.7^{Bd}	32.5 ± 0.7^{Bd}

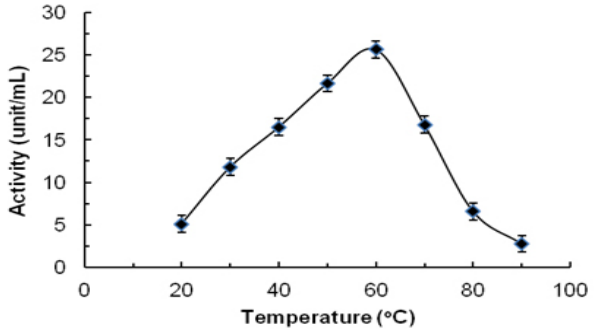


Figure 1. Effect of temperature on activity of GP

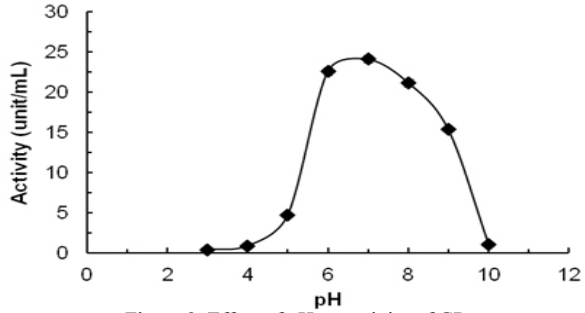


Figure 2. Effect of pH on activity of GP

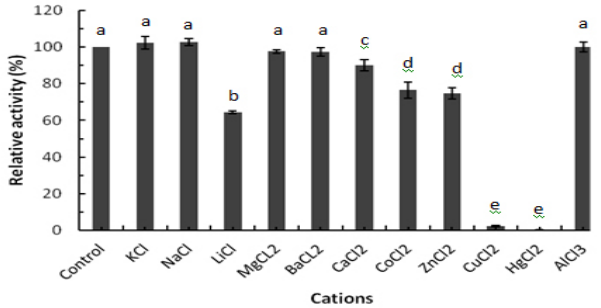
Owusu-Apenten, 2005; Su *et al*., 2009). In the present study, the presence of stabilizers during extraction resulted in an increase in the level of GP in the extract where the combined presence of cysteine and EDTA improved activity by nearly 3 times compared to the control (extract without any stabilizer) (Table 1). Moreover, the control was found to lose activity rapidly during storage at 4°C where more than half of the initial activity was lost after 1 day of storage and after 4 days of storage, only 10.4% of the initial activity remained. Ascorbic acid alone showed an improvement in proteolytic enzyme stability compared to the control. However, the proteolytic activity also decreased significantly during storage and only 66.9% remained on the fourth day. Ascorbic acid in combination with EDTA resulted in better stability of the enzyme than the used of ascorbic acid alone (Table 1). Stability during storage is also improved where the loss in activity was only 10.5% after 4 days of storage. As shown in Table 1, cysteine in combination with EDTA afforded the highest proteolytic activity of the enzyme and its activity was stable during storage at 4°C, retaining 93.9% after 4 days. The presence of EDTA assists in the binding of any metal ions responsible for attack on sulfhydryl group in the active site and causing the inactivation of cysteine protease. According to the "Memorandum of United States Environmental Protection Agency 20460 dated January 26, 2004", permissible levels of EDTA calcium disodium salt in food may range from 25 to 800 ppm and acceptable daily intake of 2.5 mg/kg was established by the 'Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1973. EDTA and its salts are eliminated from the body, 95% via the kidney and 5% by the bile. Hence, 5 mM EDTA used in the preparation of the extraction buffer should be considered safe for food processing. Cysteine has been reported to maintain the stability of protease by preventing the autolysis that is likely to happen during extraction (Adulyatham and Owusu-Apenten, 2005).

The extraction and drying methods used in the study yielded 0.94 g GP powder per 100 g fresh rhizome with a specific activity of 27.6 ± 0.1 Unit/mg protein. This recovery is higher than the method employed by Qiao *et al*. (2009) who extracted protease from Chinese ginger rhizome using acetone precipitation followed by 60% ammonium sulfate precipitation and lyophilization. The resulting yield was 0.6% crude enzyme powder. The specific activity of GP from the current study is also higher than those of two local varieties of Indian ginger extracted with acetone (12.0 Unit/mg) (Bhaskar *et al*., 2006). Cysteine in combination with EDTA effectively protected the protease from oxidation during extraction and, therefore, retaining a high specific activity.

Properties of GP

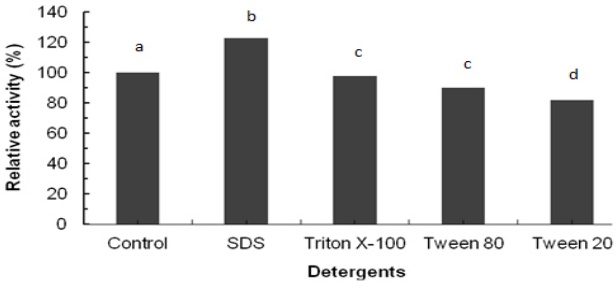
The activity of GP increased when the reaction temperature was raised from 20 to 60°C (Figure 1) where the optimum temperature was 60°C. However, at temperatures higher than 60°C, its activity declined drastically, most probably due to denaturation of the enzyme. With a high optimum temperature (60°C), GP may have a viable potential application in the food industry especially in the dairy or meat processing industry where high processing temperatures are often necessary (Hashim *et al*., 2011). GP extracted from Chinese ginger rhizomes (*Zingiber officinale* cv. Laiwu Shandong) also exhibited an optimum temperature at 60°C (Hashim *et al*., 2011). Other plant cysteine proteases have also been reported to have optimum temperature at 60°C and include proteases from *Euphorbia amygdaloides* (Demir *et al*., 2005) and capparin from *Capparis spinosa* (Demir *et al*., 2008).

GP acted optimally at pH 7.0 and maintained its activity between pH 6-8 (Figure 2). This suggests



[a,b,c] Different of letter shows a significantly different (P < 0.05)

Figure 3. Effect of cations on activity of GP



[a,b,c] Different of letter shows a significantly different (P < 0.05)

Figure 4. Effect of detergents on activity of GP

that GP is active in neutral, mildly acidic, and mildly alkaline conditions. It is possible that the crude enzyme extracted in the present study might contain more than one type of proteases which lead to a broad range of optimum pH. With a wide range of effective pH, GP is more adaptable to pH changes which might occur during food processing. Previous studies reported that pure GP showed an optimum pH ranging from pH 4.5 to 6.0 (Thompson *et al*., 1973; Hashim *et al*., 2011). The difference in pH could be due to factors such as variety, types of proteolytic enzymes present in the source and degree of purity of enzyme used in the characterization process.

The influence of cations on the activity of GP is shown in Figure 3. The enzyme was highly inhibited by heavy metal cations such Hg^{2+} and Cu^{2+}. This inhibition was most likely due to the sulfhydryl group in the GP locus forming a complex with these heavy metal cations (Chen *et al*., 2009). Monovalent cations (K^+ and Na^+) except for Li^+, and some divalent cations (Mg^{2+}, Ba^{2+} and Ca^{2+}) either showed no significant inhibitory effect ($P \leq 0.05$) or slight inhibition. The results obtained are in agreement with the effect of cations on other ginger proteases (Ohtsuki *et al*., 1995; Hashim *et al*., 2011). The sulfhydryl group tends to be bound with divalent cations i.e Co^{2+} and Zn^{2+} (Gavel *et al*., 2008) and therefore GP's activity reduced significantly.

The effect of detergents on the activity of GP is depicted in Figure 4. Based on the results, 0.25 mM SDS increased the activity of GP by 23%. Demir *et al*. (2008) found that 0.1 mM SDS also increased the activity of protease extracted from capsules of Caper (*Capparis spinosa*) by 47%. However, this activity was completely abolished by high concentrations of SDS at 1 to 10 mM. SDS at low concentrations has been claimed to attract hydrogen atom from the sulfhydryl group of cysteine (Voet and Voet, 2011) where the ionized cysteine possibly assisted the substrate-enzyme binding reaction and therefore increased the activity of GP. Triton X-100, a nonionic detergent, and the emulsifiers (Tween 20 and Tween 80) reduced the activity of GP slightly. This reduction was most probably caused by a change in the enzyme's conformation when its inner site was disturbed by non polar site of those detergents (Voet and Voet, 2011).

Table 2. Effect of inhibitors on activity of GP

Protease inhibitors	Relative activity (%)
Control	100.0 ± 0.0^a
o-Phenanthroline	67.8 ± 1.2^b
PMSF	60.5 ± 0.6^c
EDTA	101.8 ± 1.1^a
N-Ethylmaleimide	2.4 ± 1.0^d

[a,b,c,d] Means within a coloumn with different letters differ (P < 0.05)

N-Ethyl maleimide (NEM) (Table 2) was found to inhibit the activity of GP strongly. Therefore, GP can be grouped under the cysteine proteases. Hashim *et al*. (2011) has also considered GP to be a cysteine protease based on inhibition by Iodoacetamide, E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane), PCMB (p-chloromercuribenzoic acid) and N-terminal sequence. From the current study, phenyl methyl sulfonyl fluoride (PMSF), a serine protease inhibitor and o-phenanthroline, a metallo-enzyme inhibitor inhibited the activity of GP moderately between 32.2-39.5% inhibition (Table 2). This inhibition was most probably caused by PMSF attacking serine residues, and o-phenanthroline binding alanine or tryptophan residue at non-locus (non-active) site of GP. It caused the conformational tetrahedral distortion of GP, and therefore reduced its proteolytic activity (Voet and Voet, 2011). It is also possible that these inhibitors affected more than one type of proteases in the extract, leading to some loss in activity. Hashim et al. (2011) also found that PMSF caused a 25% inhibition of GP.

Conclusion

A new cysteine proteolytic enzyme was extracted from Malaysian ginger by a simple extraction method. Stabilizers are necessary during extraction and storage in order to improve the stability of the enzyme. The enzyme displayed an optimum activity at 60°C with a broad pH range of 6 to 8. The enzyme, considered as a cysteine protease due to inhibition by NEM and Hg^{2+} and Cu^{2+} and retains its activity

in the presence of monocations except Li^+ and some detergents. Information on the properties of this enzyme should facilitate its application in food and other industries.

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