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Proteases from Latex of *Euphorbia* spp. and Its Application on Milk Clot Formation

Protease dari Latex Euphorbia spp. dan Aplikasinya dalam Penggumpalan Susu

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Abstract

Crude proteases were extracted from Euphorbiaceae family, i.e. *E. milii* var *imperata*, *E. trigona*, and *E. maculata*. Among those three crude proteases, the activity of protease from *E. trigona* was the highest (812.50 U/ml), whereas *E. milii* and *E. maculata* crude proteases activity were 298.60 U/ml and 95.80 U/ml, respectively. *E. maculata* protein concentration was the highest among those three crude enzymes (1.206 mg/ml). The optimum pH and temperature of the enzymes were pH 7.0, pH 6.0, pH 6.5 and 60 °C, 50 °C, and 50 °C, respectively. Crude protease from *E. milii* var *imperata*, *E. trigona*, and *E. maculata* retained proteolytic activity over a wide range of pH (5.0–9.0) and temperature (up to 65 °C) with casein as substrate. All crude proteases showed milk clotting activity ranged from 0.58 U/ml to 1.01 U/ml. Thus, these crude proteases are potential to be applied in dairy industries. However, further study on enzyme purification and characterization are necessary to obtain high purity of proteases before its application.

Abstrak

Protease kasar berhasil diekstrak dari tanaman family Euphorbiaceae, yaitu E. milii var imperata, E. trigona, dan E. maculata. Diantara ketiga protease tersebut, aktivitas protease tertinggi diperoleh dari E. trigona (812,50 U/ml), sedangkan aktivitas protease dari E. milii dan E. maculata adalah 298,60 U/ml dan 95,80 U/ml, berturut-turut. Konsentrasi total protein tertinggi terdapat pada protease kasar E. maculata (1,206 mg/ml). pH dan suhu optimum ketiga enzim tersebut adalah pH 7.0, pH 6.0, pH 6.5 dan suhu 60 °C, 50 °C, and 50 °C, berturut-turut. Protease kasar dari E. milii var imperata, E. trigona, dan E. maculata menunjukkan aktivitas proteolitik pada rentang pH 5.0–9.0 dan rentang suhu sampai 65 °C menggunakan kasein sebagai substrat. Semua protease kasar menunjukkan aktivitas penggumpalan susu dengan rentang dari 0,58 U/ml sampai 1,01 U/ml. Berdasarkan hasil yang diperoleh, protease kasar dari ketiga jenis tanaman ini berpotensi untuk diaplikasikan dalam industri olahan susu. Meskipun demikian, studi lanjut mengenai purifikasi dan karakterisasi sangat diperlukan untuk memperoleh protease murni sebelum aplikasi dalam industri makanan, khususnya pada industri olahan susu.

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INTRODUCTION

Proteases are hydrolytic enzymes; they also named proteolytic enzymes or proteinases. These enzymes act by hydrolyzing the peptides bonds, transform proteins into shorter peptides for several purposes (Mahajan and Badgujar, 2010). Since proteases involve in many functions extending from cellular level to the organ and organisms level, they have a first place in the world market of enzymes (Leary et al., 2009). The proteases account for approximately 60% of the total industrial enzymes market (Rao et al., 1998). Furthermore, proteases occupy a critical position, they have potential application in commercial fields, such as in food, dairy, alcoholic beverage (Mahajan and Badgujar, 2010), beer clarification, meat tenderization, soy sauce industry, brewing cheese elaboration, bread manufacture, flavor improvement, protein modifications, cleaning detergents, leather processing, molecular biology, textile and pharmaceutical industries (Kumar and Takagi, 1999).

There are many sources of proteases as the main sources for protease production, e.g. plants, animals and microorganisms. Plant proteases were used for long time ago, as antihelmintic, milk-clotting and cheese making (Siota and Villa, 2011). The great majority of commercial enzymes have been obtained mainly from microbial sources because of industrial application of enzymes requires low cost in their production as well as large scale production. Recently, plant enzymes are becoming progressively more important, with applications in industrial processes, biotechnology and pharmacology. Proteolytic enzymes derived from plants are very attractive since they can be active over a wide range of temperatures, pHs, in presence of organic compounds as well as other additives and also have broad substrate specificity (Dubey et al., 2007). Hence, these all have stimulated the research works on plant proteases. Recent biotechnological developments and, particularly, protein engineering predict the appearance in the near future of plant proteases with more and improved industrial properties (Siota and Villa, 2011). Based on the MEROPS database there are seven classes of proteases i.e. Serine, Cysteine, Aspartic, Glutamic, Asparagin, Metallo and Threonine (Rawlings et al., 2010). According to International Union of Biochemistry and Molecular Biology (IUBMB), the enzyme subclass of proteases (EC 3.4) is in turn divided into sub-subclasses: enzymes belonging to subsubclass EC 3.4.21 (serine proteases) possess a Ser residue in the active site; those belonging to EC 3.4.22 (cysteine proteases) have a Cys residue instead; those belonging to EC 3.4.23 (aspartic proteases) depend on an Asp residue for their catalytic activity; and those belonging to EC 3.4.24 (metalloproteases) use a metal ion (normally $Zn^{2+)}$ in their catalytic mechanism (Reviewed in Antao and Malcata, 2005). However, in plants, five classes of endoproteases have been described: Serine, Cysteine, Aspartic, Metallo and Threonine (Rawlings et al., 2010).

Proteases have been found and extracted from distinct parts of plant, ranging from latex, fruits, and seeds (Costa et al., 2010). Moreover, they have been isolated from stems, flowers, leaves, roots, storage roots, and sprouts (Antao and Malcata, 2005). Proteases have been identified and studied from the latex of several plant families such as Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae and *Euphorbia*ceae (Domsalla and Melzig, 2008). Plant latex contain substances, mixture of organic and inorganic compounds (Silva et al., 2003), waxy materials and hydrolytic enzymes (Yagami et al., 1998). Protease from latex of Carica candamarcensis is used as a protective agent during DNA extraction (Genelhu et al., 1998). Thermostable serine proteases named 'wrightin' from the latex of the plant *Wrightia tinctoria*, 'Carnein' from the latex of the weed *Ipomoea carnea fistulosa* (Morning glory) and 'Milin' from the latex of *Euphorbia milii*, have found applications in food and other biotechnology industries (Yadav et al., 2006; Patel et al., 2007; Tomar et al., 2008).

Euphorbia is a widely cultivated genus, and certainly more species can be found in occasional cultivation, 21 species are naturally grow Thailand, most of them introduced, and at least 7 cultivated ones (Esser, 2010). The genus *Euphorbia* characterized by the milky white latex, contains lipids, rubbers, resins, sugars, proteins and enzymes (Ko et al., 2003). The presence of proteolytic activity in the latex of *Euphorbia* exhibits stability over a broad range of pHs, temperatures, as well as at higher concentrations of chemical denaturants, and low susceptibility to autodigestion at ambient temperature. Hence, these enzymes may have potential for use in biotechnology application, such as in the food industries.

The scopes of this research were collection and characterization of crude proteases from *Euphorbia* spp.. The crude proteases were characterized on its biochemical characteristics e.g.: protease activity and protein concentration; lastly, the crude proteases were applied in milk clotting.

RESEARCH METHODS

Euphorbia spp. plants purchasing

Tree samples of *Euphorbia* spp.. were purchased from local shop. In this study, *Euphorbia* species were *Euphorbia milii* var. *imperata*, *Euphorbia maculata* and *Euphorbia trigona.*

Latex preparation from *Euphorbia* spp.

Before latex collection, 20 mM Tris-HCl buffer pH 8 was prepared for crude enzymes and caseine substrate. *Euphorbia* plants secrete latex immediately when the leaves, stems and fruits are injured. The latex bleeding proceeds for a few minutes until a clot form around the wounded area. The coagulation process is vital for plant defense against possible pathogen attack (Pereira *et al.*, 2001). The isolation and purification process from *Euphorbia* spp.. performed by collection of the latex in the beginning. The crude latex was collected in a clean tube by breaking the twigs and petioles of the *Euphorbia milii* plant, by superficial incisions on stems of the *Euphorbia trigona* plant, lastly by breaking the young leaves, stems and apical tender part of *Euphorbia maculata* plant.

The latex was collected in 20 mM Tris HCl buffer at pH 8.0, the collection of latex were done early in the morning to get more latex amount and provide cool temperature. The latex was collected in a clean tube. Then, latex was frozen at −20 °C for 24 h. The freezing method has a purpose to pack the rubbery materials and insoluble materials in order to make them removed easily from the crude enzymes by centrifugation method. Subsequently, latex was thawed and centrifuged at 24,000 ×g for 45 min to remove the gum and other insoluble materials. The optimum objective of centrifugation in enzyme purification is to obtain a tightly packed precipitate and a clear supernatant (Scopes, 1982).

Latex collection of *E. maculata* plants, the latex collection was using a soaking method. The *E. maculate* plants were cut into small pieces and soaked in 20 mM Tris-HCl buffer pH 8.0 at 4 °C. After soaking process, the solution was filtered and then filtrate containing *E. maculata* latex was frozen at −20 °C for 24 h. Subsequently, filtrate was thawed and centrifuged at 24,000 ×g for 45 min to remove the rubbery materials and other insoluble materials. Then, the clear supernatant was dialyzed overnight against the same buffer.

Determination of protein concentration of crude enzyme

Protein concentration of the crude enzymes was determined spectrophotometrically (absorbance at 595 nm) as well as Bradford assay (1976) using BSA as the standard.

Measurement of protease activity

Activity of *Euphorbia* spp.. proteases were determined using casein as the substrate, 100 µl of crude enzymes was added to 900 µl of 1% casein solution in 20 mM TrisHCl buffer, pH 8.0. Then, the mixture was incubated at 37 °C for 20 min, and the reaction was stopped by addition of 10% Cl_3CO_2H (1 ml). After a period of 20 min at room temperature, the tubes were centrifuged 8000 ×g for 5 minutes at 4 °C. The supernatant was collected and the absorbance was determined by spectrophotometric measurement at 280 nm using a spectrophotometer. One unit of enzyme activity was defined as an amount of enzymes that gives increasing of one unit absorbance at 280 nm under the assay condition and used L-tyrosine as the standard.

Dependence of enzyme activity on pH and temperature

The effect of pH on the enzymatic activity of the purified enzyme were determined within the range of pH 2.0–12.0. The buffers used were glycine–HCl (pH 2.0–3.5), sodium-acetate (pH 4.0–5.5), sodium-phosphate (pH 6.0–7.5), Tris (pH 8.0–10.0) and glycine (pH 10.5–12.0). Denatured casein was dissolved in the corresponding buffer of required pH, will be used as substrate to measure the activity of the enzyme. Denatured casein was used as substrate in the enzyme assays at low pH insoluble below pH 4.0. Activity measurements were conducted as described above. Similarly, an analysis of the effect of temperature on the caseinolytic activity of the enzyme was conducted to determine the temperature optimum. Enzyme samples were incubated at different temperatures in the range of 10–80 ^{0}C for 15 min and an aliquot was used for activity measurement at the same temperature.

Milk clotting activity assay

Low heat skim milk powder was dissolved in 10 mM calcium chloride at pH 7.0 to a final concentration of 10%. Enzyme sample was added in the proportion of 0.1 ml per ml of the milk. The end point was noted by appearance of milk clots. One milk clotting unit of activity was defined as the amount of enzyme of plant latex that coagulates 1 ml milk at 37 °C in one minute (Arima *et al.,* 2000).

RESULTS AND DISCUSSION

The genus *Euphorbia* characterized by the milky white latex, contains lipids, rubbers, resins, sugars, proteins and enzymes (Ko *et al.,* 2003). The presence of proteolytic activity in the latex of *Euphorbia* exhibits stability over a broad range of pH, temperature, as well as at higher concentrations of chemical denaturants, and low susceptibility to autodigestion at ambient temperature. The enzyme may have potential for use in biotechnology application, such as in the food industries. The food industries, i.e. cheese, protein hydrolisate, bioactive peptides, yoghurt and beverage employ proteases in the process. The present study was performed to characterize milk clotting activities of various *Euphorbia* spp.. crude enzymes.

Protein concentration of crude enzyme

The protein concentration of crude enzymes was determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in the assay. The results are shown in Table 1. It can be seen that *E. maculata* had the highest protein concentration in the crude enzyme solution. *E. maculata* latex collection method was a soaking method, therefore, the protein from another plant tissues were extracted together with latex. Not only latex but also plant tissues proteins were extracted to the buffer. In fact, there are many plant protein contents such as plastid, photosynthetic protein, pigment and secondary metabolite (Wang *et al.,* 2008). The latex characteristic of *Euphorbia milii* var. *imperata* compared with latex of *E. trigona* was more sticky and easier to remove the rubbery white materials after freezing process. *E. trigona* has more slurry latex and difficult to remove rubbery white materials to obtain clear supernatant after centrifugation process. The rubbery white materials content with chemicals and proteins such as alkaloids, terpenoids, cardenolide, rubber, phenolics, proteases, oxidase, lectins, lipase and etc (Konno, 2011) which may inhibit the enzyme functions. Therefore, it is required to perform further purification process.

Proteases activity

Activity of *Euphorbia* spp.. proteases were determined using casein as the substrate. The summary of *E. milii, E. trigona,* and *E. maculata* proteases activity are presented in Table 2. Little caseinolytic activity was detected in the homogenate from the latex, stems and leaves of *E. maculata.* The latex secreted from the stems and leaves, the other plant proteins like pigment, plastids, inhibitor and photosynthetic proteins were extracted and dissolved in the buffer. These proteins act to inhibit protease activity of *E. maculata* crude enzyme homogenate. The highest protease activity was found in *E. trigona* crude enzyme, followed by *E. mili* var. *imperata* protease. Quantitative measurements of enzyme activity are based on rate assays. Hence, all experimental parameters that may affect the rate of an enzyme-catalyzed reaction, including pH, ionic strength, buffer composition and temperature need to be further defined.

In the earlier work, Yadav *et al.* (2006) purified a highly stable glycosilated serine protease from *Euphorbia milii* with potential applications in food industry. Fonseca *et al.* (2010) purified and characterized the biochemical of Eumiliin (serine protease from *E. milii)* by combination of ion-exchange chromatographic steps using DEAE-Sephacel and gel filtration with Sephadex G-75, Eumiliin has fibrinogenolytic activity, it may has

Table 1. Total protein of crude enzymes of *Euphorbia* spp

No.	Name of *Euphorbia* species	Total protein of crude enzyme (mg/ml)	Total protein of crude enzyme (mg)	Purification fold
1	*E. milii* var. *imperata*	0.261	26.10	1.00
2	*E. trigona*	0.647	64.70	1.00
3	*E. maculata*	1.206	120.60	1.00

Table 2. Proteases activity of crude enzymes of *Euphorbia* spp

No.	Name of *Euphorbia* species	Total activity of crude enzyme (U)	Specific activity of crude enzyme (U/mg)	Purification fold
1	E. milii var. imperata	298.60	11.44	1.00
2	E. trigona	812.50	12.55	1.00
3	E. maculata	95.80	0.79	1.00

an important applications as antithrombic drugs.

Enzyme activity on various pHs and temperatures

The optimum pH and temperature of crude proteases from *E. milii, E. trigona,* and *E. maculata* were determined by assaying their activity under broad range of pHs and temperatures. The results of optimum pH determination of three crude enzymes are shown in Figure 1. Protease from *E. milii* and *E. trigona* retained proteolytic activity over a wide range of pH, whereas *E. maculata* protease significantly lost its activity in acid and base environment. Crude enzyme of *E. milii* showed an optimum activity in pH 7.0, it indicated that *E. milii* protease could be possible as a neutral protease. *E. trigona* crude protease showed a broad range of activity; however, it showed highest protease activity at pH 6.0. Also, it retained activity in acid and base condition. *E. maculata* crude protease showed its highest activity at pH 6.5. Among those three crude proteases, *E. maculata* presented the lowest protease activity.

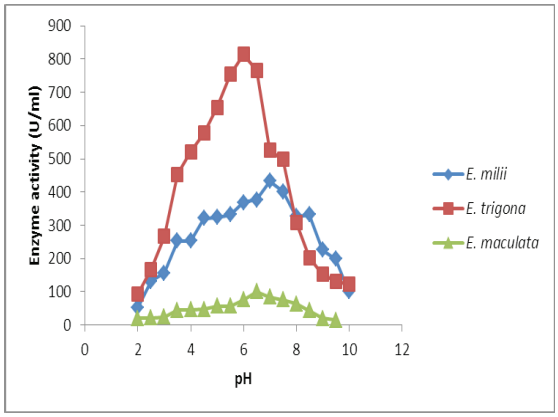

Figure 1. Effect of pH on activity of crude proteases from *E. milii, E. trigona,* and *E. maculata.*

The results of optimum temperature assay of three crude enzymes are shown in Figure 2. Protease from *E. milii* and *E. trigona* retained proteolytic activity over a wide range of pH, whereas *E. maculata* protease significantly lost its activity in acid and base environment. Crude enzyme of *E. milii* showed an optimum activity in 60 °C, it indicated that *E. milii* protease could be stable in warm environment. *E. trigona* crude protease showed a broad range of activity; however, it showed highest protease activity at 50 °C. *E. maculata* crude protease showed its highest activity at 50 °C.

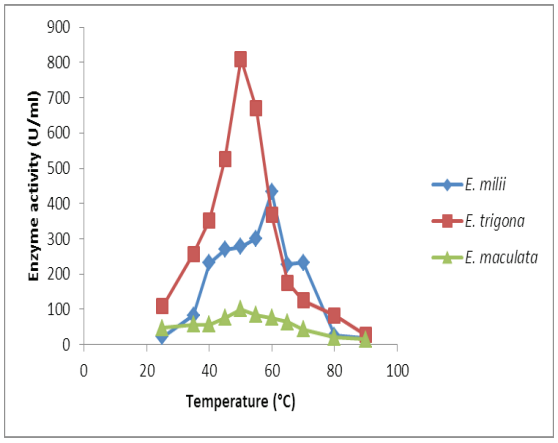

Figure 2. Effect of temperature on activity of crude proteases from *E. milii, E. trigona,* and *E. maculata.*

Based on the previous report, a new serine protease 'milin' was purified from the latex of the medicinally important plant *Euphorbia milii* using a single step on cation exchange chromatography on SP-Sepharose fast flow (Yadav *et al.,* 2006). The molecular mass (SDSPAGE), optimum pH and temperature of the enzyme were 51 kDa, pH 8.0 and 60 °C, respectively. Milin retains full proteolytic activity over a wide range of pH (5.512) and temperature (up to 65 °C) with casein and azoalbumin as substrates. In this study, crude protease from *E. milii* var. *imperata* showed the same pattern of optimum temperature with 'milin' from the previous study. The studies of protease from *E. trigona* and *E. maculata* have not been reported so far. Therefore, further study of their protease characteristics is promising.

Protease from *Euphorbia* plant latex could be possible to be applied as detergents additive. It depends on its compatibility with the detergents over wide ranges of pH and temperature. An ideal detergent enzyme should be stable and active in the detergent solution for a long period of time and should have enough temperature stability to be effective over a wide range of washing temperatures. *E. milii, E. trigona* and *E. maculata* proteases were found to be promising with regard to pH and temperature stability. It required further purification and characterization to study their detergent compatibility, stability to surfactants and oxidizing agents and for its application in detergent formulations.

Milk clotting activity

In this study, the milk clott formation process performed by *Euphorbia* spp.. proteases were determined. The milk clotting activity (U/ml) were determined by adding crude enzymes into

skim milk solution. The results of milk clott formation assay are shown in Table 3 and Figure 1. Milk clotting process by the actions of enzymes usually stated that the casein is changed to paracasein. There are three stages of enzymic coagulation, i.e. primary stage, secondary stage, and third stage. In the first stage, the enzyme (rennet) cuts off a specific fragment of one of the caseins, namely, -casein. At the natural pH of milk, about 80% of -casein must be cleaved to permit aggregation of the micelles to proceed. The next stage is the physical process of aggregation of casein particles (micelles) to form a gel. After losing its water soluble tail, -casein can no longer keep the casein particles separated, so they begin to form chains and clusters. The clusters continue to grow until they form a continuous, three dimensional network which traps water inside, and forms a gel, something like jelly. Lastly, the third stage refers to an ongoing development of the gel network (Hagel, 2008).

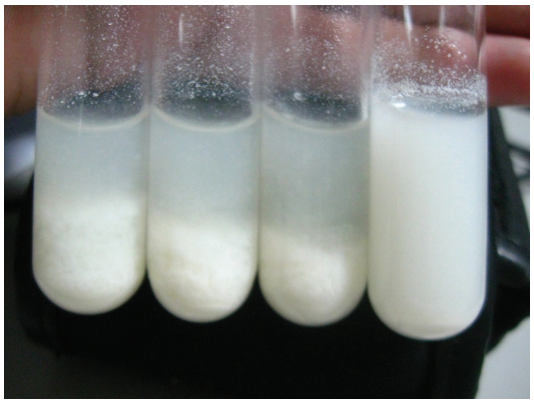

Figure 3. Milk clotting activity assay, from left to right: *E. trigona, E. milii, E. maculata,* and control only buffer without enzyme

Dahot *et al.*. (1990) screened some Pakistani plants for milk-clotting activity, include in the study was *Euphorbia caducifolia* that has higher ratio of milk clotting in comparison to other plants. In the study of *Euphorbia amygdaloides* latex, it was also researched whether purified and characterized protease can be collapsed to milk. It was determined that protease enzyme can collapse milk and it can be used to produce cheese (Demir *et al.,* 2005). In the earlier study of various laticiferous plants, the highest ratio of milk clotting activity to caseinolytic activity was found in the latex of *Euphorbia nivulia* followed by *E. milii, E. prunifolia* and *E. hirta,* respectively (Badgujar and Mahajan, 2009). *Euphorbia neriifolia* protease called neriifolin also has milk-clotting activity. The ability of this enzyme to produce milk curds could make it useful as milk coagulants (Yadav *et al.,* 2011).

Concerning about safety of consuming *Euphorbia* plants, they have been used as a traditional medicine since long time ago. Many of these indigenous medicinal plants are used as spices and food plants.

They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 2001). One such medicinal plant is *Euphorbia*, a well-known genus having therapeutic efficacy (Natarajan *et al.,* 2007). Plants of *Euphorbia* species show anticarcinogenic activity due to the presence of several terpenes, anthocyanins, alcohols and steroids; diterpenoid ingenol 3,20-dibenzoate and phorbol 12-tiglate 13-decanoate isolated from *Euphorbia*ceae plants show antileukaemic activity against the P-388 lymphocytic leukaemia in mice (Kupchan *et al.,* 1976). *Euphorbia neriifolia* Linn. (*Euphorbia*ceae) plant is traditionally used in the treatment of abdominal troubles, bronchitis, tumours, leucoderma, piles, inflammation, and enlargement of spleen (Bigoniya and Rana, 2009). *Euphorbia hirta* Linn. is one of such herbs belonging to the family *Euphorbiaceae* which is a very popular herb amongs practitioners of traditional medicine. The herb *E.* hirta can be used as source of oral drugs to fight infections caused by susceptible bacteria (Abubakar, 2009).

Tenderness has been identifed as the most important factor affecting consumer satisfaction and perception of taste. There are several means of tenderizing meat either chemically or physically. Treatment by proteolytic enzymes is one of the popular methods for meat tenderization. Proteolytic enzymes derived from plants, such as papain, bromelain and ficin have been widely used as meat tenderizers in most parts of the world. Moreover, ginger and cucumis can be used as an effective alternative to papain (Naveena *et al.*, 2004). Ramezani *et al.* (2003) investigated the water holding capacity of ficin tenderized meat and evaluated the effect of ficin on meat protein by

Table 3 Milk clotting activity assay of *Euphorbia* spp crude enzymes

No.	Name of *Euphorbia* species	Milk clotting time	Milk clotting activity (U/ml)
1	*E. milii* var. *imperata*	17 min 05 sec	0.58
2	E. trigona	09 min 56 sec	1.01
3	E. maculata	23 min 20 sec	0.43

gel electrophoresis and concluded that solubility of meat protein increased when ficin was used as meat tenderizer. However, *Euphorbia* plant proteases and its utilization as a meat-tenderizing agent are not fully appreciated and the literature available is scanty. Based on the evidences of *Euphorbia* spp milk clotting activity and their uses for medicinal purposes, *Euphorbia* species proteases have potential characteristics to be applied in food industry, especially in the manufacture of cheese and as a meat tenderizer.

CONCLUSION

Based on the study results, it can be concluded that all crude proteases exhibited caseinolytic activity and milk clotting activity. Further purification and characterization are necessary before enzymes application in dairy industry.

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