Purification and Characterization of Rennin Like Protease *Enterococcus faecium* 1.15 Isolated from Indonesia Fermented *Bakasam*

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Abstract.

The protease enzyme produced by Lactic Acid Bacteria (LAB) with characteristics similar to rennin is called Rennin Like Protease (RLP). The purpose of the study was to obtain information on the biochemical characteristics of RLP produced by Enterococcus faecium 1.15 as a basis for the utilization of the enzyme. The first stage of the study was observation of the growth curves and the potential of the isolates to produce RLP, biochemical characterization. Rennin Like Protease (RLP) produced by Enterococcus faecium 1.15 which has been purified using Sephadex G-50 has a molecular weight of 42 kDa with biochemical characters, with an optimum temperature of 40 ℃ and pH of 4.5. The comparison between MCA/PA with a value of 3.74 which is close to the MCA value of Calf Rennet is 1.

Keywords: *Enterococcus, milk clotting activity, purification, characterization.*

I. INTRODUCTION

The production of rennin (rennet) from cattle is still constrained by the rarity of cattle being slaughtered at a young age because it is not profitable for both cattle breeders and entrepreneurs, so that the production of rennin or rennet from calf abomasum does not develop in Indonesia. Renin enzymes from ruminants are few in number, expensive and limited market availability [16]. So we need an alternative enzyme as a substitute for rennin from other sources. An enzyme that has milk clotting activity is also known as Rennin Like Protease (RLP). RLP from microbes has the advantage of high availability, can be produced in a relatively short time, and no bitter flavor is produced as in the use of the enzyme papain and bromelin. Protease extract from the herbaceous plant *Cynanchum otophylum* used as milk coagulant to make traditional cheese from China with molecular weight 14 and 27 kDa [10].

Proteases produced extracellular from Lactic Acid Bacteria have advantages because they can also be used as starters in fermentation. A novel Lactic Acid Bacteria reported produce RLP were isolate *Lactobacillus plantarum* 1.13 from Indonesia traditional fermented meat *Bakasam* [13], *L. paracasei* 2.12 isolated from fresh milk of Ettawa goats in Bogor Regency[14]. Microbial source had prospective application for cheese industry [17]. The domestic industry of rennin products from microbes is undeveloped and less attractive to cheese businesses, possibly due to their limited availability, and the quality is still inferior to microbial rennin products from abroad. This presents a challenge to export deeper the potential of existing microbes.Efforts to explore Lactic Acid Bacteria which have the big potential to produce RLP from fermented traditional food products such as *Bakasam* which is a typical Lampung fermented meat have great potential as an alternative to rennin. The research aims to determine the biochemical characteristics of RLP *E. faecium* 1.15 and this information is needed as a basis for the application of enzymes as coagulating agents in cheese making.

II. MATERIALS AND METHOD

MCA Activity using Skim Milk Agar [15]

To ensure milk clotting activity, isolate *E. faecium* 1.15 was grown on MRSA containing 3% skim, incubated at 37°C for 24 hours. *E. faecium* 1.15 with MCA activity will show casein clumping around the colony.

Growth Rate of *E faecium* 1.15

Observation of the growth curve of isolate *E. faecium* 1.15 was carried out every 5 hours for 50 hours of incubation time. In the first stage, 2 ose *E. faecium* 1.15 were cultivated in 5 ml MRS broth and incubated at 37°C for 24 hours. Furthermore, 1 ml (1%) of the first stage of culture was cultivated in 100 mL of MRS broth and analyzed for OD, pH, MCA every 5 hours for 50 hours of incubation at 37°C.

Milk Clotting Activity (MCA)

RLP activity is in line with the speed of the milk (casein) coagulation reaction. Skimmed milk with a concentration of 10% and 10 mM CaCl2 with a total volume of 10 mL, then added 1 mL of the tested RLP enzyme and recorded the time of milk coagulation at 37°C. MCA was calculated as: SU (Soxhlet Unit) = 2400× 5×D/T×0.5 [11,12], in which T is milk-clotting time (s), and D is dilution of the enzyme.

Protease Activity

The activity of extracellular protease (PA) was measured based on the amount of tyrosine product produced from the reaction using the modified method of Cupp-Enyard. In the initial stage is to make a Tyrosine standard curve. Furthermore, 6 µl of RLP enzyme sample was added with 6 µl of Tris buffer HCl (pH 7.4, 25 mM), then mixed with 6 µl of 1% (w/v) casein solution. Homogenization was carried out and then incubated at 37°C for 30 minutes. To stop the reaction, 12 µl of trichloroacetic acid (TCA) was added. Then centrifugation was carried out at 10,000 g for 1 min at 4 C. The supernatant was transferred to the well and 143 µl of reagent A (a mixture of Na_2CO_3 solution and $CuSO_4.5H_2O$ solution at a ratio of 5:1) and 29 µl of follin ciocalteau reagent was added, and further incubated for 15 min before being measured at 540 nm. Calculation of protease activity based on the Tyrosine standard curve that has been made.

Protein Concentration

In the early stages of measuring the concentration of dissolved protein (mg/mL), that is making the BSA standard curve. Furthermore, the reagent preparation, namely the Working reaction was prepared using a mixture of reagents A and B (50:1) Pierce® BCA. A mixture of sample and working reaction (1:20) was transferred into a 96-well microplate, incubated at 37 °C for 30 min, then read using a multi-scan tool at a wavelength of 540 nm.

Enzyme Purification

The initial stage is the production of crude RLP from E faecium 1.15 which has been grown in 100 mL of MRS broth incubated at 37C for 25 hours without shaking. Next, centrifugation was carried out at 10.000 x g for 30 min at 4 °C to separate the cell biomass and the supernatant which was the crude enzyme. The second stage is the precipitation of ammonium sulfate and the optimum concentration is obtained, namely 45%. To remove the salt residue, dialysis was carried out in cellulose membrane of MWCO 12.5 kDa (Sigma-Aldrich). The final stage of purification is using Filtration Gel with Sephadex G-50. Proteolytic activity, protein concentration and MCA were measured in each fraction

SDS-PAGE and Zymography

Molecular weight analysis was carried out using SDS PAGE (Sodium Dodecyl Sulphate-Poly Acrilamide Gel Electrophoresis) brand Novex-Tris Glysin. The 15 µL sample and 10 µL loading dye were mixed and heated at ± 95 ºC for 10 minutes to denature the protein. The denatured sample was put into the gel well then the electrophoresis machine was run at 110 volts for 1 hour. After the protein separation was complete, the gel was removed and the staining was carried out using Simplyblue. Then destaining using aquadest and then documenting it. Precision Plus Protein Dual Xtra Standard (2-250 kD) protein markers were used to predict the size of the protein bands that appeared. To ensure that the protein band is an enzyme, a Zymogram analysis is carried out. Polyacrylamide gel is added with casein substrate. Samples that had been run on the gel were renatured by immersing them in Triton X-100 2.5% (v/v) for 40 minutes. Then the gel was soaked in 25 mM Tris HCl, 10 mM pH 6, 10 mM CaCl2 buffer at 37 °C for 12 h. Furthermore, the staining and clear band stages were carried out to show enzymes that had activity to hydrolyze the substrate contained in the gel.

Milk Clotting Activity (MCA) Characterization

Determination of optimum temperature and pH as well as stability against pH and temperature based on milk clotting activity and the percentage of curd produced.

III. RESULTS AND DISCUSSION

Growth curve of *E. faecium* 1.15

The growth curve of *E. faecium* isolate 1.15 (Fig.1) at an incubation temperature of 37ºC for 50 hours is presented in Fig.2. *E. faecium* 1.15 isolate was isolated from *Bakasam* which is a traditional Indonesian fermented meat product. The characteristics of LAB isolates on agar showed the presence of casein clumps around the colony. This shows that the isolate has the potential to produce extracellular protease enzymes with characteristics similar to rennin namely milk clotting activity.

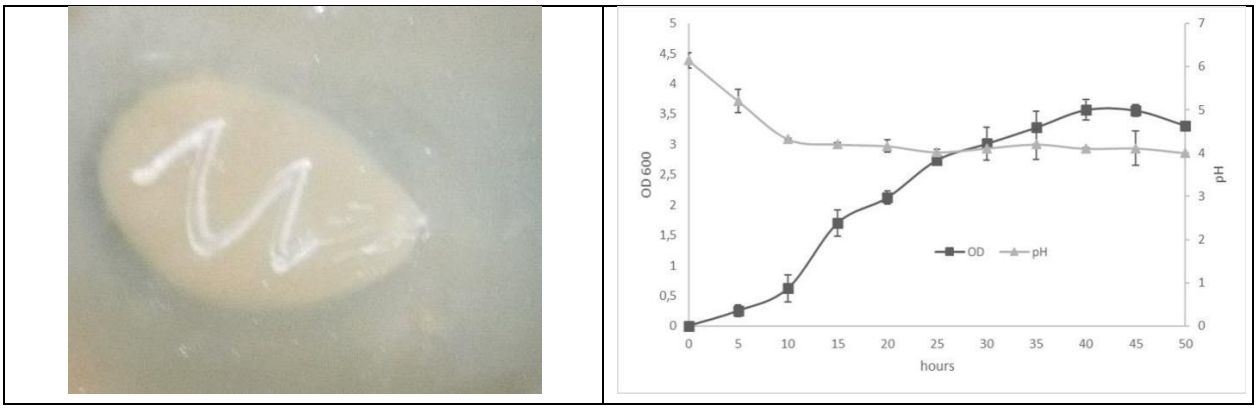

Fig 1. Local isolate *E. faecium* 1.15 and Value of Optical Density (OD) and pH of the media on growth *E. faecium* 1.15 on MRS broth at 37 ℃ incubation temperature

The first observation was made starting at the 5th hour and it was shown that there had been an increase in the number of bacterial cells with an increase in OD_{600} value of 0.25 and the pH of the media had decreased from 6.14 to 5.20. This shows that lactic acid has formed at the beginning of the growth (lag phase) of the bacteria. At the 10th hour, there was a significant growth acceleration up to the 35th hour (log phase), and after that there was a decrease in the growth speed or steady state (stationary phase) and at the 50th hour it was seen that there was a decrease in OD values indicating that it had cell death occurred. The decrease in pH of MRS broth media at the 50th hour reached pH 4.00.

LAB produces lactic acid as the end of sugar (carbohydrate) metabolism. Lactic acid produced in this way will reduce the pH value of the media (MRS broth). During the fermentation process there is a decrease in pH, a decrease in pH of the media due to the production of lactic acid produced during the fermentation process. The lactic acid produced is the result of the breakdown of glucose through the glycolysis pathway for homofermentative lactic acid bacteria (*L. acidophilus, L. bulgaricus*) and through the 6 phosphogluconic or phosphoketolase (6-PG/PK) pathway for hetero fermentative lactic acid bacteria. Lactic acid bacteria is bacteria that produce large amounts of lactic acid as the end result of metabolism in sugars. Lactic acid production is the most important biochemical process that occurs during fermentation. Factors that influence fermentation are acid, alcohol, microbes, temperature, oxygen, and salt.

Purification of RLP from *E. faecium* 1.15

Purification stages included the production of crude RLP extracts, 45% ammonium sulfate precipitation, dialyzed, and than Sephadex G-50. The results and efficiency at each purification stage are presented in Table.1.

Table 1. Summary of purification on RLP from *E. faecium* 1.15

Purification step	Volume (ml)	Protein (mg/ml)	MCA (SU/ml)	Spesific MCA (SU/mg)	PA (U/ml)	Spesific PA (U/mg)	Purity (MCA)	Yield (%) MCA	Purity (PA)	Yield (PA)	MCA/PA
Crude Extract	100	3,64	95,23	26,16	17,57	4,83	1,00	100	1,00	100,00	
45% Amonium sulfat	2,0	8,106	175	21,59	23,51	5,80	0,83	3,68	1,20	2,68	
Dialisis	0,8	6,72	200,02	29,76	53,18	6,33	1,14	1,68	1,31	2,42	
G-50 Sephadex	0,5	1,41	250,05	177,34	66,84	47,40	6,78	1,31	9,82	1,90	**3,74**

Table 1 shows that in the first stage of precipitating ammonium sulfate purification, MCA activity increased from RLP *E. faecium* 1.15 to 0.83 (23.51 U/mL), with a yield of 3.68 percent. When compared to *Moringa oleifera* purified by 1.01 folds using precipitation ammonium sulfate (40%) with milk clotting activity of 384 SU/ml [5]. Furthermore, the purification using Sephadex G-50 can increase the purity to 6.71 with a yield of 1.31%. The resulting comparison of the MCA/PA value is 3.74. This shows that RLP does not have too high proteolytic activity but has milk clotting activity. Proteolytic activity that is too high will over hydrolyze casein, causing the resulting curd to decrease [7]. Comparison of MCA and PA values is used as a reference to compare potential enzyme activity as an alternative to renin [8]. The purified enzymes from *Moringa oleifera* ratio MCA/PA of 155,47 [5]. The results of other studies reported that purified enzymes from Dregea sinensis had an MCA/PA ratio of 27.31 [19].

Enzymes that are used as alternatives to rennin must meet the requirements of purity, safety and free of antibiotics. Furthermore, in case that the enzyme used as a substitute for rennin (rennet substitute), several parameters that need to be considered are the ratio between MCA (Milk Clotting Activity) and PA (Proteolytic Activity). Proteolytic activity is expected not to be too high so it protects the gel structure formed from the proteolytic activity, avoiding the loss of dissolved protein and fat in whey which causes the low percentage of curd obtained. Likewise in the ripening process, if rennet replacement enzyme activity has a high proteolytic activity, bitter falvor can be formed.

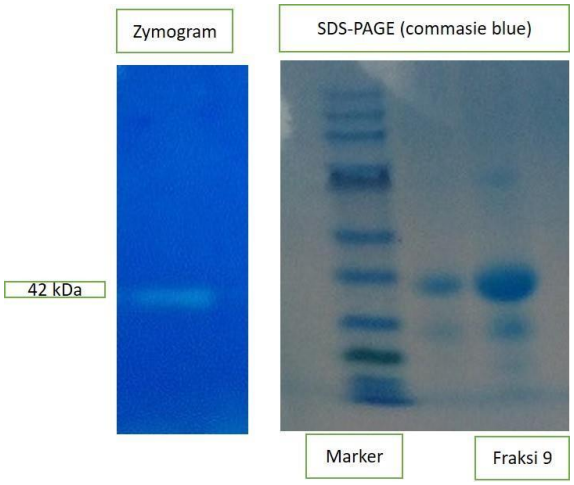

Fig 2. Sodium dodecyl sulfate polyacrilamide gels electrophoresis from the results purification of RLP from *E. faecium* 1.15. Lane M, molecular weight marker protein and zymogram (casein)

To determine the molecular weight of the protein from RLP *E. faecium* 1.15, SDS-PAGE analysis was carried out and after going through the purification stage, the highest MCA activity was obtained in fraction-9 and showed a molecular weight of 42 kD (Fig.2). This was confirmed by the results of the zymogram which showed clear bands of the same size. When compared with other studies, namely purified protease from *Gammarus bakhteyaricus* had a molecular weight of protein of 25 kDa [2].

Characterization of Milk Clotting Activity (MCA)

Optimum Temperature

RLP from *E. faecium* 1.15 has an optimum temperature at 40ºC with an MCA value of 99, 18 SU/mL and the percentage of curd obtained reaches 16.4% as illustrated in Fig.3.a When compared with MCA *B. lechiniformis* 545 activity has the highest MCA at 75 ºC [1]. Crude enzyme from thermophilic fungus *Thermomucor* indicae-seudaticae N31 has the optimum MCA at 70 ºC [2]. *Pediococcus acidilactici* SH is optimum at MCA at 50ºC and loses MCA activity at 60 ºC. Whereas *Bacillus stearothermophillus* showed optimum MCA activity at 60 ºC [1]. Calf rennet has optimum MCA activity at a temperature of 40-42ºC [1]. So that RLP *E. faecium* 1.15 has an optimum temperature with a value that is almost the same as calf rennet.

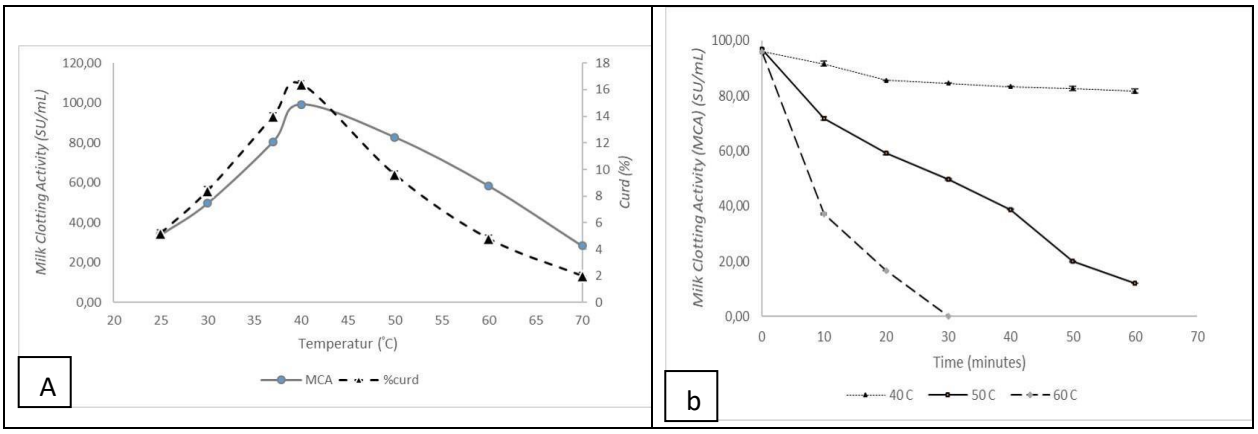

Fig 3. Optimum temperature of MCA RLP from *E. faecium 1.15*

The RLP from *E. faecium* 1.15 enzyme is classified as a thermolable (non-heat resistant) enzyme as illustrated in Fig.9.b. Based on the data obtained, RLP from *E. faecium* 1.15 has optimum activity at a temperature of 40 ℃. Whereas as the temperature increases, the enzyme activity will decrease and lose activity at 70 ℃. Based on the data obtained, RLP from *E. faecium* 1.15 has optimum activity at a temperature of 40 ℃. Whereas as the temperature increases, the enzyme activity will decrease and lose activity at 70 ℃. A temperature that is too high will affect the substrate conformational changes so that the reactive side of the substrate has obstacles to enter the active side of the enzyme and this causes the enzyme activity to be low. High temperatures will cause damage to no covalent interactions which maintain the three-dimensional structure of the enzyme so that the enzyme is denatured.

pH Optimum

RLP from *E. faecium* 1.15 shows the optimum MCA activity at pH 4.5 with a value of 99.18 SU/mL and the percentage of curd obtained by 16.8% (Fig.4a). MCA activity decreases as the pH increases. So that RLP from *E. faecium* 1.15 can be classified as an acid protease. The RLP produced by *Bacillus steoarothermophillus* is also an acid protease group with optimum MCA activity at pH 4.5 [1], while commercial RLP from the *Bacillus* group is generally an alkaline protease group.

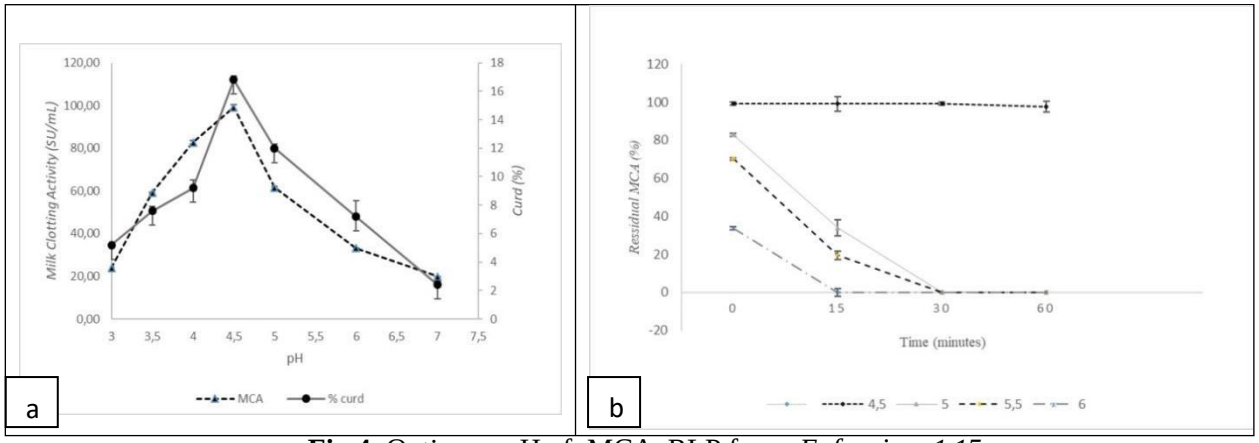

Fig 4. Optimum pH of MCA RLP from *E. faecium 1.15*

The enzyme will show the highest activity at a certain pH buffer. This phenomenon is a characteristic of enzymes. Changes in pH on a small scale deviation can cause decreased enzyme activity due to changes in ionization of functional groups. The ionic group plays an important role in maintaining the conformation of the active side of the enzyme to bind and convert the substrate into a product. On large scale deviation changes, changes in pH will result in enzymes undergoing denaturation due to interference with various noncovalent interactions that maintain stability of the three-dimensional structure of the enzyme. Along with the increase of pH, there is a decrease in MCA activity. At pH 6 and incubation time of 30 minutes RLP from *E. faecium* 1.15 has lost MCA activity. Whereas *Bacillus subitilis* loses MCA activity at pH 5 [15]. Changes in pH change the conformation of the enzyme and affect the binding of the enzyme substrate and catalytic activity on the active side of the enzyme. The results showed that RLP from *E. faecium* 1.15 had stable MCA activity at pH 4.5 to 60 minutes incubation (Fig.4b).

IV. CONCLUSION

Rennin Like Protease (RLP) from *E. faecium* 1.15 has a molecular weight of 42 kDa with optimum activity at pH 4.5 and temperature of 40 ℃ and a MCA/PA ratio value of 3.74. This indicates that RLP has a proteolytic activity that is not too high but has an ability to coagulate milk (casein).

V. ACKNOWLEDGMENTS

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