Proteomics study of extracellular fibrinolytic proteases from *Bacillus licheniformis* RO3 and *Bacillus pumilus* 2.g isolated from Indonesian fermented food

Diana Nur Afifah[1], Ninik Rustanti[1], Gemala Anjani[1], Dahrul Syah[2], Yanti[3], Maggy T. Suhartono[2]

[1]Department of Nutrition Science, Faculty of Medicine, Diponegoro University, Indonesia.
[2]Department of Food Science and Technology, Faculty of Agricultural Technology Bogor Agricultural University, Indonesia.
[3]Faculty of Biotechnology, Atma Jaya Catholic University, Indonesia.

Email: d.nurafifah.dna@fk.undip.ac.id

Abstract. This paper presents the proteomics study which includes separation, identification and characterization of proteins. The experiment on Indonesian fermented food such as extracellular fibrinolytic protease from *Bacillus licheniformis* RO3 and *Bacillus pumilus* 2.g isolated from *red oncom* and *tempeh gembus* was conducted. The experimental works comprise the following steps: (1) a combination of one- and two-dimensional electrophoresis analysis, (2) mass spectrometry analysis using MALDI-TOF-MS and (3) investigation using protein database. The result suggested that there were new two protein fractions of *B. licheniformis* RO3 and three protein fractions of *B. pumilus* 2.g. These result has not been previously reported.

1. Introduction

Proteomics is defined as a comprehensive protein analysis to a particular cell or organism. This study includes separation, identification and characterization of proteins. One of the most effective methods to separate the proteins contained in the complex mixture is by using two-dimensional (2D) gel electrophoresis [1]. This method is employed to separate proteins with two following steps (1) isoelectric point (pI) in the first dimension (IEF, *isoelectric focusing*) and (2) molecular weight (MW) in the second dimension (SDS-PAGE, *sodium dedocyl sulfate polyacrilamide gel*)[2].

2D electrophoresis have ability to separate a large number of proteins, including post-translational modifications. The 2D electrophoresis caused the changes in the molecular charge or weight and unique forms of proteolysis-resulted proteins [3,4]. This technique employed *Immobilized pH Gradient* (IPG) strips made from copolymerization of acids and bases derivatives with different concentrations of acrylamide in polyacrylamide matrix [5].

2D electrophoresis is used to separate the complex protein into single polypeptide component which is the major analysis in proteomics study. To date, 2D electrophoresis technique is the only method for separating thousands of proteins in a single separation stage. Few samples for the analysis are used such as biofluid, tissues, cells, and organelles. A comparison analysis of sample pairs , for

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example comparing normal cells with cells experiencing transformation or comparing multiple cells at different growth stages is presented by [2]. This technique can be used for proteomic study of cancer and a variety specific gene expression analysis from mixed community of prokaryotic microorganisms in the environment [6]. The combination of the method and mass spectrometry analysis of *Matrix Assisted Laser Desorption Ionization Time-of-Flight* (MALDI-TOF), two extracellular serine protease from *Bacillus subtilis* 168, namely WprA (52 kDa) and Vpr (68 kDa) can be detected. These proteins havefibrinolytic activity [7].

Bacillus subtilis produces several extracellular p0roteases in the late exponential growth phase [8]. The main extracellular proteolytic enzymes are subtilysin and neutral protease which are coded as apr and npr gene [9]. Other extracellular proteases are *bacillopeptidase* F, EPR, and Mpr. *Bacillopeptidase* F [10] and protein Epr, which are coded as bpr and epr genes [11], both are serine proteases, while Mpr, coded as mpr gene, include metalloprotease [12].

In this study, proteomics study on extracellular enzymes of *Bacillus licheniformis* RO3 and *Bacillus pumilus* 2.g isolated from Indonesian fermented food, red oncom and tempeh gembus was conducted.

2. Materials and Methods

2.1 Microorganisms

Bacillus licheniformis RO3 (AB968524) was isolated from fermented food of red oncom while *B. pumilus* 2.g (AB968523) was isolated from tempeh gembus [13].

2.2 Preparation of samples

2.2.1 B. licheniformis RO3

B. licheniformis RO3 was grown in ½ *Luria-bertani broth* (LB) + 1% oncom flour (w/v), hereinafter called LBO, for 0, 12, 24, 36, 48, 60, and 72 hours on 120 rpm shake incubator at 37°C. Extracellular enzyme was obtained by 6000 g centrifugation for 15 min at 4°C. The enzymes was then freeze-dried to increase the protein concentration. The protein concentration in the sample was calculated by the method of Bradford [14]. Protease activity was calculated with the method of Bergmeyer et al. [15] with casein substrate. Before conducting mass spectrometry analysis using MALDI-TOF-MS, the protein fractions of extracellular enzyme of this *B. licheniformis* RO3 were analyzed using one-dimensional electrophoresis (SDS-PAGE) and two-dimensional (2D) electrophoresis.

2.2.2 *B. pumilus* 2.g

B. pumilus 2.g was grown in a medium (nutrient broth) (NB) for 72 hours at 120rpm shake incubator at 37°C. Extracellular enzyme was obtained by 12,000 g centrifugation for 10 min at 4°C. Enzymes were then purified with 80% ammonium sulfate precipitation, ion exchanger chromatography with *CM-Sephadex* C-50 (Amersham Pharmacia Biotech, Uppsala, Sweden), and hydrophobic interaction chromatography with *Phenyl Sepharose 6 Fast Flow* (Amersham Pharmacia Biotech, Uppsala, Sweden). At every stage, dialysis was conducted. Pure enzyme was then freeze-dired and ready for further analysis. The protein concentration in the sample was calculated by the method of Bradford [14]. Fibrinolytic activity was calculated by the method of Jeong et al.[16] with fibrin substrate. Before conducting mass spectrometry analysis using MALDI-TOF MS, the protein fractions of pure enzyme were only analyzed with one-dimensional electrophoresis (SDS-PAGE).

2.3 One-dimensional electrophoresis (SDS-PAGE) and zimogram

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with separator gel with concentration of 12% and barrier gel of 4% [17]. Zimogram was conducted with separator gel with concentration of 12 containing 0.02% fibrinogen or fibrin.

2.4 Two-dimensional (2D) electrophoresis

Electrophoresis method conducted in this study was performed according to *Protean* IEF *Cell Instruction Manual* from BioRad.

Rehydration of Immobilized pH gradient (IPG) strips. A total of 50 µl samples with protein concentrations up to 1 mg mixed with 150 mL of rehydration buffer (8 M urea, 2% CHAPS, 0.2% Bio-Lytes, and 50 mM DTT). The sample mixture was inserted into afocusing tray . Protected surface of IPG strip sheet was opened with tweezers and then inserted into the sample mixture solution, location of positive and negative poles in IPG strip and focusing tray were adjusted, and the surface of IPG strip was facing down. The position of the IPG strip on the *focusing tray* must be balanced. IPG strip wasthen coated with mineral oil as much as 2.5 ml. Furthermore, wicks paper soaked with ddH_2O was pasted on the electrode surface of focusing tray. After ascertaining that there was no bubbles, focusing tray was sealed and IPG strips were incubated for 16 hours in the *Protean* IEF *cell*.

Isoelectric Focusing (IEF). After 16 hours, *running* IEF was performed with the following settings: Voltage I: 250 V, 30 minutes; Voltage II: 4000 V, 1 hour; Voltage III: 4000 V, 3 hours at 10^{o}C. After *running* IEF for 4.5 hours, IPG strips was removed and preparation before the two-dimensional electrophoresis was done.

Prior to one-dimensional electrophoresis (SDS-PAGE) IPG strips were incubated for 15 minutes in 2.5 ml equilibration buffer I with composition as follow: 6 M urea, 0.375 M Tris-HCl, 2% SDS, 20% glycerol, 2% DTT).The incubation process was followed by second incubation process for 15 minutes in 2.5 ml of equilibration buffer equilibrasi II with composition as follow: 6 M urea, 0.375 M Tris-HCl, 2% SDS, 20% glycerol, 2.5% iodoacetamida. During the incubation process in the equilibration buffer, polyacrylamide separator gel was prepared initially for SDS-PAGE without using barrier gel. Furthermore, equilibration buffer was discarded and IPG strip was ready to be running on a second dimension with SDS-PAGE electrophoresis at a voltage of 100 volts for 2 hours in electrophoresis buffer. After electrophoresis, staining with *Coomassie Brilliant Blue* R-250 dye solution was conducted.

2.5 Spectrometric analysis of MALDI-TOF-MS

Digestion from proteins point resulted from 2D Electrophoresis or protein ribbons resulted from SDS-PAGE in gel was performed using the method of Lee et.al. [18]. All mass spectrometry analyzes were performed using MALDI-TOF-MS Voyager Biospectrometry Workstation (PE Biosystems, Foster City, CA, USA). Spectra was analyzed with data Explorer software (PE Biosystems) and compared to databases fromNational Center of Biotechnology Information (NCBI) using *MASCOT peptide mass fingerprinting* program (http://www.matrixscience.com). Protein score more than 74 was stated as identical to particular protein ($p < 0.05$).

2.6 Estimation of molecular weight (MW) and isoelectric point (pI)

The molecular weight of protein fraction was calculated based on the standard curve equation of protein molecule weight, the protein fraction isoelectric was calculated based on the standard curve equation of isoelectric point. The calculation results of MW and pI were used to verifythe results of MALDI-TOF-MS, i.e. by entering the calculation result of MW and pI into ExPASy-TagIdent program (http://web.expasy.org/tagident/).

3. Results and Discussion

The result of one-dimensional electrophoresis study (SDS-PAGE) of extracellular protease from *B. licheniformis* RO3 with LBO production media is depicted in Figure 1. The result shows that the protein fraction ribbon was not visible in 0 hour fermentation.Some ribbons began visible in the 12th hour fermentation. The significant appearance of the protein ribbons was observed during the 24th hour fermentation. However, a number of protein fraction ribbons was stable during the 36th to 72nd hour fermentation..

Critical parameter that determines the success of proteomics study is the ability to obtain a single protein in a complex mixture.

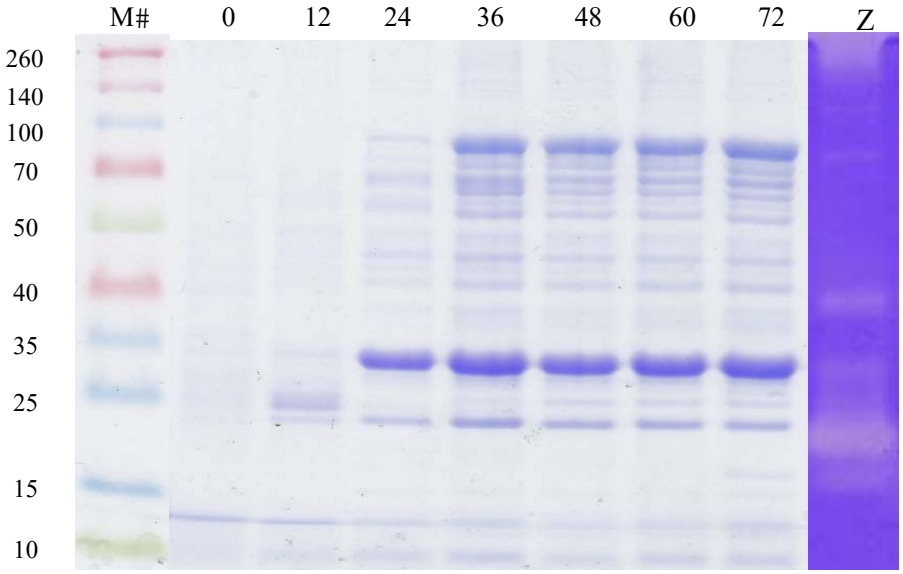

Figure 1. The results of one-dimensional electrophoresis (SDS-PAGE) of extracellular protease from *B. licheniformis* RO3 with LBO media. M: Fermentation marker, Z: zimogram results with fibrinogen substrate

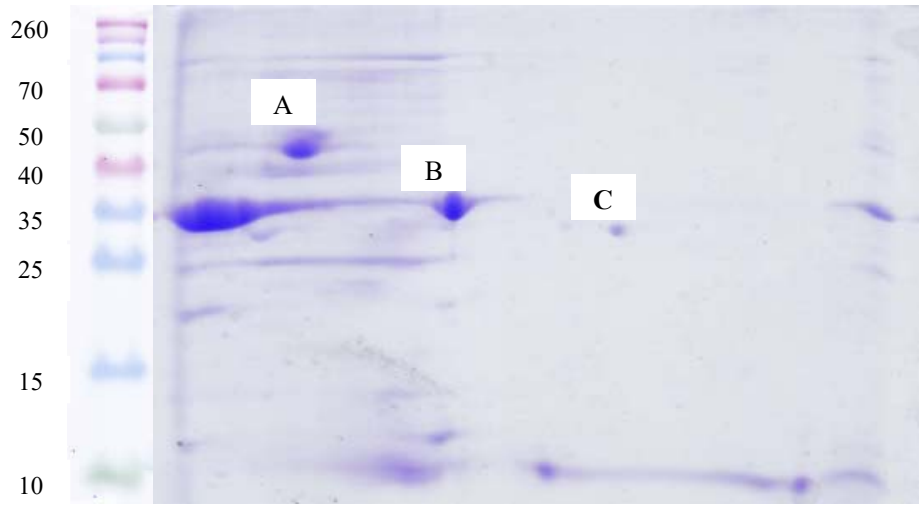

Figure 2. The results of two-dimensional electrophoresis of *B. licheniformis* RO3 with LBO media

One of the most effective ways to separate proteins in a complex mixtures is by using two-dimensional electrophoresis. In this study, two-dimensional electrophoresis was performed on extracellular protease which was fermented in LBO media for 48 hours, which was when they achieved the highest enzyme activity, 0.283 U/mg and several fibrinogenolitik extracellular protease

enzymes were formed (Figure 1). Identification using MALDI-TOF resulted from two-dimensional electrophoresis was applied to three protein points: A, B, and C (Figure 2).

The results of MALDI-TOF-MS, followed by the identification of proteins showed fromthe three points that were tested, only one point which had protein score of more than 74, i.e point B with protein score of 120 and it was identical to *flagellin* of *Bacillus licheniformis* DSM 13. *Flagellin* is globular protein that regulates itself in the hollow cylinder to form filaments in bacterial flagellum. *Flagellin* has molecular weight of 30,000 to 60,000 dalton. Flagellin is a major substituent of bacterial flagella, and presents in large numbers in almost all flagelled-bacteria [19].

Table 1. Comparison of m/z protein of protease *B. licheniformis* RO3 with database

Code	MW(kDa)	pI	Similarity				
			Accsess Code	Protein Score	Name	MW (kDa)	pI
A	48	4.80	A4IQG0	31	*Superoxide dismutase* [*Geobacillus thermodenitrificans* NG80-2] YP_001126309.1 [a]	52	4.96
			G4NXJ1	29	*Response regulator aspartate phosphatase* F [*Bacillus subtilis* subsp. *spizizenii* TU-B-10] YP_004879327.1 [a]	46	5.34
			Q65JX1	27	*Bacillopeptidase* F [*Bacillus licheniformis* DSM 13 = ATCC 14580] YP_006713127.1 [a]	155	5.18
B	37	5.38	Q65EB8	120	*Flagellin* [*Bacillus licheniformis* DSM 13 = ATCC 14580] YP_006715058.1 [a]	33	5.41
C	33	5.99	C8WV29	43	*Short-chain dehydrogenase/reductase* SDR [*Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* DSM 446] YP_003184407.1 [a]	29	6.02
			L0BRU6	29	*Hypothetical protein* B938_15200 [*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3] YP_007187718.1 [a]	13	5.08

[a] NCBI *Reference Sequence*

Points A and C which are resulted from 2D electrophoresis have certain similarities with particular protein, with protein score of less than 74. Point A, hereinafter referred to as fraction A is possibly *bacillopeptidase* DSM F because it has similarities with bacillopeptidase F of *B. licheniformis* DSM 13 with protein score of 27. Based on the result of molecular weight (MW) and pI with standard curves, fraction A has MW of 48 kDa and pI of 4.80. While bacillopeptidase F from *B. licheniformis* DSM 13 has MW of 155 kDa and pI of 5.18.

Bacillopeptidase F was first discovered by Roitsch & Hageman [10] which was serine endopeptidase excreted by *Bacillus subtilis* 168 after the end of exponential growth. The enzyme is active at pH 10 and inhibited by PMSF inhibitor. There were two bacillopeptidase F enzymes which

were successfully isolated, one of them hasmolecular weight of 33 kDa, and the other had molecular weight of 50 kDa. Both forms of enzyme is glycoprotein, which is smaller and having pI of 4.4, while the larger has pI of 5.4. Bacillopeptidase F successfully isolated by Roitsch & Hageman [10] evidentlydifferent from all the other proteases from *B. subtilis. Bacillopeptidase* F of *B. licheniformis* KJ-31, because it was fibrinolytic as identified by Hwang et al. [20] and it hasMW of 37 kDa. *Bacillopeptidase* F of B. *subtilis natto* that was fibrinolytic also has been identified by Hitosugi et al. [21] and it hasMW of 34.1 kDa.

Point C, hereinafter referred to as fraction C is probably uncharacterized proteins because it is similar to *hypothetical protein* from *B. amyloliquefaciens* subs. *plantarum* AS43.3 with protein score of 29. Fraction C has MW of 33 kDa and pI of 5.99.

The result of identification using MALDI-TOF-MS also verifiedby comparing the result of the molecular weight (MW) and pI calculations using ExPASy-TagIdent program (http://web.expasy.org/tagident/).

It turns out that fractions A and C do not have similarities to proteins that exist in UniProtKB/Swiss-Prot database. These results indicate that both protein fractions in extracellular protease of *B. licheniformis* RO3 is new or different from those already reported.

In one-dimensional electrophoresis study (SDS-PAGE) of extracellular protease from *B. pumilus* RO3 with NB production medio, some protein fractions characterized by the presence of several ribbons were visible. However, only three factions that were continued to be identified. The measured fibrinolytic protease activity of this enzyme is 1,442.3 U/mg.

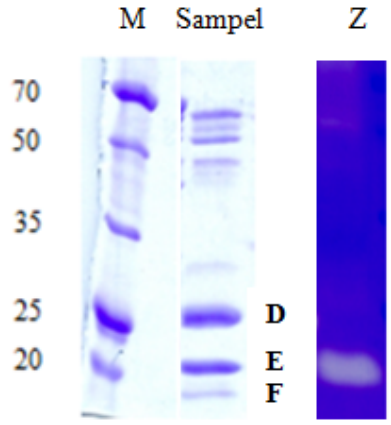

Figure 3. Extracellular fibrinolytic protease of *B. pumilus* 2.g
M: marker, Z: zimogram with fibrin substrate

The result of identification showed that of the three ribbons that were tested, none of theprotein score more than 74. Thus, none of the ribbon has similarities to the existing proteins database. This is inline with the characterization results of fibrinolytic enzyme produced by *B. pumilus* 2.g [22]. Fibrinolytic enzyme is a serine protease but has MW of 20 kDa. Generally, protease serine having fibrinolytic nature has MW of 27 kDa [23,24]

A number of researchers has presenteda genomic study to *Bacillus subtilis*. Moreover, the researchers has identified 11 different extracellular proteases [25]. Study conducted by Park et.al. [7] was able to detect two extracellular serine protease having fibrinolytic activity of *Bacillus subtilis* 168, i.e. WprA (52 kDa) and VPR (68 kDa).

In this study, one protein fractions namely flagellin was identified. However flagellin is not extracellular protease. Thus, the other potential protein to be tested are new protein fractions which

have not been reported and have fibrinolytic protease activity. This new protein fraction may be *bacillopeptidase F* and other extracellular proteases.

Table 2. Comparison of m/z protein of protease from *B. pumilus* 2.g with database

Code	MW (kDa)	Similarities				
		Protein score	Access Code	Name	MW(kDa)	pI
D	23	52	C8WXL1	*Septum site-determining protein* MinD [*Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* DSM 446] YP_003185221.1 [a]	29	6.02
		42	M4HMH2	*Hypothetical protein* BCK_26788 [*Bacillus cereus* FRI-35] YP_006599369.1 [a]	21	8.80
		41	F8IDH7	*Septum site-determining protein* MinD [*Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* Tc-4-1] YP_005518349.1 [a]	29	6.85
		40	G4NRL1	*Hypothetical protein* GYO_1184 [*Bacillus subtilis* subsp. *spizizenii* TU-B-10] YP_004876473.1 [a]	25	7.58
E	20	49	Q65117	*Zn-finger protein* [*Bacillus licheniformis* DSM 13 = ATCC 14580] YP_079408.1 [a]	19	4.83
		43	K0G197	CTP *synthase* [*Bacillus thuringiensis* MC28] YP_006831249.1 [a]	47	6.24
F	15	25	F2F9E3	*Permease* [*Solibacillus silvestris* StLB046] YP_006462816.1 [a]	94	9.48

[a] NCBI *Reference Sequence*

It has been mentioned earlierthat the most important thing of proteomics is in the separation process of complex proteins into a single protein fraction. Referring to the further study, it is needed to ensure the existence of some new protein fraction, especially on the stage of protein separation.

4. Conclusion

An experimental study of extracellular fibrinoltic proteases proteomics from *Bacillus licheniformis* RO3 and *Bacillus pumilus* 2.g isolated from Indonesian fermented food has been presented. The *Bacillus licheniformis* RO3 and *B. pumilus* 2.g can produce several extracellular proteases. The result of identification using MALDI-TOF-MS shows that there are five new protein fractions contained in extracellular fibrinolytic protease produced by *Bacillus licheniformis* RO3 (2 fractions) and *B. pumilus* 2.g (3 fractions).

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