Characterization of Protease Crude Extract from Indigenous Lactic Acid Bacteria and the Protein Degradation Capacity in Local Tuber and Cereal Paste Flour

Tatik Khusnati1, Nanda Sabbaha Nur Kasfillah2, Vilya Syafriana3, Resti Sofia Zahara3, Padmono Citoreksoko4, Sulistiani1 and Trisanti Anindyawati4

1) Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences, JL. Raya Bogor Jakarta Km 46, Cibinong 16911, Indonesia
2) Pharmacy Faculty, National Science and Technology Institute, Jakarta
3) Pharmacy Study, Pharmacy and Industrial Technology High School, Bogor
4) Research Center for Biotechnology, Indonesian Institute of Sciences

*Corresponding author: tatikkhusni@yahoo.com

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Abstract
Protease hydrolyzed protein in flour in order to more digest by human ulcer. Lactobacillus plantarum B110 and Lactobacillus satsumensis are indigenous lactic acid bacteria that produce protease. The objective of this research is to characterization of protease crude extract from indigenous lactic acid bacteria and the protein degradation capacity in local tuber and paste flour. Tuber and cereal flour used were purple sweet potato (Dioscorea alata), cassava (Manihot esculenta), rice (Oryza sativa), corn (Zea mays) and wheat (Triticum) as comparison. Protease activity was tested by Horikoshi method (1971) and protein degradation was by formol titration. Research results showed that optimum activities and stabilities of Lactobacillus plantarum B110 were at pH: 7.5, 45°C and pH: 5.0-8.0, 35-50°C, while that L. satsumensis EN 38-32 were at pH: 7.0, 40°C and pH: 6.0-8.0, 20-45°C. Increases in protein degradation capacity of the flour protein additional proteases crust extract from L. plantarum B110 were 0.0838% (purple sweet potato), 1.3299% (cassava), 0.5834% (corn), 0.7499% (rice) and 1.5511% (wheat as comparison); while that L. satsumensis EN 38-32 were 0.20% (purple sweet potato), 0.32% (cassava), 0.87% (corn), 1.17% (rice). Based on increases in protein degradation capacity, protease crude extract from L. plantarum B110 and L. satsumensis EN 38-32 were sequentially better to hydrolyze protein of cassava and rice paste flour than that other tuber and cereal.

Keywords:
degradation protein, protease, L. plantarum B110, L. satsumensis EN 38-32, paste flour of local tuber and cereal

I. INTRODUCTION
Protease can be used to hydrolyze protein in tuber and cereal flour to peptide in order to more digest by human ulcer. Lactic acid bacteria, such as: Lactobacillus plantarum S31 and Lactobacillus delbrueckii subsp. lactis CRL 581 produced protease (Budiarto et al., 2016; Villegas and Brown, 2014)

The protease activities in hydrolyzing protein in tuber and cereal flour were affected by the type of the bacteria producing protease (Sawant and Nagendran, 2014; Thiele et al., 2002). Furthermore, the different type of tuber and cereal flour caused the different concentration of protein hydrolyzed by microbial proteases in those two flour (Li et al., 2012; Adeniji, 2013).

The protein in tuber and cereal flour were hydrolyzed by the microbial protease activities to simple compounds of peptides (Endo and Okada, 2005; Ganzle et al., 2008). The peptides produced depend on the type of tuber and cereal flour used.
Some species of Lactic acid bacteria (LAB) had ability to produce protease, such as: Lactococcus lactis, Lactococcus cremoris and Lactococcus garviae (Adi and Guessas, 2016), Lactococcus lactis 1598, Streptococcus thermophilus t3D1, Lactobacillus lactis 1043 and L. delbrueckii subsp. bulgaricus b38, b122 and b24 (Atanasova et al., 2014). However, LAB species of Lactobacillus plantarum B110 and Lactobacillus satsumensis EN38-32 producing protease which have potency to degrade protein in local tuber and cereal flour haven’t been known yet.

The objective of this research was to characterization of protease crude extract from indigenous lactic acid bacteria and the protein degradation capacity in local tuber and cereal paste flour

2. EXPERIMENTAL

2.1. Production of protease (Tennalli et al., 2012 modified)

The 2% inoculum culture was poured into 50 mL media of nutrient broth with addition of 1% casein and it was incubated at temperature 37°C for 48 hours. The production media was then centrifuged at 3500 rpm for 15 minutes. Supernatant found was protease crude extract.

2.2. Optimization of protease activity in various pH (Tennalli et al., 2012 modified)

Optimization of protease activity was conducted in pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The activity test was conducted at temperature: 37°C and incubation time for 10 minutes. The highest protease activity in certain pH was stated as optimum protease activity.

2.3. Optimization of protease activity in various temperatures (Tennalli et al., 2012 modified)

Optimization of protease activity was conducted in various temperatures: 20, 25, 30, 35, 40, 45, and 50°C. The activity test was conducted in optimum pH, with incubation time for 10 minutes. The highest protease activity in certain temperature was stated as optimum protease activity.

2.4. Stability of protease in various pH (Eijsink et al., 2005; Moran et al., 2012)

Stability of protease in various pH were conducted by measuring protease relative activities in pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The activity test was conducted in optimum temperature with incubation time for 60 minutes. Protease relative activities with values: ≥ 50% showed that those activities were in stable condition.

2.5. Stability of protease in various temperatures (Eijsink et al., 2005; Moran et al., 2012)

Stability of protease in various temperatures was conducted by measuring protease relative activities in temperatures: 20, 25, 30, 35, 40, 45 and 50°C. The activity test was conducted in optimum pH with incubation time for 60 minutes. Protease relative activities with value: ≥ 50% showed that those activities were in stable condition.

2.6. Production of tuber and cereal paste flour additional protease

Tuber and cereal flour used were purple sweet potato (Dioscorea alata), cassava (Manihot esculenta), rice (Oryza sativa), corn (Zea mays) and wheat (Triticum) as comparison. One (1) gram for each flour from wheat, purple sweet potato, cassava, rice, and corn was soluted in 10 mL aquadest, heated at temperature 70°C with agitation. Heating was conducted up to paste flour formed. Each supernatant (1 unit crude protease) from L. plantarum B110 and L. satsumensis EN 38-32 was added into 10 mL paste flour and incubated in shaker incubator with 100 rpm, at temperature 40°C, for 24 hours. Paste flour treated was then analyzed its protein degradation.
2.7. Protease activity (Horikoshi modified, 1971)

Protease activity test was conducted by method of Horikoshi (1971) modified. The 0.2 mL protease was poured into reaction tube, added 0.4 mL 2% casein and 0.4 mL buffer phosphate 0.05 M pH 8. The mix was incubated at temperature 37°C for 10 minutes, added 1 mL 20% TCA and homogenized. Incubation was continued at temperature 37°C for 10 minutes, and solution was centrifuged by rotation 3500 rpm for 5 minutes. Control was made. One unit protease was defined as the amount of mL protease needed to produce 1 μmol tiroisin every minute with casein as substrate.

2.8. Protein degradation (U.S.P., 1989 in Nilsang et al., 2005).

Protein degradation was tested by formol titration. Sample of 10 mL treated paste flour was added phenolphthalein, and neutralized by NaOH 0.1 N solution. The 10 mL of 37% Formaldehyde was added into the solution, titrated by standard solution of NaOH 0.1 N up to the color change to pink. The protein degradation was then calculated as % Nitrogen x Fc (Conversion Factor).

3. RESULTS AND DISCUSSION

Research results showed that the values of protease activities of Lactobacillus plantarum B110 in various pH 4.5-8.0 were in the range 0.4488 – 0.8995 U/mL (Table 1) and in various temperatures: 20-50°C were 0.4415 - 1.0357 U/mL (Table 2) with relative activities at pH 4.5-8.0 were in the range 47.56 – 100% (Table 3) and at temperatures: 20-50°C were 40.57 - 100% (Table 4).

The optimum protease activity of Lactobacillus plantarum B110 was reached at pH: 7.5 (0.8995 U/mL)(Table 1) and temperature: 45°C (1.0357 U/mL) (Table 2) with stabilities at relative activity ≥ 50% were at pH 5.0-8.0 (Table 3) and temperatures: 35-50°C (Table 4).

| Table 1. Protease Activity from L. plantarum B110 in Various pH |
|----------------------|----------------------|
| pH       | Protease Activity (U/mL) |
|----------|--------------------------|
| 4.5      | 0.4488 a                |
| 5.0      | 0.4966 a                |
| 5.5      | 0.5408 b                |
| 6.0      | 0.7432 c                |
| 6.5      | 0.7542 c                |
| 7.0      | 0.8848 d                |
| 7.5      | 0.8995 d                |
| 8.0      | 0.6567 bc               |

| Table 2. Protease Activity from L.plantarum B110 in Various Temperatures |
|----------------------|----------------------|
| Temperature | Protease Activity (U/mL) |
|-----------------|--------------------------|
| 20               | 0.4415 b                |
| 25               | 0.4893 b                |
| 30               | 0.5545 b                |
| 35               | 0.5868 b                |
| 40               | 0.6070 b                |
| 45               | 1.0357 *                |
| 50               | 0.7910 b                |

| Table 3. Protease Relative Activity from L. plantarum B110 in Various pH |
|----------------------|----------------------|
| pH       | Protease Activity (U/mL) | Protease Relative Activity (%) |
|----------|--------------------------|-------------------------------|
| 4.5      | 0.4230                   | 47.56                         |
| 5.0      | 0.4893                   | 55.01                         |
| 5.5      | 0.5390                   | 60.60                         |
| 6.0      | 0.7082                   | 79.62                         |
| 6.5      | 0.7229                   | 81.27                         |
| 7.0      | 0.8701                   | 97.82                         |
| 7.5      | 0.8895                   | 100                           |
| 8.0      | 0.6457                   | 72.59                         |

| Table 4. Protease Relative Activity from L. plantarum B110 in Various Temperature |
|----------------------|----------------------|
| Temperature | Protease Activity (U/mL) | Protease Relative Activity (%) |
|-----------------|--------------------------|-------------------------------|
| 20               | 0.4157                   | 40.57                         |
| 25               | 0.4304                   | 42.01                         |
| 30               | 0.4525                   | 44.16                         |
| 35               | 0.6052                   | 59.07                         |
| 40               | 0.6144                   | 59.97                         |
| 45               | 1.0246                   | 100                           |
| 50               | 0.7744                   | 75.58                         |
The values of protease activities of *L. satsumensis* EN 38-32 in various pH 4.5-8.0 were in the range 0.2032 – 0.4574 U/mL (Table 5) and in various temperatures of 20-50°C were 0.2385 – 0.7214 U/mL (Table 6) with relative activities at pH 4.5-8.0 were in the range 34.10 - 100% (Table 7) and at temperatures: 20-50°C were 42.92 - 100% (Table 8).

**Table 5. Protease Activity from *L. satsumensis* EN 38-32 in Various pH**

| pH  | Protease Activity (U/mL) |
|-----|--------------------------|
| 4.5 | 0.2032 *a*               |
| 5.0 | 0.2482 *a*               |
| 5.5 | 0.3383 *b*               |
| 6.0 | 0.3834 *d*               |
| 6.5 | 0.3737 *ed*              |
| 7.0 | 0.4574 *e*               |
| 7.5 | 0.3480 *hc*              |
| 8.0 | 0.3222 *b*               |

**Table 6. Protease Activity from *L. satsumensis* EN 38-32 in Various Temperatures**

| Temperature (°C) | Protease Activity (U/mL) |
|------------------|--------------------------|
| 20               | 0.2385 *a*               |
| 25               | 0.3222 *b*               |
| 30               | 0.4896 *c*               |
| 35               | 0.5637 *c*               |
| 40               | 0.7214 *d*               |
| 45               | 0.3866 *b*               |
| 50               | 0.3576 *b*               |

**Table 7. Protease Relative from *L. satsumensis* EN 38-32 in Various pH at 60 minutes incubation time**

| pH  | Protease Activity (U/mL) | Protease Relative Activity (%) |
|-----|--------------------------|--------------------------------|
| 4.5 | 0.0430                   | 34.10                          |
| 5.0 | 0.0478                   | 37.90                          |
| 5.5 | 0.0575                   | 45.60                          |
| 6.0 | 0.0773                   | 61.30                          |
| 6.5 | 0.0870                   | 68.99                          |
| 7.0 | 0.1261                   | 100.00                         |
| 7.5 | 0.0795                   | 63.05                          |
| 8.0 | 0.0816                   | 64.71                          |

The optimum protease activity of *L. satsumensis* EN 38-32 was reached at pH: 7.0 (0.4574 U/mL) (Table 5) and temperature: 40°C (0.7214 U/mL) (Table 6), with stabilities at relative activities ≥ 50% were at pH 6.0 - 8.0 (Table 7) and temperatures: 20 - 45°C (Table 8).

The different optimum activities and stabilities between protease from *Lactobacillus plantarum* B110 and *L. satsumensis* EN 38-32 were due to the different species of both lactic acid bacteria producing protease. It has been reported that the different species of bacteria may have resulted in the different characteristics of protease produced (Eijink, 2005; Hayek and Ibrahim, 2013, Naidu, 2011, Sulthoniyyah et al., 2015). The optimum temperature and pH of protease *B. subtilis* were in the range from 40°C to 50°C and pH 8, respectively (Naidu, 2011), while that *Lactococcus* species were 37°C and pH 7.2 (Addi and Guessas, 2016).

The protein degradation concentration of the tuber and cereal paste flour with addition of *L. plantarum* B110 protease crude extract were 1.2570% (purple sweat potato), 1.8077% (cassava), 1.9305% (rice), 1.7506 (corn), and 1.5551% (wheat as comparison) (Table 9).

The increases of protein degradation concentration of the tuber and cereal paste flour additional *L. plantarum* B110 crude protease were 0.0838% (purple sweat potato), 1.3299% (cassava), 0.7499% (rice), 0.5834% (corn), and 1.5551% (wheat as comparison) (Table 9).

The increasing of protein degradation concentration from the treated cassava paste
flour was highest than that the other local tuber and cereal paste flour (Table 9).

**Table 9.** Protein Degradation Concentrations of Local Tuber and Cereal Paste Flour With and Without *L. plantarum* B110 EN 38-32 Protease

| Tuber Paste Flour | Protein Degradation Concentration (%) | Increase of Protein Degradation (%) |
|-------------------|---------------------------------------|-------------------------------------|
| Wheat             | Control*: 1.2771                      | Sample**: 2.8322                    | 1.5551                             |
| Purple Sweet Potato| Control*: 1.1732                      | Sample**: 1.2570                    | 0.0838                             |
| Cassava           | Control*: 0.4778                      | Sample**: 1.8077                    | 1.3299*                            |
| Rice              | Control*: 1.1806                      | Sample**: 1.9305                    | 0.7499                             |
| Corn              | Control*: 1.1672                      | Sample**: 1.7506                    | 0.5834                             |

Notes:
* Paste flour without addition of *L. plantarum* B110 protease
** Paste flour with addition of *L. plantarum* B110 protease

The protein degradation concentration of the tuber and cereal paste flour additional *L. satsumensis* EN 38-32 crude protease were 0.7300 (purple sweet potato), 0.7900 (cassava), 2.1900% (rice), 1.9600 (corn), and 1.5551% (wheat as comparison)(Table 9).

The increases of protein degradation concentration of the tuber and cereal paste flour additional *L. satsumensis* EN 38-32 crude protease were 0.20% (purple sweet potato), 0.32% (cassava), 0.87% (corn), 1.17% (rice) and 1.83% (wheat as comparison)(Table 10).

The concentration increase of protein degradation of the treated rice paste flour was highest than that the other local tuber and cereal paste flour (Table 10).

The different increases of protein degradation concentration between the tuber (purple sweet potato and cassava) and cereal (corn and rice) paste flour with wheat paste flour (as comparison) additional crude proteases from *L. plantarum* B110 and *L. satsumensis* EN 38-32 were due to the different protease activities from those two lactic acid bacteria in protein hydrolysis of those two flour. It has been reported that the protease activities from the different bacteria species may have resulted in the different hydrolysis of flour protein (Adeniji, 2013; Gupta et al., 2002; Sawant and Nagendran, 2014). The LAB of *Lactococcus lactis* 1598, *Streptococcus thermophilus* t3D1, *Lactobacillus lactis* 1043 and *L. delbrueckii* subsp. *bulgaricus* b38, b122 and b 24 had high proteolytic activity in the formation of peptides with molecular weight between 5 and 10 kDa (Atanasova et al., 2014).

**Table 10.** Protein Degradation Concentrations of Local Tuber and Cereal Paste Flour With and Without *L. satsumensis* EN 38-32 Protease

| Tuber Paste Flour | Protein Degradation Concentration (%) | Increase of Protein Degradation (%) |
|-------------------|---------------------------------------|-------------------------------------|
| Wheat             | Control*: 1.2771                      | Sample**: 3.8322                    | 1.5551                             |
| Purple Sweet Potato| Control*: 0.5300                      | Sample**: 0.7300                    | 0.2000                             |
| Cassava           | Control*: 0.4700                      | Sample**: 0.7900                    | 0.3200                             |
| Rice              | Control*: 1.0200                      | Sample**: 2.1900                    | 1.1700*                            |
| Corn              | Control*: 1.0900                      | Sample**: 1.9600                    | 0.8700                             |

Notes:
* Paste flour without addition of *L. satsumensis* EN 38-32 protease
** Paste flour with addition of *L. satsumensis* EN 38-32 protease

4. CONCLUSION

The characterization of protease crude extract from indigenous lactic acid bacteria and its protein degradation capacity in local tuber and cereal paste flour showed that the optimum activity and stability of proteases from *Lactobacillus plantarum* B110 were at pH: 7.5, 45°C and pH:5.0-8.0, 35-50°C, while that *L. satsumensis* EN 38-32 were at pH: 7.0, 40°C and pH:6.0-8.0, 20-45°C. The maximum increase of protein degradation concentration of the tuber and cereal paste flour (with wheat paste flour as comparison) additional proteases crude extract from *L. plantarum* B110 was at cassava with value of 1.3299%, while that *L. satsumensis* EN 38-32 was at rice with 1.1700%. Based on the increases of protein degradation concentration, proteases crude extract from *L. plantarum* B110 and *L. satsumensis* EN 38-32 were sequently better to hydrolyze protein of cassava and rice.
paste flour than that the other tuber and cereal paste flour.

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