Isolation and Biochemical Characterization of Extracellular Proteolytic Enzyme From Lactic Acid Bacteria Isolated From Various Indonesian Traditional Fermented Product (Bakasam)

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Abstract. Local isolates of lactic acid bacteria isolated from Bakasam had potential to producing extracellular proteases. The research was carried out with the purpose of knowing the biochemical character of protease produced from isolates of lactic acid bacteria. The results showed extracellular protease isolates BAL 2 (Lactobacillus acidophilus) has a specific activity i.e. of 0.89 U/mg. Biochemical characterization of proteases indicates optimum pH 5.5, and is inhibited by chelating agent EDTA. The result of the purification with Sephadex G-100 column showed a band on SDS-PAGE with the size of molecular weight approximately 90 kD.

1. Introduction

Food processing industry in Indonesia is heavily depends on importing enzyme protease, so it inhibits the development of the livestock product food industry. Development of enzyme technology to replace the independence is necessary to support national food industry. The enzyme protease produced by the microbes can be used in lieu of enzymes from plant sources. One of the microbes that can be utilized is lactic acid bacteria. Indonesia has a germplasm microorganisms which are priceless. Lactic acid bacteria (LAB) as "friendly bacteria" has a great potential in the development of biotechnology-based food industry.

LAB has the ability to produce proteolytic enzymes around the cell wall, membrane, the cytoplasm within the cell, and Lactobacillus acidophilus has the capability of generating extracellular protease [1]. Lactobacillus acidophilus, during the fermentation process, utilize peptides or free amino acids for its growth, this bacteria are also capable of hydrolysing casein by using protease which is excreted in the vicinity of the surface of the cell wall [2].

Some research has been conducted to find out the biochemical characters of intracellular protease of some lactic acid bacteria such as Lactobacillus bulgaricus and Streptococcus thermophilus, while biochemical characters of intracellular protease as well as extracellular of isolates originating from Indonesia have not been much researched. Protease is an enzyme capable of hydrolysing peptide bonds in proteins. Based on Nomenclature Committee of The International Union of Biochemistry, protease are classified in hydrolase enzymes (EC.3.4). Some commercial proteases producers are genus Bacillus (B. cereus, B. pumilus, B. subtilis, B. licheniformis, B. stearothermophilus, and B. polymixa. Another group of bacteria are Aeromonas, Lactobacillus, Pseudomonas, Serratia, Streptomyces, and Staphylococcus. The aim of the study was to isolate and biochemical characterization of extracellular proteolytic enzyme from lactic acid bacteria isolated from various indonesian traditional fermented product (Bakasam).
2. Material and methods

2.1. Screening the proteolytic activity of LAB Isolates
Isolates grown in MRS media that had been added 2% skimmed milk. Subsequently incubated 37 °C for 24 hours. Isolates that had a high activity of extracellular proteolytic will show a clear zone surrounding colonies. Isolates that show width clear zone will be taken as isolates to do testing of their proteolytic activity quantitatively and their protein levels.

- Purification and Biochemical
- Characterization of Extracellular
- Proteolytic Enzymes

2.2. Protein Concentration Assay (Bradford, 1976)
Protein concentration was measured using a standard protein Bovine Serum Albumin (BSA) with the concentration of 0.1 to 1 mg/ml. As many as 100 µl of the sample plus 5 ml of Bradford reactant, homogenized and incubated for 5 minutes at 37 °C and then its absorbance was measured with a spectrophotometer at 595 nm.

2.3. Proteolytic activity assay (Walter,1984)
The activity of the protease was measured based on the method [3], single unit activity is expressed in number of enzymes that are able to generate one micro mol tyrosine per minute.

2.4. Ammonium Sulfate Precipitation
To precipitate protein, gradually added ammonium sulfate to supernatant results of centrifugation while stirring with a magnetic stirrer with a concentration (30%, 40%, 45%, 60%) and left on for one night at 4oC.

2.5. Gel Filtration Chromatography
Equilibrated gel filtration chromatography column of Sephadex G-100 with the stream buffer Tris HCl 10 M as much as 200 ml and rest overnight at a temperature of 4 °C. 2 ml Protease solution added with dropper vertically into column right above the surface of the gel. Enzymes were allowed to flow slowly until the entire enzyme was under the surface of the gel. The column is filled with buffer Tris HCl pH 8 and 10 mM as elution. As many as 20 oil fraction by as much as 70 drops on each fraction using the fraction collector.

2.6. The optimum pH of extracellular protease enzymes
Determined by changing the universal buffers to the desired pH on 50 ºC. pH value tested between 6-10. Enzyme activity is measured by [3].

2.7. Determination of Protease Molecular Weight
Molecular weight of highest activity protease and high activity antimicrobial proteins determined with SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrilamide Gel Elektrophoresis)

2.8. Identification with 16S rDNA
The identification was done molecularly using specific primer and amplification of DNA fragments using PCR (Polymerase Chain Reaction). Electrophoresis has done on the results of amplification fragments using agarose and sequencing was done on isolated fragments. Analysis has done on the result of the sequencing with BLAST software.
3. Results and Discussion

3.1. Screening the proteolytic activity of LAB Isolates
Isolation and purification of LAB isolates from various animal food source which was a protein-rich substrates, so it was expected that obtained LAB isolates have a high potential in generating extracellular protease. Isolation from fermented food products namely Bakasam (made from beef and chicken meat). Retrieved two LAB isolates potential with proteolytic activity, BAL 1 (source isolation Bakasam beef) and BAL 2 (source isolation Bakasam chicken meat).

Figure 1. (A) LAB Isolate 1; (B) LAB Isolate 2.

3.2. The Growth of The BAL 1 Isolates and Its Proteolytic Activity
Growth and protease production, observations was carried on the cell suspension turbidity data (wavelength 600 nm) measured in every certain time lapse, beside that, activity of proteases and protein levels on cell suspension was measured.

Figure 2. (A) Isolate Growth BAL 2 And The Specific Activity Of Proteases Produced.
Based on the data in Figure 2. It was seen that the growth curve beginning with the initial phase (lag) which was a period of microbes adjustment. On that phase enzyme synthesis was occurred by cell used for metabolic pathway. After the initial phase was completed, cellular reproduction started to happen. Cellular concentration was increasing, slowly it was increasing until reaching maximum and logarithmic growth occurred or exponential. After substrate and certain compounds which was required for bacterial growth in media culture run out and product inhibitor occurred, then the rate of the growth of bacteria declined. Decline phase was characterized by a reduced number of live cells (viable) in the media due to the occurrence of death (mortality). BAL 2 isolates execrated extracellular protease with the highest activity after 18 hour (0.83 U/mg) of the bacteria growth period (logarithmic growth phase). The data production time of data can be used to do enzymes production in a larger scale.

3.3. Ammonium sulfate precipitation and Dialysis
Before purification by gel filtration, protease was concentrated using ammonium sulfate. Precipitation used salt based on the solubility of proteins which polar interact with water molecules, ionic interactions of proteins with salt, and repulsive force from same charged protein. The solubility of proteins (in particular temperature and pH) increasing in the concentration of salt (salting in). The increasing in the solubility of the protein will increase the power of ion solution. The addition of certain salts would cause protein solubility decrease (salting out). Water molecules that bind with the salt ions was increasing that eventually led to the withdrawal of the sheath that surrounded the water surface, thus causing the protein, aggregated, and then settled. Ammonium sulfate is a salt that is most often used to precipitate proteins because it has high solvency in water, relatively inexpensive.

![Figure 3. The Precipitation of Protease Isolates BAL 2 with Ammonium Sulfate.](image)

Protease BAL 2 Isolates which was result from 45% ammonium sulfate precipitation; showed the highest specific activity of proteases, namely 1.42 U/mg, so that was used for purification. The impact of mineral salts and excess of ammonium sulfate in the enzyme was removed by using the dialysis method.

3.4. Gel Filtration Chromatography
The selection of the column chromatography method based on the nature of separated enzyme proteins. Information about the characterization of the enzymes was needed, such as the approximate molecular weight, degree of hydrophobicity, and the sulfidril bond.

Results of Sephadex G-100 column produced a widen protein peak. The fraction that had the highest protease activity was the 7th fraction with a specific activity of 4.10 U/mg. Purification of protease BAL 2 Isolates with Sephadex G-100 column gives the level of purity to 4.60 times rough extract with the rate of 32.8%. The rate of under 50% estimated because the levels of a protein that had a protease activity
is lower than the other cell excreted protein. Protein loss during purification could occur because autolysis (Scopes, 1987), and this happens because of the effect of dilution of the enzyme.

### Table 1. Purification of protease Isolates BAL 2.

| Purification Step     | Volume (ml) | Total protein (mg) | Total Activity (Unit) | Specific Activity (Unit/mg) | Purity (%) | Yield (%) |
|-----------------------|-------------|--------------------|-----------------------|----------------------------|------------|-----------|
| Crude                 | 100         | 29.67              | 26.4                  | 0.89                       | 1          | 100       |
| (NH4)2SO4 45%         | 15          | 13.17              | 18.7                  | 1.42                       | 1.59       | 70.8      |
| Gel Filtration        | 2           | 2.11               | 8.67                  | 4.1                        | 4.60       | 32.8      |

**Figure 4.** The Precipitation of Protease Isolates BAL 2 with Ammonium Sulfate.

**3.5. SDS-PAGE**

The level of purity of the enzyme could be known by using the technique of polyacrylamide gel electrophoresis, SDS-PAGE. Gel prepared by acrylamide and N,N'-methylene-bis-acrylamide which polymerizes through a free radical mechanism with the help of catalysts for N,N,N',N'-tetrametilen diamine (TEMED) and initiator amoniumpersulfat (APS). Principle analysis of SDS-PAGE is protein separation that is based on the size of the molecule.

On the well is the result of purification with Sephadex G-100 column that shows a Ribbon with molecular weight 90 kD.

**Figure 5.** SDS-PAGE protease Isolates BAL 2.
3.6. The optimum pH of extracellular protease enzymes
pH which changes on a small deviation scale could lead to decreasing enzyme activity due to ionization changes of its functional group. Ionic bonds played an important role in maintaining the active side of the conformation of the enzyme to bind and change the substrate into product. Changes onn large deviation scale, changes in pH will result in enzyme undergoes denaturation due to disruption of various non-covalent interactions that maintain the stability of the three-dimensional structure of the enzyme [4].

![Figure 6. Effect Of pH On The Activity Of Protease Isolates BAL 2 (Temperature of 37°C).](image)

3.7. BAL 2 Isolates Identification
BAL 2 Isolates: Lactobacillus acidophilus (identity 99%). Identifications results showed that BAL 2 isolates Lactobacillus acidophilus.

![Figure 7. Lactobacillus acidophilus (identity 99%).](image)

4. Conclusion
Extracellular proteases BAL 2 isolates (Lactobacillus acidophilus) had a specific activity i.e. of 0.89 U/mg. Biochemical characterization of proteases indicated of optimum pH 5.5, and was inhibited by chelating agent EDTA. The result of the purification with Sephadex G-100 column shows a Ribbon on SDS-PAGE with the size of molecular weight approximately 90 kD. Based on the results of the characterization could provide information for further research in the field of food biotechnology and further studied were needed to review more about the character of the gene that encodes the protease.

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References
[1] Zhao R, Sun J, Mo H, Zhu Y 2007 Analysis of Functional Properties of Lactobacillus acidophilus World J. Microbiol. Biotechnol. 23: 195–200
[2] Thomas T D, Pritchard G G 1987 Proteolytic enzymes from dairy starter cultures Fed. Eur. Microbiol. Soc. Microbiol. Rev. 46: 245
[3] Walter H E 1984 Method with haemoglobin, casein, and azocoll as substrate In. Bergmeyer HU (ed) Methods of enzymatic analysis Verlag Chemie Deerfield Beach Florida Basel.
[4] Hames B D, Hooper N M 2000 Biochemistry The instant Notes Ed ke-2. Hongkong Spinger Verlag. pp. 83-84