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Properties of an Extracellular Protease of *Bacillus megaterium* DSM 319 as Depilating Aid of Hides

BUDIASIH WAHYUNTARI[1]* AND HENDRAWATI[2]

[1]*Division of Biocatalyst Technology, Center for Technology of Bioindustry, Agency for the Assessment and Application of Technology, Jalan MH Thamrin 8, BPPT Bld II/15, Jakarta 10340, Indonesia;*

[2]*Faculty of Science and Technology, Syarif Hidayatullah Islamic State University, Jalan Ir H Juanda 95, Jakarta 15412, Indonesia*

Properties of a *Bacillus megaterium* DSM319 extracellular protease which are related to its application for depilating hides were investigated. The properties observed were optimum temperature and pH, the type of protease, and type of the protein which could be hydrolyzed by the enzyme. The enzyme was produced in a 3.5 liters LKB jar fermentor in a medium containing (2.0% v/v molasses and 1.3% w/v urea at 37 °C, pH 7.5, aeration 1 vvm, agitation 250 rpm for 24 h). The enzyme solution was concentrated by means of membrane ultrafiltration followed by ammonium sulfate precipitation at 70% w/v saturation. Optimal temperature and pH were observed at 30 °C and pH 8, respectively. Two mM PMSF and EDTA inhibited the enzyme leaving level of activity of 84.5 and 4.3% correspondingly, indicated that the crude enzyme might be a metal requiring serine protease. The presence of 0.5, 2.0 and 3.5 mM $CaCl_2$ caused an increase of the enzyme activity of 73, 88, and 79% respectively. The enzyme was able to hydrolyze Na-Benzoyl-DL-Arginine p-Nitro Anilide, a specific amino acid sequence cleaved by trypsin at a reaction rate of 0.024 absorbance value at 405 nm per min. The enzyme was capable of hydrolyzing bovine serum albumin, hemoglobin, and gelatin, and to hydrolyze alkaline soluble collagen and keratin. The K_m value of the enzyme for hydrolysis of bovine serum albumin and gelatin was 3.44 and 1.65 mg mL^{-1}, whereas V_{max} values were 8.09 and 55.24 mg mL^{-1}, respectively. The experimental data showed that the crude enzymes have potential for dehairing of cow hides.

Key words: depilating, metalloprotease, protease, serine

Penelitian ini mengamati sifat-sifat protease ekstrasel *Bacillus megaterium* DSM 319 yang berkaitan dengan pemanfaatannya sebagai penghilang bulu pada kulit ternak. Sifat-sifat yang diamati adalah suhu dan pH optimum, tipe protease dan jenis protein yang dapat dihidrolisis oleh enzim tersebut. Enzim protease diproduksi dalam fermentor LKB 3,5 liter menggunakan medium dengan komposisi sebagai berikut: molase 2,0%, urea 1,3% b/v pada 37 ºC, pH 7,5 dengan aerasi 1 vvm, agitasi 250 rpm selama 24 jam. Larutan enzim kasar dipekatkan dengan membran ultra filtrasi dilanjutkan pengendapan dengan ammonium sulfat pada tingkat kejenuhan 70% b/v. Suhu dan pH optimal enzim teramati masing-masing pada 30 ºC dan pH 7,5. PMSF dan EDTA 2 mM menghambat aktivitas enzim hingga aktivitasnya masing-masing tinggal 84,5 dan 4,3%, yang mengindikasikan bahwa larutan enzim kasar antara lain mengandung protease serin yang memerlukan logam untuk aktivitasnya. Adanya $CaCl_2$ 0,5 mM, 2,0 mM, dan 3,5 mM meningkatkan aktivitas protease masing-masing 73,88 dan 79%. Protease yang diamati mampu menghidrolisis Na-Benzoyl-DL-Arginine p-Nitro Anilide suatu deret asam amino spesifik yang dipotong oleh tripsin dengan laju reaksi sebesar nilai absorbansi 0,024 pada λ 400 nm per menit. Protease ini juga mampu menghidrolisis bovine serum albumin, hemoglobin dan gelatin dan juga collagen dan keratin yang terlarut dalam basa. Nilai K_m enzyme dalam menghidrolisis bovine serum albumin dan gelatin masing-masing 3,44 dan 1,65 mg mL^{-1}, sedangkan V_{max} nya masing-masing 8,09 dan 55,24 mg mL^{-1}.

Kata kunci : metalloprotease, penghilang bulu, protease, serin

Skin (hide) contains several substances including collagen protein which is associated with keratin, elastin, albumin, globulins, mucoids, lipids, carbohydrates and mineral salts (Joseph 1989). Those substances exist in skins or hides in three layers. On the surface of intact skin are the keratinous epidermal layers which consist of epidermis, hairs, and hair root sheaths. Below the epidermal layers is the basement membrane which is attached to the dermis or corium. The dermis consists of a special type of collagen as well as various glycoproteins and protein glycans which interlinked in a close network (Frendrup 2000). In leather processing, the corium is transformed into leather by removing all non collagenous substances. The common beam house operations are soaking, dehairing, liming, deliming, bating and pickling. The use of enzymes in the soaking and dehairing operations was reported to accelerate the processes. Some microbial proteolytic enzymes have been reported as useful in leather processing (Joseph 1989, Dayanandan

*Corresponding author, Phone: +62-21-7560536, Fax: +62-21-7566922 ; E-mail:budiasih.wahyuntari@bppt.go.id

et al. 2003; Tang *et al.* 2004; Macedo *et al.* 2005; Pillai and Archana 2008). The important factors in choosing enzymes for leather processing includes the fact that the enzyme should not attack collagenous materials and should stable under processing conditions. This experiment was to study the properties of a protease produced by *B. megaterium* DSM 319 that is related to its utilization in leather processing. The properties investigated were optimal pH and temperature of the enzyme activity, type of protease, and hydrolytic activity of the enzyme towards different kinds of proteins.

MATERIALS AND METHODS

Microorganism. *B. megaterium* DSM319 was used throughout the experiment. Stock cultures were maintained in 50% (v /v) glycerol.

Materials. Molasses ("Gunung Madu", Lampung, Indonesia) and technical grade urea were used for enzyme production. All chemicals used for enzyme activity, protein assay and enzyme stability study were analytical grade.

Enzyme Preparation. Crude enzyme of *Bacillus megaterium* DSM319 was produced in 2 L media containing 2% w/v molasses and 1% w/v urea in a Biostat fermentor. Fermentation conditions were: agitation at 250 rpm, aeration 1 vvm, at 37 °C and pH 7.5 for 24 h. The bacterial cell was separated by means of microfiltration and the enzyme solution was concentrated by membrane ultrafiltration followed by $(NH_4)_2SO_4$ precipitation at 70% saturation.

Assay of Proteolytic Activity. Proteolytic activity was assayed by modification of the Walter (1984) method. 400 µL of 1% w/v casein (Hammarsten, Merck) dissolved in Tris/Cl buffer pH 8 was incubated with 50 µL crude enzyme at 30 ºC for 10 min. Units of enzyme activity were defined as the amount of enzyme that produced an equivalent to one mole of free tyrosine under standard reaction conditions (temperature and pH). Relative enzyme activity is defined as % ratio of enzyme activity at certain conditions (various pH, temperature, inhibitors, ions) to the optimal condition (pH & temperature) without addition of inhibitor or ions).

Protein Determination. Protein assays were performed according to the Bradford method (1976) using bovine serum albumin V fraction as protein standard.

Effect of pH and Temperature on Enzyme Activity. The temperature-activity profile was observed by assaying the activity in a temperature range of 20-60 ºC at pH 7.5 (the pH of enzyme production); whereas the pH-activity profile was studied by assaying protease activity in a pH range of 6-11 in universal buffer at 30 ºC (the optimal temperature resulted from previous experiment-the temperature-activity profile).

Effect of Protease Inhibitor on Enzyme Activity. To determine the type of protease, prior to assaying activity, the enzyme was pretreated with protease inhibitors at room temperature (~28 °C) for 1 hour. The inhibitors used were phenyl-methyl-sulfonyl-flouride (PMSF) and ethylene diamine tetra acetic acid (EDTA). PMSF is a serine protease inhibitor and EDTA is a metalloprotease inhibitor. The concentrations of inhibitors tested were 2 and 5 mM.

Effect of Divalent Ions on Enzyme Activity. The effect of various divalent metal ions on enzyme activity was studied by incubating the enzyme in the presence of different concentrations of metal ions at room temperature and the residual activity was determined after 1.0 h. The metal salts studied were $CaCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$ and $FeCl_2$ at final concentration of 0.5 , 2 , 3.5, and 5 mM.

Hydrolytic Activity of Enzyme Toward Trypsin-Specific Substrate, some Proteins, and Kinetics Parameters of the Enzyme. The hydrolytic ability of the enzyme cleaving a trypsin chromogenic substrate and various proteins was observed. The chromogenic substrate used for the experiment was benzoyl-DL-arginine *p*-nitroanilide (BAPNA). A 100 mg aliquot of BAPNA was dissolved in 100 ml 50 mM phosphate buffer pH 7. A 50 µL aliquot of crude enzyme was then added to 450 µL of substrate solution. The reaction mixture was incubated at 30 ºC and every 2 min the absorbance (A) of the reaction mixture in 10mm cuvette was measured at λ 405 nm. The activity of the enzyme increased the absorbance (A_{405}).

The proteins studied were bovine serum albumin, casein, gelatin, collagen, hemoglobin and keratin. Casein, albumin and gelatin were dissolved in Tris-Cl buffer at pH 8. Collagen, keratin and hemoglobin were dissolved in 1 % w/v urea in 0.1N NaOH and the pH was then adjusted to pH 8 using 0.1 N HCl. An 800 µL aliquot of 1% protein suspension was combined with 200 µL enzyme solutions and then the mixture was incubated at 30 ºC for 1 h. The reaction was stopped by immersing the reaction mixtures in boiling water. The controls were run by combining the protein suspension with buffer solution (without extract enzymes) in the same proportion as the reaction mixtures. The

hydrolytic activity was observed by SDS-PAGE by comparing the bands of proteins of the enzymatic reaction mixtures with their respective controls.

Enzyme Kinetics Using Gelatin and Albumin. Gelatin and albumin concentrations used in the experiments were 0.7, 0.8, 1.0, 2, and 4.0 mg mL^{-1}. The enzymatic reactions were carried out under optimal conditions based on previous experiments. Lineweaver-Burk plots of the experimental results were made.

Application of the Protease on Dehairing Cow Hide. At our laboratory, the cow hide was immersed in enzyme containing phosphate buffer pH 8 for 2 h at 30 ºC, in a shaking incubator. Then the mixture was kept at room temperature overnight. The amount of enzyme added was 5 % of the hide wet weight and the final enzyme concentration used was 10 U mL^{-1}. The hair was then removed mechanically. The experiment was carried out without any Na_2S addition.

The enzyme was also tested in commercial leather processing at "UD.Sumber Kulit" in Magetan, East Java. The dehairing process was in a horizontal drum with a capacity of 250 kg salted cow hide. The hide was added with water and 1% v/v enzyme (the enzyme final concentration used was 10 unit mL^{-1}) and the drum was rotated for 1 hour. After this 1.5% w/v CaO then added with rotation for 30 minutes. Then 0.5% w/v N_2S (50% of normal concentration used) was added with rotation for 1 h, then we added 1.5% CaO w/vand rotated for 30 min, finally added with 100% water and kept overnight and washed in the following day.

RESULTS

Effect of Temperature and pH on Enzyme Activity. The assay was done at pH 7.5, the same pH as the initial pH of the fermentation (enzyme production conditions). The optimum activity was at 30-36 ºC. Increasing the temperature up to 50 ºC resulted in decreasing relative enzyme activity down to 40% (Fig 1). Based on experimental data (Fig 1), when the enzyme was incubated at temperature above 55 ºC, the activity was not measurable. At 30 ºC, the optimum pH of the enzyme activity was observed at pH 8 (Fig 2). The enzyme was more stable at pH 7-8. At pH higher than 8, the enzyme assayable activity decreased drastically.

Effect of Protease Inhibitors and Divalent Ions on the Enzyme Activity. Phenyl methyl-sulfonyl-flouride (PMSF), a serine inhibitor, at the concentration used, only partly inhibited the enzyme (Fig 3). However ethylene diamine tetra acetate (EDTA) at 5 mM almost totally inhibited the enzyme (Fig 3). At 2.0 mM PMSF decreased the enzyme activity to 84.5%, whereas 5 mM PMSF decreased the activity to 47.7%. In the presence of 2 mM EDTA, residual enzyme activity was only 4.3%.

Effect of Some Divalent Cations on the Protease Activity. All divalent ions tested except Ca^{2+} ion decreased enzyme activity (Fig 4). The calcium ion at 0.5 mM and 2.0 mM increased the enzyme activity to 63.3 and 68.7%. However, at 3.5 and 5.0 mM the Ca^{2+} ion decreased the enzyme activity to 71.0 and 72.0% respectively.

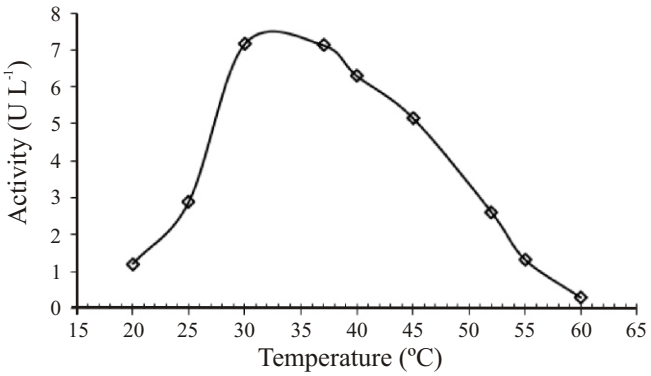

Fig 1 Effect of temperature on protease activity in 50mM Tris-Cl buffer pH 7.5.

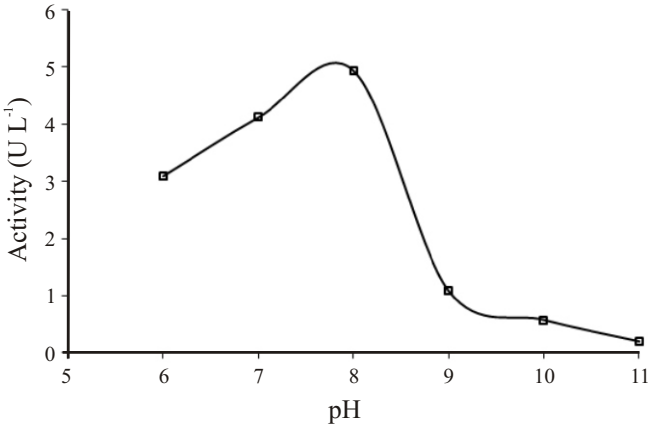

Fig 2 Effect of pH on proatease activity at 30 ºC in universal buffer.

Hydrolytic Enzymic Activity on Chromogenic Substrate of Trypsin and Some Other Proteins. The crude protease was able to digest specific chromogenic substrate for trypsin. The equation of *p*-nitroanilide release was $Y = 0.024\ t + 0.664$, $R^2 = 0.971$ (Fig 5).

Bovine serum albumin (BSA) and gelatin were completely dissolved in Tris-Cl buffer at pH 8 (Fig 6 SDS/PAGE well 1a and 3a). Hemoglobin was

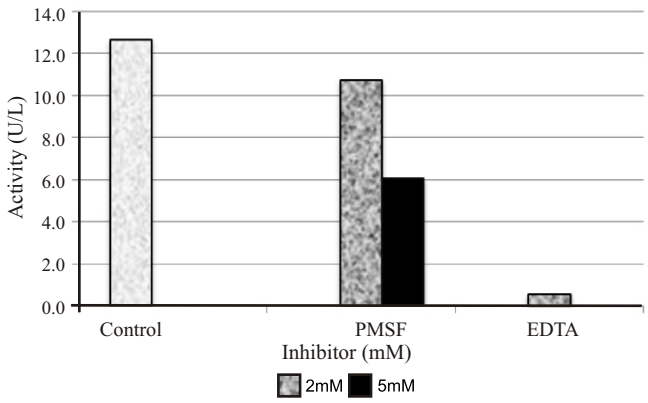

Fig 3 Effect of phenyl methyl sulfonyl flouride (PMSF) and ethylene diamine tetra acetate (EDTA) on enzyme activity at pH 8, 30 ºC.

completely dissolved in 1% urea in 0.1N NaOH pH 8 (Fig 6 SDS/PAGE well 6a), but collagen and keratin were only partly dissolved (Fig 6 SDS/PAGE well; 2a and 4a). The enzyme was able to hydrolyzed bovine

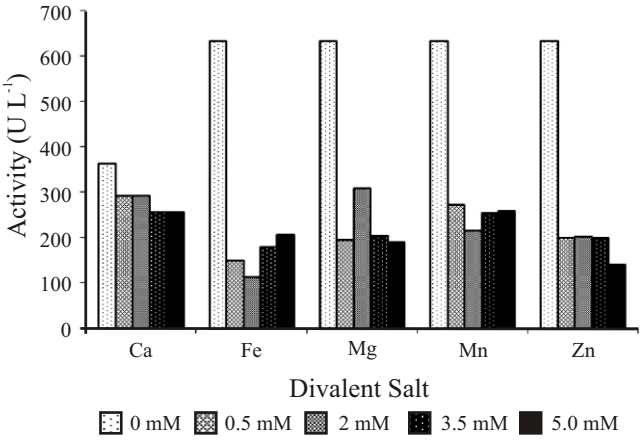

Fig 4 The effect of divalent ions on protease activity.

serum albumin, gelatin, and hemoglobin completely (Fig 6 SDS/PAGE well 1a-1b; 3a-3b and 6a-6b). Dissolved collagen and keratin was also hydrolyzed by the enzyme (Fig 6 SDS/PAGE well 2a-2b and 4a-4b), but an insoluble fraction remained intact in the reaction tube after enzymatic hydrolysis. Crude proteases extract (Fig 6 SDS/PAGE well 5) was used as a control protein bands.

Enzyme Kinetics on Gelatin and Albumin. The hydrolytic rate of the enzyme towards albumin and gelatin was observed. The experimental results, shown in indicates that hydrolysis rate of the protease on albumin [1/V=160.73(1/S)+55.235, R^2=0.92] was much higher than that of on gelatin. [1/V=49.072(1/S)+8.0948, R^2= 0.99] (Fig 7).

Application of the Protease on Dehairing of Cow Hide. Fig 8 shows the experimental enzyme

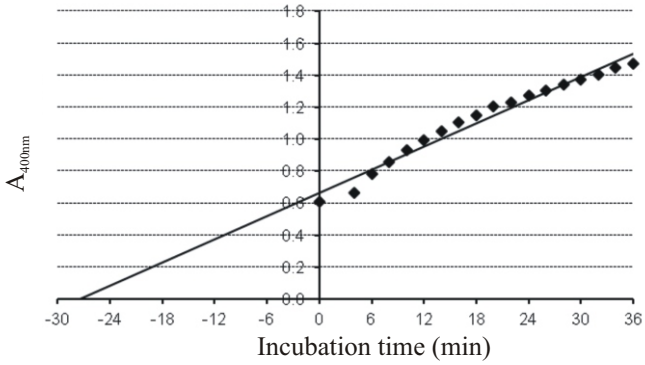

Fig 5 Protease hydrolyzed specific chromogenic substrate for trypsin, Y= 0.024 t + 0.664, R2 = 0.971.

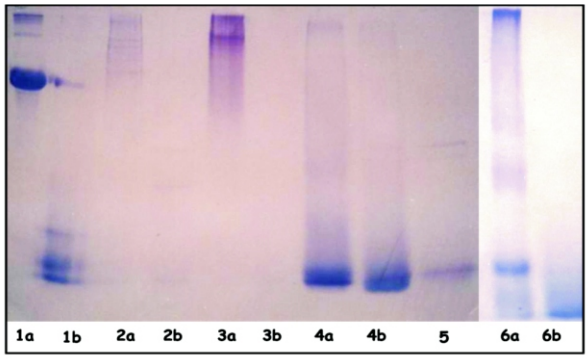

Fig 6 SDS/PAGE of hydrolysis products of proteases from *B. megaterium* DSM 319 on some proteins. 1a, BSA; 1b, hydrolyzed BSA; 2a, collagen; 2b, hydrolyzed collagen; 3a, gelatin; 3b, hydrolyzed gelatin; 4a, keratin; 4b, hydrolyzed keratin; 5, Protease protein (control); 6a, hemoglobin; 6b, hydrolyzed hemoglobin.

application for cow hide dehairing at our laboratory, the hide was soaked with enzyme only, without addition of N_2S. There were some hairs remaining on the hide.

Experimental results of dehairing in commercial leather processing at "Sumber Kulit" in Magetan, East Java showed that the hide was completely dehaired (Fig 8A2, B2).

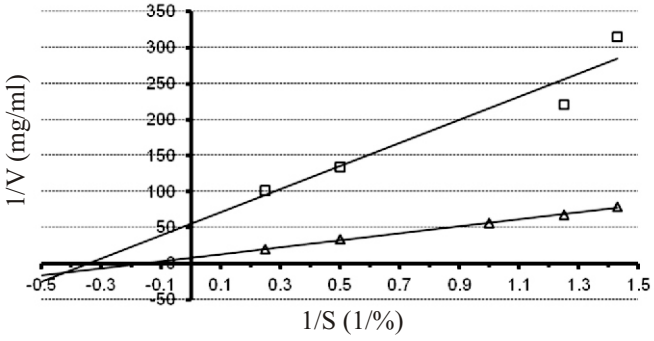

Fig 7 Kinetics of B. megaterium DSM319 proteases on gelatin (Δ) and albumin (□). The Lineweaver-Burk equation, r: 1/V=49.072(1/S)+8.0948, R2= 0.99; £: 1/V=160.73(1/S)+55.235, R2=0.92.

DISCUSSION

Experimental results (Fig 1 and 2) showed that the enzyme was a neutral protease. It was active in the temperature range of 30-45 °C and pH 7-8, with optimum activity at 30 °C, pH 8. Therefore the enzyme can be used at room temperature with slightly alkaline conditions. In tropical areas the soaking of hides is usually carried out at ambient temperature, which is around 28-32 ºC. Since the enzyme activity decreased drastically at pH's higher than 8, the application of the enzyme should be done before alkalization (swelling) of the hides or in soaking process. A similar process has been practiced for more than a decade as reported by Bezak *et al.* (1989) and Brady *et al.* (1990). Recent studies reported that a slightly alkaline protease produced by *Pseudomonas aeruginosa* MCM B-326 had the potential to be used for depilating buffalo hide (Zambare *et al.* 2011; Pandeeti *et al.* 2011). This enzyme was active in the pH range of 7-9 and temperature range of 20-50 °C, with optimum activity at pH 8 and temperature 35 °C.

Inhibition studies of our enzyme showed that the protease produced by *B. megaterium* DSM 319 was slightly inhibited by PMSF. However, the enzyme was totally inhibited by EDTA, which indicates that the crude enzyme contains serine proteases and that the enzyme needs a metal cofactor for its activity. The effect of some divalent ions on enzyme activity was observed. Only the calcium ion enhanced protease activity, whereas other divalent ions (Fe, Mg, Mn, and Zn) decreased activity (Fig 4). Protease produced by *Bacillus pumilus* that used for dehairing was also reported as a serine protease which needed a metal cofactor (Wang *et al.* 2006).

The presence of a trypsin like protease in the crude extract was also investigated. The experimental result showed that the crude protease of *B. megaterium* DSM 319 contained trypsin-like protease as shown in Fig 5. Early reports on application of enzymes on the dehairing of hides mentioned that the enzyme used was trypsin (Zugno 1992). The process of using trypsin in dehairing resulted in increased hide permeability that would promote the soaking process and so produce leather which would have a very clean hair pocket and grain.

The most important consideration in selecting protease for dehairing of hide, that the enzyme should digest the epidermis and basement membrane but should not attack the collagen of the hide (Dayanandan *et al.* 2003; Wang *et al.* 2007). The basement membrane consists of globular glycoproteins, proteoglycan and non fibrous collagen which are target of proteolytic enzymes in the depilatory process (Brady *et al.* 1990).

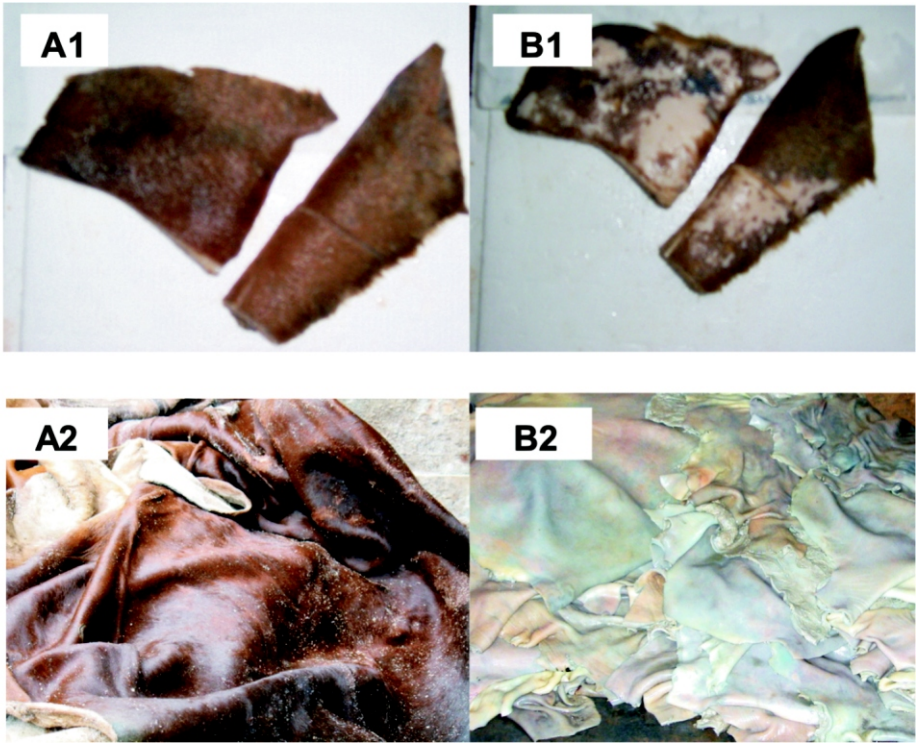

Fig 8 Application of proteases from *B. megaterium* DSM 319 in dehairing of cow hide. A: raw hides, B: dehaired hides; A1 & B1: at our laboratory; A2 & B2 at Sumber Kulit in Magetan, East Java.

The experiment results showed that proteases produced by *B. megaterium* DSM 319 were able to digest albumin, gelatin, hemoglobin and urea soluble collagen (Fig 6).

Application of the enzyme on the dehairing of cow hide showed that mechanical removal of the hair after soaking the hide with crude enzyme, without addition of N_2S, could not remove the hair completely (Fig 8 B1). The trial application of the crude enzymes in commercial leather processing, addition of 50% N_2S of normal used could remove the hair satisfactorily.

Recent study on the use of protease for depilation of hide show these proteases mostly belong to the keratinolytic group (Macedo *et al*. 2005; Pillai and Archana 2008; Cai *et al.* 2008) and elastases (Pandeeti *et al.* 2011). The keratinolytic enzymes were reported that they could be used as hair-save depilating agent without addition of Na_2S (Macedo *et al*. 2005 ; Cai *et al*. 2008), whereas our enzymes for removing the hair completely still need addition of Na_2S.

Based on the data and dehairing trial at commercial leather processing in Magetan, East Java, the crude protease produced by *B. megaterium* DSM319 can be applied for dehairing of cow hide.

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