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Full Length Research Paper

Isolation and partial purification of lysozyme from saliva of Bali cattle (*Bos sondaicus*) using an aqueous mixture of polyethylene glycol (PEG) with sodium sulfate

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Lysozyme from saliva of Bali cattle (*Bos sondaicus*) was successfully isolated through an aqueous mixture system of polyethylene glycol (PEG 4000) and salt (Na_2SO_4). About 100 µg/ml lysozyme of 14.2 kDa, determined through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), per 4800 µg/ml saliva was obtained. Based on turbidity assay of *Staphylococcus aureus*, it was revealed that inhibition activities of 40 µg/ml isolated lysozyme were comparable to 800 µg/ml of a commercial hen egg white lysozyme.

Key words: Lysozyme, Bali cattle, *Bos sondaicus*, PEG, aqueous mixture system.

INTRODUCTION

Lysozyme (EC 3.2.1.17, N-acetylmuramic-hydrolase) with a molecular weight around 14.3 kDa is one of the components of the animal innate immune systems (Janeway et al., 2005; Cegielska-Radziejewska et al., 2008). It is usually present in the body tissues such as in the lungs (Travis et al., 1999) and body fluids such as tears, breast milk and in saliva (Hankiewicz and Swierczek, 1974). In human saliva, lysozyme was found to be about 20 to 80 µg/ml, and has the ability as an innate defense system (Tenovuo, 1989). As a natural immune system, lysozyme also has the ability to hydrolyze peptidoglycan of bacterial cell walls (Araki et al., 2003; Islam et al., 2006). Studies on lysozyme in saliva have been done extensively, especially in human saliva (Tenovuo, 2002a) in relation to oral health, as well as the overall body health, considering the mouth as one of the entrance of various pathogens. In line with this, lysozyme has also been used not only for the treatment of dry-mouth (xerostomia) for instance, but also for gastrointestinal infections (Tenovuo, 2002b). Moreover some researchers are interested in revealing the links between levels of salivary lysozyme and several diseases such as odd hypertension (Qvarnstrom et al., 2008).

It is now evident that, aside from the lysozyme bacteriolytic action, a dimeric form of lysozyme exhibits therapeutic, antiviral and anti-inflammatory properties (Cegielska-Radziejewska et al., 2008). Increasing demand for the availability of lysozyme from various natural sources, both for industry and for research purposes has opened up opportunities to explore of new sources of lysozyme. One of these sources is the saliva of Bali cattle (*Bos sondaicus*). This paper presents partial purification and characterization of lysozyme isolated from the saliva of Bali cattle using polyethylene glycol (PEG)/Na_2SO_4 aqueous mixture. Successes in isolating lysozyme from saliva of Bali cattle do not only open up

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Abbreviations: **PEG**, polyethylene glycol; **ddW**, double distillated water; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

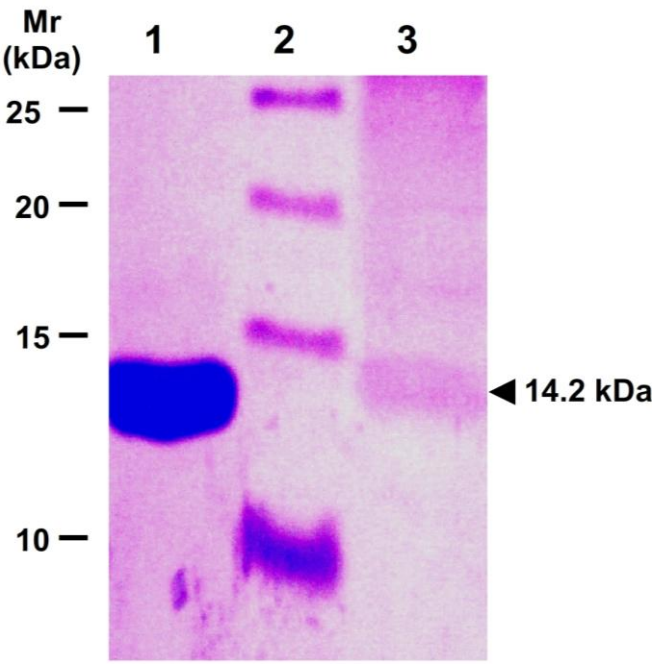

Figure 1. Lane 1, Commercial hen egg white lysozyme; lane 2, molecular weight marker; lane 3, representation of SDS-PAGE profile of lysozyme isolated from Bali cattle (*Bos sondaicus*) saliva by aqueous mixture of PEG4000/Na_2SO_4.

opportunities to study the role of lysozyme in the diversification of species, but also open up additional sources of lysozyme.

MATERIALS AND METHODS

Collection of sample

A total of 35 ml saliva was collected from oral cavity of *B. sondaicus* using a 20 ml syringe without needle. The saliva was collected from three bulls and two cows, age range between 1.5 and 2.5 years old, with good health condition based on exterior examination. The cattle were raised traditionally on a farm, fed with native grass and local roughage with water *ad libitum* in West Lombok District, Lombok Island, Indonesia. The saliva was centrifuged (3,000 × g) for 5 min at 4°C. Supernatant was collected, added with 0.2% (v/v) of NaN_3, aliquoted and kept at 4°C until used within 2 weeks.

Isolation and purification of lysozyme

The method adopted for isolation of the lysozyme was from Su and Chiang (2006). This method was based on PEG/sodium sulfate aqueous two-phase system. The stock solution of PEG 4000 (50%, w/w) and sodium sulfate (30%, w/w) were prepared in double distillated water (ddW). To avoid protein precipitation, polymers, buffer, salt and ddW were mixed before adding saliva. The pH of the system was nine and the total weight was 10 g. The phases were mixed gently for 60 min at room temperature followed by centrifugation at 1500 × g for 20 min. The formed fractions were dialyzed separately against phosphate buffered saline (PBS) pH 7, and then analyzed for the protein concentration and the lysozyme activity.

Estimation of total protein

Total protein in saliva, as well as the isolated lysozyme, was estimated by measurement of the absorbance at 280 nm in a UV-Vis spectrophotometer (Shimadzu UV-160, Japan) using a BSA standard curve.

Determination of molecular weight of the enzyme

Molecular weight of the enzyme was determined by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a commercial lysozyme as standard. The gel was stained using coomassie brilliant blue staining system (Volker et al., 1985).

Inhibition of lysozyme on *Staphylococcus aureus*

A reduction on turbidity was used for determining the lysozyme inhibition on *S. aureus* as modified from Jenzano et al. (1986) and Kumar et al. (2001). *S. aureus* was cultured in Luria-Bertani broth media, pH 7 at 37°C. Isolated lysozyme (40 µg/ml) was added into the culture and as a comparison, a commercial hen egg white lysozyme (800 µg/ml) was also included. *S. aureus* cultured with LB medium alone was considered as control. Optical density (Abs) at 600 nm was observed at zero hour of incubation, then the optical density was observed at 2 and 6 h. Finally, percent inhibition was determined as percentage reduction in turbidity compared to control, calculated by the formula;

$$\text{Inhibition (\%)} = \frac{\text{Abs of control } \textit{S. aureus} - \text{Abs of treated } \textit{S. aureus}}{\text{Abs of control } \textit{S. aureus}} \times 100\%$$

RESULTS AND DISCUSSION

As a natural antimicrobial, lysozyme has been used both in food and cosmetics industry. This has led to the increase in the demand for lysozyme from year to year. So far lysozymes derived from hen egg white are the most widely used sources of natural lysozyme (Cegielska-Radziejewska et al., 2008). Exploration of natural lysozyme from various species has been done extensively, but rarely from saliva of domestic cattle such as *B. sondaicus*. In this study we succeeded in isolating the lysozyme from saliva of Bali cattle with aqueous mixture of PEG 4000/Na_2SO_4, a method modified from Su and Chiang (2006).

Following mixing and centrifugation of the aqueous phases, three layers were observed designated as F1 on top, F2 in the middle and F3 at the bottom part. With this method, lysozyme was obtained in the F1 phase. The concentration of isolated lysozyme was about 100 µg/ml from 4800 µg/ml total protein of post-dialysis of original saliva. The lysozyme concentration was comparable to those of human saliva lysozyme; 20 to 80 µg/ml (Tenovuo, 1989). The purity and molecular weight specifications of this lysozyme (42 kDa) were verified on the results of SDS-PAGE analysis (Figure 1). In addition, the inhibitory activities of the salivary lysozyme are presented in Figure 2. In this study, 40 and 800 µg/ml of isolated saliva lysozyme and commercial hen egg white

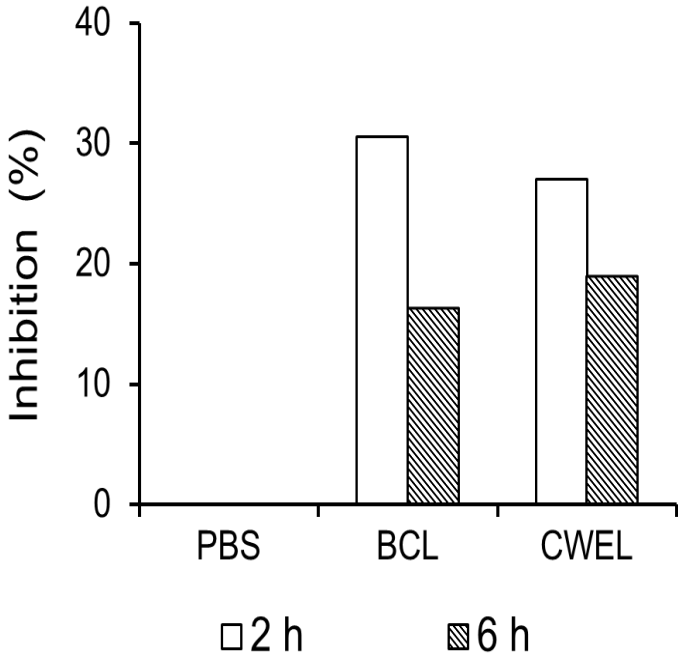

Figure 2. BCL, Percentage inhibitions of lysozyme isolated from Bali cattle (*Bos sondaicus*); CWEL, commercial hen egg white lysozyme; PBS, phosphate buffered saline without lysozyme as control, based on turbidimetric assay of *S. aureus* during 2 and 6 h cultures. Data obtained from the two independent experiments were carried out in duplicate.

lysozyme were used respectively, for turbidimetric assay of *S. aureus*.

The results of this study indicate that lysozyme isolated from Bali cattle saliva has a potent ability to lyse the cell wall of *S. aureus* even though *S. aureus* with their peptidoglycan layer has been reported resistant to the hydrolysis effects of lysozyme (Bera et al., 2005). Interestingly, Ibrahim et al. (1994) as well as Cegielska-Radziejewska et al. (2008) revealed that the modified lysozyme enhanced its hydrolysis effects both against gram-negative bacteria (*Escherichia coli* K12) and gram-positive bacteria (*S. aureus*). Although the present works give a rough estimation of the sensitivity of isolated lysozyme (40 µg/ml) compared to 800 µg/ml of commercial lysozyme, it was quite worthy to speculate that saliva of Bali cattle may contain a unique natural lysozyme. However, whether the uniqueness of the inhibition of Bali cattle salivary lysozyme in this study is similar to the modified lysozyme reported by Ibrahim et al. (1994) and Cegielska-Radziejewska et al. (2008), needs to be further elucidated.

In conclusion, the present work shows that the lysozyme from saliva of Bali cattle is capable of isolation using aqueous mixtures of PEG 4000/Na_2SO_4. The lysozyme has a unique potential as an anti-bacterial. Moreover, the natural role of this lysozyme in saliva or in the oral cavity and how the prospect of its application remains to be further investigated.

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