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## Resistance of Immobilized Lactoperoxidase Activity from Bovine Whey Against Storage Solutions

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### ABSTRACT

Lactoperoxidase (LPO) could be simply obtained from whey through immobilization using a cation exchange resin of SP Sepharose. LPO received high attention since the antimicrobial properties of LPO system (LPOS) that are consisted of LPO,  $\text{SCN}^-$  and  $\text{H}_2\text{O}_2$  was able to generate  $\text{OSCN}^-$  for strong antimicrobial agents. This study was done to analyze the immobilization efficiency of LPO onto two types of sepharose: SP-Sepharose Fast Flow (SPFF) and SP-Sepharose Big Beads (SPBB). The remaining of LPO's activity (%) against storage solution was also observed. The whey was obtained from bovine skimmed milk that was coagulated using rennet and acid lactic. The LPO was obtained from whey using SPFF. To analyze the resistance of enzyme activity, the immobilized LPO was stored in pure water, phosphate buffer, milk and whey at  $10^\circ\text{C}$ . The activity of LPO was monitored for 10 days. The result indicates that the LPO could be purified from whey and single band has been detected using electrophoresis method. The obtained LPO ( $35 \text{ U mL}^{-1}$ ) was attached onto SPFF and SPBB. Maximum immobilization efficiency has been achieved by 0.6 g SP-FF and 0.9 g SP-BB. LPO activity of the immobilized LPO were able to be kept until 5 days when it was stored in whey. Other storage solution remained various LPO activity during storage. This study concluded that IE of LPO attached onto sepharoses might be reached variously depended on the sepharose type and whey as LPO source might be used for maintaining LPO activity.

**Key words:** Lactoperoxidase, SP sepharose fast flow, SP sepharose big beads, immobilization, remaining activity

### INTRODUCTION

Lactoperoxidase (LPO), together with  $\text{SCN}^-$  and  $\text{H}_2\text{O}_2$  have been understood to generates intermediate product of  $\text{OSCN}^-$  as antibacterial agent that has a broad spectrum of antimicrobial effects against bacteria, fungi and viruses. This antibacterial agent could be produced if these three components exist in the medium (Seifu *et al.*, 2005). LPOS has been widely used as a preservative in dairy products and nondairy products (Seifu *et al.*, 2004; Touch *et al.*, 2004; FAO/WHO, 2005; Boots and Floris, 2006; Oghaiki *et al.*, 2007; Fweja *et al.*, 2008; Al-Baarri *et al.*, 2011a).

It has been understood that whey contains large number of LPO therefore purification method of LPO from whey has been well developed (Touch *et al.*, 2004; Zhou and Lim, 2009;

Al-Baarri *et al.*, 2010). SP-Sepharose has been known to provide beneficial effect for the immobilization efficiency since SP-Sepharose almost completely immobilized LPO and reusable (Al-Baarri *et al.*, 2010). Although, LPO was widely used in food application but it still remained the problem of its expensiveness, therefore the immobilization of LPO was needed for the reuse of LPO.

SP Sepharose has been known as immobilization agent for capturing lactoperoxidase (Fee and Chand, 2006; Hayashi *et al.*, 2012). SP Sepharose Fast Flow (SPFF) and SP Sepharose Big Beads (SPBB) were the common resin for immobilization since they provide simple application, long term of use and easy for reuse (Amersham-Bioscience, 2001). If compared to other immobilization agent such as chitosan, SP Sepharoses showed higher captivation of lactoperoxidase (Al-Baarri *et al.*, 2012). In the other hand, SP Sepharose application for immobilization of LPO resulting in the much more expensive of the use of LPO, therefore the efficient use of SP Sepharose to immobilize LPO is required. Based on related literatures, there was no documentation for the efficient use of LPO immobilization using SP Sepharose, therefore this research has been done for analyzing the maximum captivation of LPO onto SP Sepharose. This information might provide the benefit for minimum use of SP Sepharose for LPO immobilization. Since the immobilized LPO allowed the reuse of enzyme, the appropriate storage solution for keeping the enzyme activity is needed. To answer this, this research has also been done for analyzing the remaining of LPO activity after storage.

## **MATERIALS AND METHODS**

**Materials:** SP Sepharose Fast Flow and SP Sepharose Big Beads were purchased from Amersham Pharmacia Biotech, Sweden (Lot. No. 10029743 and 10081054, respectively). Microbial derived rennet was purchased from Singapore and ABTS or 2,20-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) was obtained from Kagawa Science (Lot No. 7ROZC-EC) Tokyo Chemical, Industry Co. Ltd., Japan. Cow's milk was obtained from Faculty of Animal and Agricultural Sciences's farm, Diponegoro University, Semarang, Indonesia. The spectrophotometer (Mini UV-1800, Shimadzu, Japan) was used for analysis enzyme activity. Unless other compounds specified, all other compounds were reagent grade.

**Preparation of whey:** The whey was prepared as method performed by Al-Baarri *et al.* (2011b) without any modification.

**LPO immobilization from whey:** The procedure for immobilization of LPO was conducted as the method that was performed by Al-Baarri *et al.* (2010) with minor modifications. SP Sepharose Fast Flow (SPFF) was used as agent for LPO immobilization from whey. Whey at the volume of 1800 mL was eluted through a glass column (3×40 cm) filled with 60 g of SPFF. Prior to elution, SPFF was washed with 300 mL of Phosphate Buffer (PB) (pH 6.8) containing 1 M NaCl to remove unnecessary compounds. The whey was circulated through the column using feedback tubing and a peristaltic pump. The circulation was done at the flow rate of 1.0 mL min<sup>-1</sup>. After draining the whey away, the resin was washed with 300 mL of 0.4 mM NaCl in 0.1 mM phosphate buffer (pH 7.0) using fraction collector (10 mL per tube) to obtain the solution containing high concentration of LPO. Three groups of fractions (fraction number 1-10, 11-20, 21-30) were analyzed for protein profile using sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) to check the purity. Finally, based on the SPS PAGE analysis, the fraction number 21-30 was selected for LPO activity analysis (LPO activity was 35 U mL<sup>-1</sup>). Thus, this fraction was used throughout experiment.

**Determination of captured LPO onto SP sepharoses:** SPFF and SPBB (0.1-1.0 g) were washed in 1 M NaCl in PB pH 7.0 and then were placed in the column (1×10 cm). The immobilization process was started with the elution of 1 mL of LPO through column. The flow rate was set into 1 mL min<sup>-1</sup> using peristaltic pump. The output was collected for measurement of remained LPO activity in the SP Sepharoses. This experiment was repeated three times and column were wash with serial elution of 1 M NaOH and pure water, respectively. The immobilized LPO was stored in pure water, PB, milk and whey. All storage solutions were sterilized using autoclave at 110°C for 10 min. Immobilized LPO was stored at 4°C for 10 days. The remaining of LPO activity immobilized onto SP-Sepharose was calculated by eluting immobilized LPO using 1 M NaCl in PB pH 7.0. The LPO activity in the elution was analyzed spectrometrically.

**LPO activity determination:** LPO activity was performed as the following method: 450 µL of 1.0 mM ABTS in 10 mM acetate buffer (pH 4.4) and 450 µL of 0.55 mM H<sub>2</sub>O<sub>2</sub> in pure water were gently poured into the cuvette. The enzyme (50 µL) was subsequently added to cuvette. The increase of absorbance at 412 nm measured for 20 sec. One unit of LPO enzymatic activity was expressed as the amount of enzyme needed to oxidize 1 µmol ABTS min<sup>-1</sup>. The molar extinction coefficient of ABTS at 412 nm was 32.400 M<sup>-1</sup> cm<sup>-1</sup> (Touch *et al.*, 2004).

**Immobilization efficiency:** The immobilization efficiency (IE) was calculated as follows:

$$IE (\%) = \frac{E_1}{E_0} \times 100$$

where, E<sub>0</sub> is the LPO activity added to the SP Sepharoses (U mL<sup>-1</sup>) and E<sub>1</sub> is the LPO activity embedded in the SP Sepharose (U mL<sup>-1</sup>) (Al-Baarri *et al.*, 2010).

## RESULTS AND DISCUSSION

**Purification LPO:** Whey has a lot of enzymes and it is available in low cost because whey is by product of dairy manufacture so it is the challenge to use whey as enzyme sources including LPO. The LPO activity and band(s) of the solution obtained from the elution of 1 M NaCl in PB pH 7.0 through SPFF containing LPO were checked using spectrophotometer and SDS PAGE, respectively. As mention in methods, SPFF containing LPO was generated from whey that was eluted through SPFF column. The result of LPO activity was 27.7±2.9, 39.5±4.5 and 35.2±3.4 U mL<sup>-1</sup> for fraction number 1-10, 11-20, 21-30, respectively (data not presented). The highest of LPO activity was group of fraction number 11-20, however since the band of this group showed two bands indicating two protein was detected, for whole of experiment, group of fraction number 21-30 was used. This group showed single band indicating only LPO that was captured by SPFF.

In this research SPFF was used to obtain LPO since this ion exchange resin has diameter 45-165 µm resulting in the wider of surface area than SPBB (Amersham-Bioscience, 2001). In line with this result, Touch *et al.* (2004) used SPFF for purifying LPO from whey resulting in the good

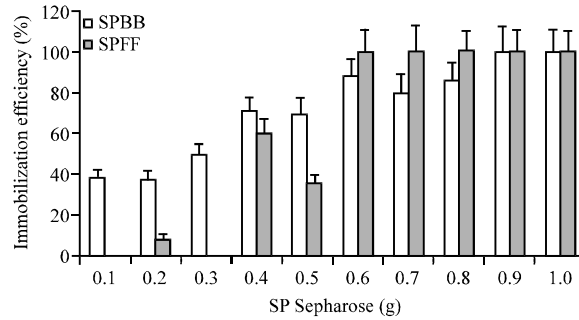


Fig. 1: Immobilization efficiency (IE) of LPO on two types of resin, i.e., SP Sepharose Big Beads (SPBB) and SP Sepharose Fast Flow (SPFF). Two types of resin (0.1-1.0 g) was used to immobilized 1 mL of LPO ( $35 \text{ U mL}^{-1}$ ). Flow rate has been set to  $1 \text{ mL min}^{-1}$  using peristaltic pump. Values are means of three sets of experiments, Error bars represent standard errors of the mean

ability to catch LPO ( $108 \text{ U mL}^{-1}$ ). The activity of LPO in this research was less than that of other researcher since the absence of microfiltration step in this research. It has been known that the microfiltration might concentrate the enzyme resulting in the high activity of LPO.

**Immobilization efficiency:** Immobilization efficiency plays an important role for determination of immobilization agent. This research determined Immobilization Efficiency (IE) of LPO using SPFF dan SPBB (Fig. 1). The volume of SP Sepharoses used in this experiment had a range from 0.1 to 1.0 g to catch the LPO at initial activity of  $35.2 \pm 3.4 \text{ U mL}^{-1}$ . The increase of IE was found as an increase of SP Sepharoses's weight. As describe in Fig. 1, when the 0.6 g of SPFF was employed, the IE achieved 100% indicating all of LPO employed was able to be captured by SPFF. When the weight of SPFF was increased, the IE was in steady state maximumly. An increase SPBB from 0.1 to 1.0 g elevated the IE from 38.6 to 100%. However, 0.9 g of SPBB completely captured LPO resulting the IE of 100%. One gram of SPFF was reported to have a maximum capture of LPO in 300 mL whey (equal to  $750 \text{ U mL}^{-1}$  LPO activity) (Al-Baarri *et al.*, 2010). This can be explained that the capture depended on the quantity of enzymes per mililiter. This research used high activity of LPO resulting in the loss of LPO activity.

Since the SPFF and SPBB provided the maximum IE at 0.6 and 0.9 g, respectively, thus these amounts of SP Sepharoses has been used in the rest of experiment. The LPO immobilized onto SPFF and SPBB was stored in the various storage solutions: Pure water, PB, milk and whey for 10 days at  $10^\circ\text{C}$ .

**Remaining LPO activity during storage:** The percentage of remaining immobilized LPO activity stored at  $10^\circ\text{C}$  for 10 days in various storage solutions is shown in Fig. 2a and b. LPO activity of immobilized LPO was measured after purging the LPO attached onto SP Sepharose with 1 M NaCl in PB pH 7.0. The percentage of remaining LPO activity was determined by comparing the LPO activity after storage to the initial immobilized LPO at first day of storage.

The percentage of LPO activity attached onto SPBB and SPFF is shown on Fig. 2a and b, respectively. Based on Fig. 2a, whey was able to maintain 100% enzyme activity of LPO attached onto SPBB within 4 days. The extention of storage time resulted in the remarkable

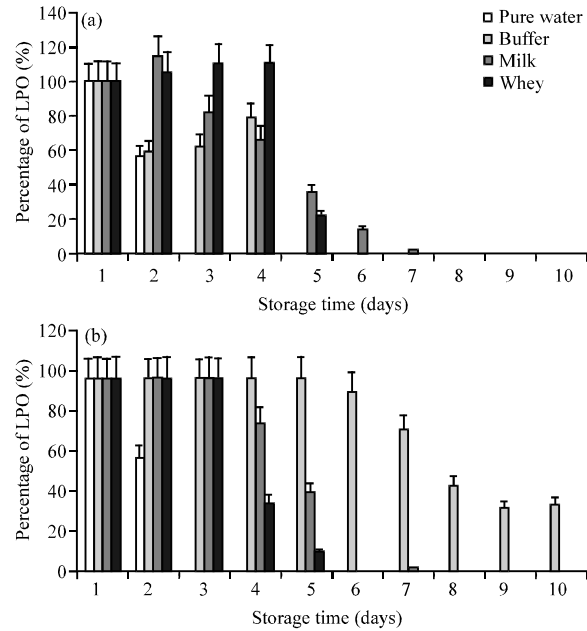


Fig. 2(a-b): (a) Percentage of remaining LPO activity attached onto SP Sepharose Big Beads (SPBB) during 10 days of storage at 10°C in various storage solutions. This experiment used 1 g of SPBB for immobilizing 1 mL of LPO (35 U mL<sup>-1</sup>). Immediately after immobilization, SPBB then was stored in sterilized pure water, phosphate buffer, milk and whey for 10 days at 10°C. Values are mean of three sets of experiments. Error bars represent standard errors of the mean and (b) Percentage of remaining LPO activity attached onto SP Sepharose Fast Flow (SPFF) during 10 days of storage at 10°C in various storage solution. This experiment used 1 g of SPFF for immobilizing 1 mL of LPO (35 U mL<sup>-1</sup>). After immobilization, SPFF was stored in sterilized pure water, phosphate buffer, milk and whey. Values are mean of three sets of experiments. Error bars represent standard errors of the mean

reduction of remaining LPO activity. Milk was able to maintain the LPO activity at seven days of storage even though the remaining of LPO activity at that time was very negligible in amount (2.3%).

As previously mentioned, whey was able to completely keep LPO activity within 4 days of storage. This can be explained that whey components support the activity of LPO. It has been studied that LPO activity might be inhibited by casein (Singh *et al.*, 2009) while the casein has been removed from whey.

The remaining of LPO activity attached onto SPFF during 10 days of storage at 10°C is shown on Fig. 2b. PB was able to maintain 100% of LPO activity within 5 days of storage. The milk and whey were able to keep 100% of LPO activity within 3 days of storage. The elevation of storage time until 5 days in whey could keep enzyme activity although the remaining enzyme activity was small amount (10.08%). Based on the availability, also since LPO was simply derived from whey, the storage of immobilized LPO in whey should keep its activity up to 5 days of storage. Therefore it is suggested that immobilized LPO should be stored in whey.

## CONCLUSION

The results can be concluded that LPO could be purified from whey using fraction number 21-30. One milliliter of LPO ( $35 \text{ U mL}^{-1}$ ) could be completely immobilized onto 0.6 g SPFF or 0.9 g SPBB (immobilization efficiency was 100%). Among various storage solution, whey was able to keep 100% of LPO activity up to 5 days of storage.

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