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# Application of Lactoperoxidase System Using Bovine Whey and the Effect of Storage Condition on Lactoperoxidase Activity

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## ABSTRACT

The purpose of this study is to investigate the potential antimicrobial effect of non-dialyzed, dialyzed and freeze-dried whey against *S. enteritidis* and the effect of storage condition on lactoperoxidase (LPO) activity. The LPO activity of all forms of whey was studied at -20, 4 and 25°C during 4 weeks storage for LPO activity. The results showed that all forms of whey had antimicrobial activity against *S. enteritidis*. The various forms of whey had no remarkable differences on the antimicrobial activity. When all forms of whey was stored at 25°C, their LPO activity terminated within 2 weeks or less. LPO activity of non-dialyzed and dialyzed whey when stored at 4°C, decreased remarkably within 4 weeks. However, the decrease of LPO activity during 4 weeks storage was not found in freeze-dried whey. All forms of whey stored at -20°C kept a stable LPO activity for 4 weeks. These results suggested that whey (non-dialyzed and dialyzed) and freeze-dried whey should be stored at 4°C or less, in order to keep LPO activity.

**Key words:** Whey, freeze-dried whey, storage condition, *S. enteritidis*, lactoperoxidase activity

## INTRODUCTION

Whey, a by-product of the cheese industry, is a source of biological and functional valuable proteins including β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, lactoferrin and lactoperoxidase (LPO) (Pescuma *et al*., 2008). Nowadays technologies allow the separation of different fractions of whey that are commercialized in the food and pharmaceutical industries. Whey may be used as ingredients in the food industry mainly due to their foaming and emulsifying properties (Ji and Hauque, 2003). Furthermore, whey proteins provide an excellent way to fortify foods increasing the nutritional quality of cheese, dairy desserts and bakery products (Whetstine *et al*., 2005; Mistry *et al*., 1996; Puangmanee *et al*., 2008).

LPO, a major enzyme in whey, is an important part of the natural host defense system in mammals, which provides protection against invading microorganisms. The mechanism of action of LPO has been explained in elsewhere (Aune and Thomas, 1977; Dajanta *et al*., 2008; Tayefi-Nasrabadi and Asadpour, 2008; Kussendrager and van Hooijdonk, 2000; Wit and van Hooydonk, 1996). The name of this mechanism is LPO system or LPOS. LPO alone will not function but must be combined with thiocyanate ion (SCN-) and hydrogen peroxide ($H_2O_2$) to make LPOS

(Seifu *et al*., 2005; Reiter, 1985; Touch *et al*., 2004). LPO catalyzes the oxidation of $SCN^-$ by use of $H_2O_2$ and generates antimicrobial products hypothiocyanite ($OSCN^-$). $OSCN^-$ is the main compound responsible for the antimicrobial properties of the LPOS due to its propensity to oxidize sulphydryl groups of microbial enzymes (Kussendrager and van Hooijdonk, 2000). This fact challenged the application of LPOS as natural preservative in various kinds food. However, LPOS is not ready yet for practical use in food industry. Several factors may inhibit the application of LPOS in food industry, including LPO cost, which is still too expensive.

Since, bovine whey is cheap and available in large amounts and various forms, we are engaged in using whey for antimicrobial agent production. Whey probably stored in the uncontrolled condition for long period due to the fact that whey is byproduct of cheese production. Thus, the objective of this study was to determine of antimicrobial effect of various forms of whey against *S. enteritidis* and to analyze its LPO activity in various condition of storage.

## MATERIALS AND METHODS

**Preparation of whey:** This experiment entirely has been done at Faculty of Agriculture, Kagawa University, Japan, from January to June 2010. Two milliliter fresh cow's milk provided by local diary farm was centrifuged at 10,300 × g at 10°C for 30 min to minimize the fat. The skim milk was treated with 0.02% (w/v) rennet and 2.0 mL lactic acid/liter milk at 30°C for 30 min. The precipitated caseins were removed by filtration through a sterilized filter cloth and then through filter paper under vacuum condition. The resultant filtrate was used as non-dialyzed whey. The non-dialyzed whey was dialyzed against 10 L of 10 mM sodium Phosphate Buffer (PB), pH 6.8, using a Spectra/Por® dialysis membrane (8 kDa) overnight. The non-dialyzed whey was freeze-dried to produce freeze-dried whey. The non-dialyzed, dialyzed and freeze-dried whey were stored at -20°C and used immediately.

**LPO activity assay of whey:** To measure LPO activity of non-dialyzed and dialyzed whey, a 100 mL of the whey was poured into 450 μL of 1 mM ABTS in 10 mM acetate buffer (pH 4.4). The enzymatic reaction for 20 sec, was started by adding 450 μL of 0.55 mM $H_2O_2$ in pure water. The absorbance of the reaction solution was monitored at 412 nm. To measure LPO activity of freeze-dried whey, the 6.5 mg of freeze-dried whey was dispersed in pure water to provide a 6.5% concentration (w/v) (the water content of whey was 93.5%). The dispersion of freeze-dried whey was used in enzymatic reaction solution instead of whey sample to measure its LPO activity. One unit of LPO enzymatic activity in whey was expressed as the amount of enzyme needed to oxidize 1 μmol ABTS/min. The molar extinction coefficient of ABTS at 412 nm was 32,400 $M^{-1}$ $cm^{-1}$.

**Antimicrobial activity of whey:** Antimicrobial activity of whey was assayed using *S. enteritidis*. This bacteria were cultured at 37°C overnight in sterile agar slants containing 1.0% polypeptone, 0.5% yeast extract, 0.3% D-glucose, 1.0% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$ and 1.5% agar at pH 7.0. The bacteria in the slants were suspended in sterile 0.88% NaCl solution. The density of bacteria was estimated from the absorbance at 600 nm using previously-established standard curve of *S. enteritidis* (CFU $mL^{-1}$) and absorbance. The sterilization of non-dialyzed and dialyzed whey was achieved by filtering through a 0.22 μm filter unit. On the other hand, the freeze-dried whey was dispersed in pure water to obtain 6.5% concentration (w/v). The dispersion of freeze-dried whey was sterilized using 0.22 μm filter unit. Antimicrobial activity of LPOS using whey was determined as follows: 400 μL of the assay mixture containing 1.0 mM KSCN, 0.2 mM $H_2O_2$, *S. enteritidis*

(ca. 5 log CFU $mL^{-1}$) and the amount of whey at 0-2.0 U $mL^{-1}$ LPO were incubated for 4 h in a water bath at 30°C (whey was observed to contain 2.6 U $mL^{-1}$ of LPO activity). Subsequently, serial dilutions of the assay mixture were prepared with a sterile 0.88% NaCl solution to enumerate the bacteria. The diluted mixture (100 μL) was spread onto Desoxycholate-hydrogen sulfide lactose agar. Plates were incubated at 37 °C for 24 h. The CFUs of microbes in the sample solution were counted on the plates. Antimicrobial activity of LPO was expressed by log $N_o/N_t$, where $N_o$ is CFU $mL^{-1}$ of the mixture with 10 mM PB (pH 7.0) instead of whey and $N_t$ is CFU per mL of the mixture with whey.

## RESULT

**Antimicrobial activity of whey:** The antimicrobial activity of various forms of whey against *S. enteritidis* was presented in Fig. 1. The antimicrobial effect against *S. enteritidis* was detected in all of whey forms: non-dialyzed, dialyzed and freeze dried whey. The antimicrobial activity increased when the concentration of whey was increased. The use of whey at the amount of 2.0 U $mL^{-1}$ resulted in the antimicrobial activity of 1.2, 1.3, 1.4 log $N_0/N_t$, for dialyzed, freeze-dried and non-dialyzed whey, respectively. The antimicrobial activity did not differ remarkably among the forms of whey.

**Various forms of whey on LPO activity:** The LPO activity of non-dialyzed, dialyzed and freeze-dried whey during 4 weeks storage at -20, 4 and 25°C has been investigated and the results are showed in Fig 2. The remarkable decrease of LPO activity was observed in the all forms of whey stored at 25°C for 2 weeks (Fig. 2a). Two weeks storage exterminated LPO activity in freeze-dried whey and one week storage disappeared LPO activity in the non-dialyzed and dialyzed whey. The extermination of LPO activity in freeze-dried whey was observed in the 10 days of storage (data not presented).

Figure 2b shows the LPO activity of all forms of whey when stored at 4°C for 4 weeks. This result shows that LPO activity of whey was retained at 4 weeks storage. No remarkable decrease of LPO activity was found in freeze-dried whey at 4 weeks storage while a noticeable decrease in LPO activity could be observed in non-dialyzed whey and dialyzed whey. The LPO activity retained 0.6 and 0.8 U $mL^{-1}$ for non-dialyzed and dialyzed whey, respectively, that represents more than

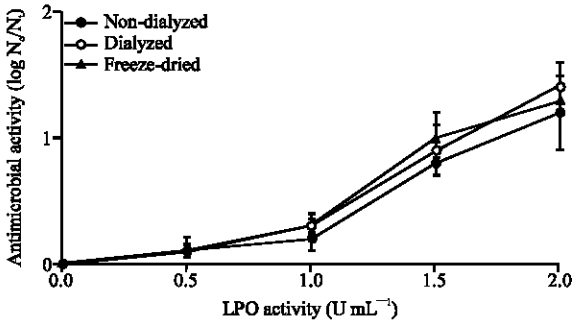


Fig. 1: Antimicrobial activity of various form of whey against *S. enteritidis*. Antimicrobial activity of non-dialyzed, dialyzed and freeze-dried whey against *S. enteritidis* was measured using initial inocula of 5 log CFU $mL^{-1}$. Data points are mean values based on triplicate determination. Error bars represent standard deviation of the mean

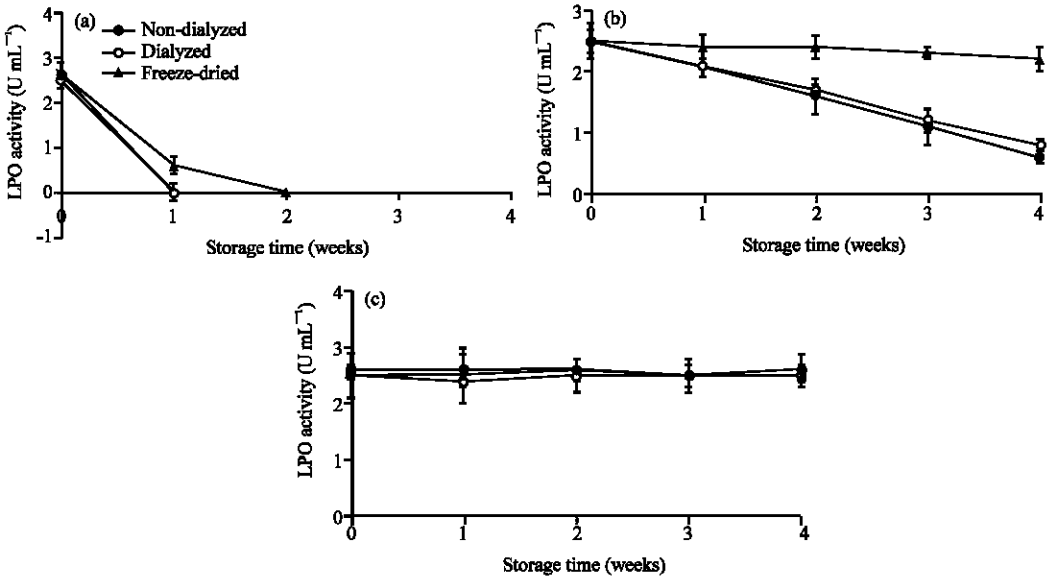


Fig. 2: LPO activity of various forms of whey stored at various conditions. The effect of storage at (a) 25°C, (b) 4°C and (c) -20°C for 4 weeks on the LPO activity of whey. LPO activity was measured in various forms of whey: non-dialyzed, dialyzed and freeze-dried whey. Data points are mean values based on triplicate determination. Vertical bars represent standard deviation of the mean

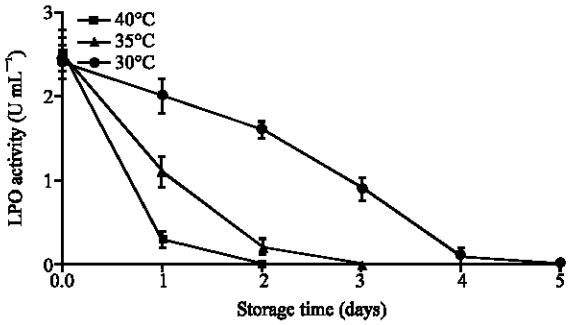


Fig. 3: LPO activity of freeze-dried whey at various temperature of storage. LPO activity was measured in freeze-dried whey stored at 30°C, 35°C and 40°C for 5 days. Data points are mean values based on triplicate determination. Vertical bars represent standard deviation of the mean

75% loss of LPO activity. When stored at -20°C, a stable LPO activity was found for all of whey's forms for 4 weeks storage (Fig. 2c).

**LPO activity of freeze-dried whey stored in various temperatures:** Whey is known as byproduct of cheese production and probably it was stored under uncontrolled temperature. To observe the LPO activity during such condition of storage, we analyzed LPO activity of freeze-dried whey when stored at 30-40°C. The results are shown in Fig. 3. LPO activity in freeze-dried whey was terminated at 5 days storage when stored at 30°C. The increase of storage temperature into

35 and 40°C resulted the rapid termination of LPO activity within 3 and 2 days of storage, respectively.

## DISCUSSION

Bovine whey constitutes about 55% of the milk nutrients including enzyme (Kosikowski, 1978; Madureira *et al*., 2007; Shah, 2000). One of them is LPO, which has ability to generate $OSCN^-$ to inhibit the growth of bacteria (Shah, 2000; Zhou and Lim, 2009; Dajanta *et al*., 2008). LPOS effectively inhibits *S. enteritidis* in fruit and vegetable (Touch *et al*., 2004) indicating that the LPOS may be applied in various kinds food including dairy and non-dairy products. However, the practical use of LPOS seems too hard to apply to food industry since LPO is expensive and the large amount is required. The utilization of whey in LPOS may significantly reduce the cost of LPOS application since it skips the LPO purification process and the whey is largely available; therefore, we challenged to use whey, which is by product of cheese manufacture to generate antimicrobial agent.

In the purification process of LPO from bovine whey, the dialysis is a procedure needed to remove low molecular weight materials and to improve the accessibility of enzyme to bound into resin (Nakano and Ozimek, 2000; Touch *et al*., 2004). On the other hand, the process of dialysis increases the cost and eventually consumes the time because this process takes more than 12 h (Touch *et al*., 2004; Wolfson and Sumner, 1993; Zhou and Lim, 2009). The potential advantages must compensate the disadvantages if a dialysis process is to be useful. Based on this result, there was no advantage of the dialysis process; thus, bypassing dialysis process shortened the procedure of whey preparation without giving any negative effect on its antimicrobial activity.

Antimicrobial activity of purified LPO against *S. enteritidis* has been reported by Touch *et al*. (2004), which showed the value of about 1.2 U $mL^{-1}$. Although this value was obtained from the different condition of LPOS, our result indicated that whey and purified LPO has comparable ability to inhibit the growth of *S. enteritidis*. Thus the whey gives an advantage for producing antimicrobial agents.

Our experiment prompted to the results that storage condition easily changes the LPO activity in whey. LPO activity in non-dialyzed and dialyzed whey stored at 4° and 25°C reduced sharply. The rapid reduction of LPO activity can be explained by the denaturation of enzyme during storage (Tamiya *et al*., 1985). Besides, the presence of natural compounds may induce the reduction of LPO activity. Lactose, as an original compound of bovine whey, has a potent inhibitor for LPO activity (Al-Baarri *et al*., 2010). Other compound such as casein and amino acid tyrosine might also inhibit LPO activity (Clausen *et al*., 2008; Fonteh *et al*., 2005). $SCN^-$ might also inhibit LPO activity (Reiter and Harnulv, 1984; Singh *et al*., 2009). This may explain the LPO activity retained in the dialyzed whey a bit higher than that of non-dialyzed whey. The inhibition of LPO activity by natural compound can be confirmed by our observation on LPO activity in milk resulted in the larger decrease of LPO activity (*ca*. 93%) during 4 weeks storage (data not presented).

A loss of enzyme activity principally due to the denaturation. The probability of denaturing the protein increases along with the raise of temperature of storage (Chattopadhyay and Mazumdar, 2000; Klibanov, 1979, 2001; Nino *et al*., 2004). This may explain the rapid decrease of LPO activity during storage at high temperature. The storage at 25°C or room temperature allows the growth of bacteria to produce protease that induce protein denaturation in whey (Drgalic *et al*., 2005; Ha and Zemel, 2003; Saw *et al*., 1998; Wlerzbicki and Kosikowski, 1973). This result might become a direct consequence for whey to store at -20°C (or 4°C for freeze-dried whey) in order to maintain

the LPO activity. The whey stored at -20°C retained LPO activity for six month without any notable change (data not presented).

In conclusion, this results showed that all forms of whey exhibited antimicrobial activity against *S. enteritidis* and the activity was comparable to those of purified LPO. This may open the gate for LPOS application in food industry. The results highlight the fact that in order to maintain the LPO activity of whey in long period of storage, the whey (non-dialyzed and dialyzed) should keep in the freezer while freeze-dried whey should be stored in refrigerator.

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