Journal of Dairy Research (2018) **85** 460–464. © *Hannah Dairy Research Foundation 2018*
doi:10.1017/S0022029918000730

Lactoperoxidase immobilization on silver nanoparticles enhances its antimicrobial activity

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Received 17 November 2017; accepted for publication 19 July 2018; first published online 23 August 2018

Lactoperoxidase (LPO) is an antimicrobial protein present in milk that plays an important role in natural defence mechanisms during neonatal and adult life. The antimicrobial activity of LPO has been commercially adapted for increasing the shelf life of dairy products. Immobilization of LPO on silver nanoparticles (AgNPs) is a promising way to enhance the antimicrobial activity of LPO. In the current study, LPO was immobilized on AgNPs to form LPO/AgNP conjugate. The immobilized LPO/AgNP conjugate was characterized by various biophysical techniques. The enhanced antibacterial activity of the conjugate was tested against *E. coli* in culture at 2 h intervals for 10 h. The results showed successful synthesis of spherical AgNPs. LPO was immobilized on AgNPs with agglomerate sizes averaging approximately 50 nm. The immobilized conjugate exhibited stronger antibacterial activity against *E. coli* in comparison to free LPO. This study may help in increasing the efficiency of lactoperoxidase system and will assist in identifying novel avenues to enhance the stability and antimicrobial function of LPO system in dairy and other industries.

Keywords: Antimicrobial activity, immobilization, lactoperoxidase, silver nanoparticles.

Lactoperoxidase (LPO) is a heme-containing antimicrobial enzyme present in human and animal secretory fluids, such as milk, saliva and tears (Sharma et al. 2013; Bafort et al. 2014). It is a member of the mammalian peroxidase family, which also includes myeloperoxidase, eosinophil peroxidase and thyroid peroxidase (Zamocky & Obinger, 2010). The antimicrobial activity of LPO is due to production of potent oxidizing and bactericidal compounds in the presence of hydrogen peroxide (H_2O_2), as LPO catalyzes the oxidation of many organic molecules including halides (Furtmuller et al. 2002) and nitrates (Van der Vlict et al. 1997). Pseudohalide thiocyanate (SCN^-) has been reported to be the most specific substrate for LPO (Sheikh et al. 2009) and oxidation of SCN^- leads to production of antimicrobial compound hypothiocyanate ($OSCN^-$) (Furtmuller et al. 2002).

LPO has been reported to have microbicidal or microbiostatic activity against a wide range of microorganisms such as viruses (Courtois et al. 1990), fungi (Popper & Knorr, 1997) and bacteria (Kussendrager & Von Hooijdink, 2000). Within the human body, LPO plays a key role in humoral defence mechanisms against microorganisms (Zamocky & Obinger, 2010) including protecting the lactating mammary gland of the mother and the intestinal tract of new born infants (Zhang et al. 2008). Lactoperoxidase system has three components, i.e. LPO, hydrogen peroxide and halides/pseudohalides, and has assumed considerable importance in the dairy industry (Lonnerdal & Lien, 2003). The use of LPO system for increasing the shelf life and preservation of milk and milk products is an easy and alternative way, particularly in areas where refrigeration facilities are not available (Garcia-Graells et al. 2000). This is done by adding small quantities of H_2O_2 to milk. Use of natural antimicrobial systems is important to improve the safety and to maintain high quality, natural freshness and healthiness of the food items (Gould, 1996). However, these systems are hampered by limitations such as selective inhibition, resistance development, and adsorption of antimicrobial components by fat, protein, and other molecules present in food items (Garcia-Graells et al. 2000).

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In view of the importance of the LPO system, it is highly advantageous to maximize the stability, activity, and recovery of LPO, thus improving the efficiency of the system. Recently, nanotechnology has been gaining tremendous importance in various fields of science and industry, and commercial applications of nanoparticles in many areas, such as chemistry, catalysis, electronics, medicine, and energy have multiplied (Masala & Seshadri, 2004). In this regard, silver in the form of nanoparticles has been known to have inhibitory and bactericidal effects (Kora & Rastogi, 2013) and has been shown to increase the antimicrobial activities of various antibiotics against bacteria (Shahverdi et al. 2007). Immobilization of LPO on AgNPs is a promising way to enhance the efficiency of LPO and is expected to be commercially beneficial because of the broad working spectrum of LPO. To our knowledge, there are no studies available on enhancing the antimicrobial activity of LPO using AgNP-based approach. Therefore, the objective of our study was to increase the antimicrobial efficiency of the LPO enzyme by immobilizing it on AgNPs. The LPO/AgNP immobilized complex was physicochemically characterized and tested for enhanced antimicrobial activity against *Escherichia coli (E.coli)* and bacterial growth both in liquid culture and agar plates was monitored and recorded at different time intervals.

Materials and methods

Purification of LPO from milk

For purification of LPO from camel milk, the procedure previously described in detail for water buffalo milk was followed (Sheikh et al. 2009). Purified LPO protein was lyophilized and used for immobilization on AgNPs.

Synthesis of AgNPs

Aqueous solution of $AgNO_3$ and purified LPO enzyme were used as salt precursor and capping agent, respectively, for AgNPs. The aqueous freshly prepared solution of $NaBH_4$ was added as a reducing agent. The colorless reaction mixture transformed into yellow color after the addition of $NaBH_4$ which was due to growth of AgNPs. The growth of AgNPs was monitored by measuring the absorbance by UV–vis spectroscopy of the reaction product using spectrophotometer at 200–800 nm. The surface morphology of the nanoparticles was studied using field emission scanning electron microscopy (FESEM; JEOL JSM-7600F, Japan). Elemental analysis was carried out using energy-dispersive spectroscopy (EDS).

Bacterial cultures and antibacterial activity

Antibacterial activities of the LPO and LPO/AgNP conjugate were determined using Gram-negative bacteria *E. coli*. The *E. coli* cells were grown in 3 culture tubes overnight in 5 ml Luria–Bertani (LB) broth containing 1·0% tryptone, 0·5% yeast extract and 1·0% sodium chloride. Each tube was inoculated aseptically with 50 µl of *E. coli* bacterial suspension (~10^4 CFUs/ml). The culture tubes were randomly kept as control, LPO alone and LPO/AgNP. The control culture tube for the *E. coli* growth was inoculated with 50 µl of *E.coli* suspension (~10^4 CFU/ml) only. The LPO alone culture tube was inoculated with 50 µl of *E. coli* suspension and also received 1 mg/ml of LPO. The LPO/AgNP culture tube was inoculated with 50 µl of *E. coli* suspension and received 2 mg/ml of LPO/AgNP conjugate. The LPO alone and LPO/AgNP culture tubes also received other two components of LPO system, SCN^- at 10 µM concentration and H_2O_2 at 5 µM in phosphate buffer at pH 7·4. All the three culture tubes were kept on horizontal shaker at speed of 200 rpm at 37 °C for 10 h. The bacterial growth was monitored by optical density (OD) at 600 nm and by number of colony forming units (CFUs) at 2 h intervals (2, 4, 6, 8, 10 h) until 10 h to analyze the inhibitory activity of the LPO and LPO/AgNP conjugate.

Results

AgNP synthesis and characterization

The AgNPs formation in the reaction mixture of AgNO3 and LPO was confirmed by the color change (colorless to yellow) supporting the reduction of Ag (I) ions to Ag (0). Further, UV–vis spectroscopy revealed a single surface plasmon absorption band of AgNP at 400 nm. The morphology and size distributions of AgNP/LPO conjugate from FESEM measurements are presented in Fig. 1. The particle shapes were spherical and the average size of the nanoparticles in the pure micelle was ~50 nm. The results of elemental analysis of silver nanoparticles using EDS are depicted in Fig. 2. The EDS spectrum showed peaks or signals of silver atoms at 2·6 and 3·0 keV in the AgNP/LPO nanoparticles. In addition, peaks for carbon and oxygen were also shown at 0·3 and 0·5 keV.

Bacterial culture and antibacterial activity

The optical density (OD) of *E. coli* cultures in control, LPO alone and LPO/AgNP is presented in Fig. 3. For control culture tube, a progressive increase in OD indicating bacterial growth is shown from 2 to 10 h (end of experiment). For the culture tube with LPO alone, the optical density remained low until 6 h and then began to increase and was high at 10 h. However, at 10 h the OD was lower than the control. In the culture tube with LPO/AgNP conjugate the OD remained low until the end of experiment (did not increase until 10 h). Similar results were also observed for the number of CFU (data not shown) with progressive increase in number of CFUs from 2 to 10 h in control and comparatively slower increase in number of CFUs from

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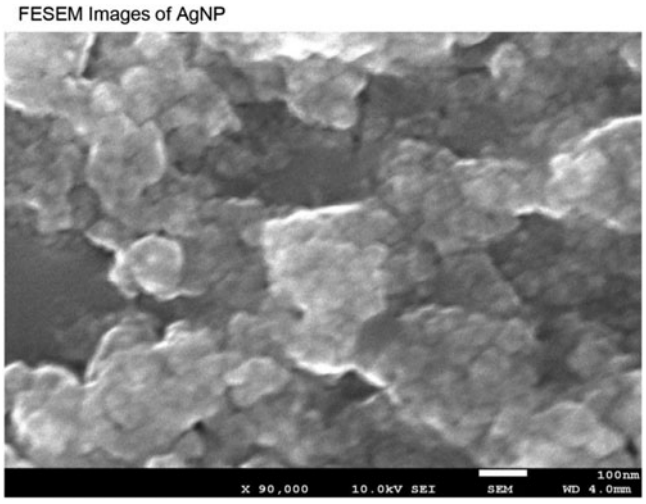

Fig. 1. Field emission scanning electron micrograph showing morphology and size distribution of silver nanoparticles and lactoperoxidase (LPO) conjugate obtained from reaction mixture of $AgNO_3$, $NaBH_4$ and lactoperoxidase. The nanoparticle shapes are spherical and the sizes in the pure micelle are ~50 nm.

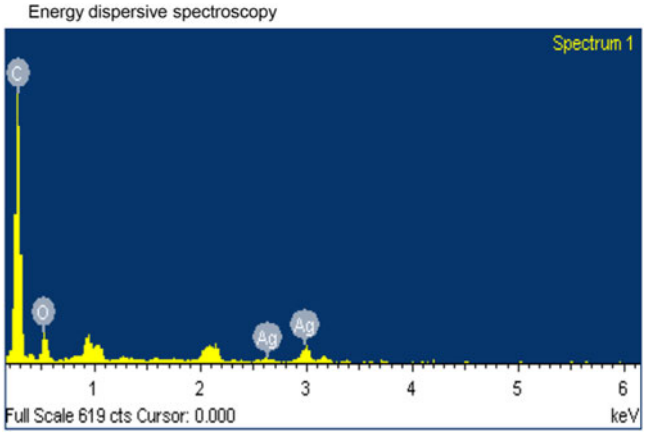

Fig. 2. Energy-dispersive spectroscopy spectrum showing elemental analysis of silver nanoparticles and lactoperoxidase (LPO) conjugate obtained from reaction mixture of $AgNO_3$, $NaBH_4$ and LPO. Peaks or signals of silver atoms at 3 keV, carbon (C) at 0·3 keV and oxygen (O) at 0·5 keV are shown.

2 to 6 h in LPO alone. The number of CFUs increased more rapidly from 8 to 10 h in LPO alone, however, the number of CFUs at 10 h was less than the control. In LPO/AgNP conjugate, the number of CFUs were already lower at 2 h in comparison to control and LPO alone, and no CFUs were observed at 4, 6, and 10 h.

Discussion

LPO is an important constituent of the lactoperoxidase system with considerable potential for industrial applications. Improvement of traditional methods and development

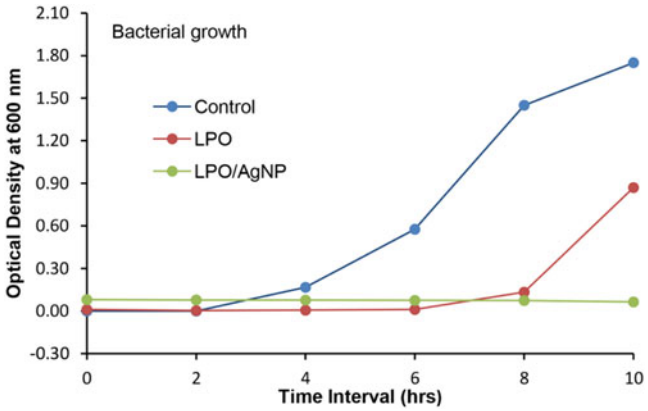

Fig. 3. Optical density of *E. coli* cultures at different time intervals in culture tubes of control, lactoperoxidase (LPO) alone and LPO conjugated with silver nanoparticles (LPO/AgNP).

of novel techniques for increasing the shelf life of milk and milk products are of high importance to commercial dairy industry. The immobilization of enzymes is being considered as an important development for increasing their process utilization and reducing operational expenses in commercial applications (Johnson et al. 2011). Immobilized enzymes show greater stability at higher temperatures for longer periods of time and in comparison to their native soluble form are more denature resistant. Nanoparticles have generated considerable scientific interest recently in view of their wide-ranging potential for use in diverse fields in industry and medicine (Rai et al. 2012). Immobilization with nanoparticles provides greater surface area that increases the enzyme loading, less fouling effect and lower mass transfer resistance

In the current study, immobilization of LPO on AgNPs resulted in enhancement of antimicrobial activity of LPO. The successful synthesis of AgNPs in our study was shown by production of yellow color of colloidal AgNPs with an absorption spectrum at 400 nm and particle sizes of approximately 50 nm. Nanoparticles emit bright colors due to oscillations of the surface electron cloud of these particles, and the interaction of combined oscillations of electrons with light of suitable energy imparts to nanoparticles a color that is specific to the particular metal to which the nanoparticles belong (Link & El-Sayed, 1999). A pale yellow color is specific for silver and indicated formation of silver nanoparticles in our study as has been reported previously in many studies (Geethalakshmi & Sarada, 2012; Huo et al. 2017). In addition, the results of UV–Visual spectroscopy showing a band at 400 nm in our study are similar to previous reports showing a silver band at 340 and 400 nm (Pal et al. 2016; Huo et al. 2017).

The LPO was assumed to be immobilized entirely on the AgNPs and FESEM imaging demonstrated homologous morphology of LPO/AgNP agglomerates with particle sizes averaging approximately 50 nm. Synthesis of diverse sizes of AgNPs and the agglomerates of AgNP conjugates with

https://doi.org/10.1017/S0022029918000730 Published online by Cambridge University Press

peptides and polypeptides have been reported previously (Dumri & Hum Anh, 2014; Brahmkhatri et al. 2015; Pal et al. 2016). The variations in the particle size probably occurred due to synthesis occurring for different times (Geethalakshmi & Sarada, 2012).

The elemental analysis by EDS also confirmed the successful synthesization of LPO/AgNPs conjugates, as the peaks showed the presence of the C, O and Ag in grown nanoparticles. The C and O peaks indicated that the enzyme (LPO) was successfully immobilized on AgNPs. The EDS spectrum peaks of 2·6 and 3·0 keV showed the presence of silver atoms in the conjugate and peaks at this optical absorption are typical for silver nanocrystallites due to surface plasmon resistance, and absorption peaks ranging from 2·5 to 4·0 keV have been reported in previous studies on AgNPs (Gardea-Torresdey et al. 2003; Geethalakshmi & Sarada, 2012; Huo et al. 2017).

Antimicrobial activity experiment using *E. coli* culture showed that AgNP conjugation with LPO resulted in increased antimicrobial activity of LPO in comparison to LPO alone. Monitoring of *E. coli* growth by taking OD at 2, 4, 6, 8 and 10 h after inoculation and counting of CFUs on agar plates inoculated with *E. coli* cultures from culture tubes at 2, 4, 6 , 8 and 10 h was done. *E. coli* cultures showed minimal growth until 6 h after inoculation in LPO alone as indicated by lack of OD increase, where as in control, OD increased progressively from 2 to 10 h. Therefore, the results indicate that LPO alone exerted and maintained its activity of inhibiting bacterial growth until 6 h. From 6 h onwards LPO started to lose its antimicrobial activity as shown by the OD increase of *E. coli* culture. This could be attributed to many factors such as temperature, protein stability etc. In the cultures containing LPO immobilized on AgNPs, the bacterial growth did not occur as shown by lack of increase in OD until the end of experiment reflecting that the immobilization of LPO on AgNPs enhanced the antimicrobial activity of LPO beyond 6 h and potentially for longer than 10 h. This indicated that LPO/AgNP conjugate retains its efficiency for longer time in comparison to free LPO enzyme. Similar conclusions were derived from number of CFUs observed in *E. coli* grown on plates.

Studies on immobilization of LPO on AgNPs are not available, whereas studies of LPO immobilization on nanoparticles of other metals are limited. In a comparative study on four different proteins including LPO, degree of coverage on silica nanoparticle surface was studied and was reported to be influenced by both protein structural stability and charge distribution at surface of protein (Turci et al. 2010). In another study (Samani et al. 2016a), LPO immobilized on silica coated magnetic nanoparticles displayed enhanced efficiency in presence of cadmium chloride (inhibitor). In a similar study (Samani et al. 2016b), LPO immobilized on Fe_3O_2 exhibited higher thermal stability in comparison to free LPO enzyme. In none of the three indicated studies, antimicrobial properties of LPO conjugates were analyzed.

Peptides, including the commercially available polymyxin B, had enhanced antimicrobial activity after conjugation with AgNPs (Ruden et al. 2009). Similarly, preparation of AgNPs using a cell penetrating 20 amino acid peptide as capping agent resulted in enhanced antimicrobial activity of the conjugate compared to AgNP alone (Liu et al. 2013). In a recent study (Pal et al. 2016), conjugation of an antimicrobial peptide with AgNPs enhanced its antimicrobial activity. The mechanisms involved in interaction of AgNP with LPO in the conjugate and increased antimicrobial activity of the LPO/AgNP conjugate are not well understood and need further studies. However, dynamic interactions of silver nanoparticles with a cysteine rich antimicrobial peptide that resulted in significantly increasing the activity and stability of the complex have been shown (Pal et al. 2016). Further, when AgNPs are in conjugation with polypeptides, the stability is enhanced and is due to the steric repulsion between proteins that does not allow nanoparticles to approach each other and thus prevents aggregation (Brahmkhatri et al. 2015). The stability of the conjugate allows its multiple recycling without losing potency as recently indicated for AgNP-ubiquitin conjugates (Brahmkhatri et al. 2015). Part of the antimicrobial activity of the LPO/AgNP conjugate is also due to AgNPs. Silver and nanoparticles of silver are known to have antimicrobial activity (Morones et al. 2005; Sharma et al. 2009; Omprakash & Sharada, 2015).

The antimicrobial action of LPO is through oxidoreductases activity involving production in situ of reactive compounds which inhibit microorganisms. LPO catalyzes oxidation of SCN^- in presence of H_2O_2 resulting in production of short-lived reactive oxidation product, ^-OSCN (Bafort et al. 2014). ^-OSCN rapidly oxidizes many biomolecules including, in relation to antimicrobial activity, the exposed sulfhydryl containing enzymes in the bacterial cell membranes causing membrane disruption, pH gradient disbalance, K ion leakage, and inhibition of solute transport. The AgNPs are thought to produce their antimicrobial effects by attaching or penetrating the bacterial cell wall and disrupting the cellular signaling by dephosphorylation of tyrosine residues on key peptide substrates (Shrivastava et al. 2007). Our opinion is that the increased antimicrobial activity of immobilized LPO with AgNPs is by increased stability of protein and increased surface area which allows for oxidation of the substrate for longer duration thus producing greater amounts of ^-OSCN. However, further studies and characterization of the interactions of AgNP with LPO and molecular mechanisms underlying the antimicrobial property enhancing mechanisms are needed.

In conclusion, an immobilized LPO/AgNP conjugate was characterized by UV–vis spectroscopy, FESEM, and EDS. We showed that AgNPs were successfully synthesized and LPO immobilized on AgNPs exhibited stronger antibacterial activity against *E. coli* in comparison to free LPO. The study will be helpful in identifying novel avenues to enhance the stability and antimicrobial function of LPO system.

https://doi.org/10.1017/S0022029918000730 Published online by Cambridge University Press

This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH) – King Abdulaziz City for Science and Technology – the Kingdom of Saudi Arabia – award number (12-BIO3082-03). The Authors also acknowledge with thanks Science and Technology Unit, King Abdulaziz University, for their technical support.

Conflict of interest

The authors have declared that no conflict of interest exists.

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