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Original Article

Protection of lactoperoxidase activity with sugars during lyophilization and evaluation of its antibacterial properties

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Abstract

The purpose of the present study was to compare the stabilizing effect of four disaccharides alone or in combination on the lactoperoxidase (LP) derived from bovine milk during lyophilization. Sucrose, lactose, maltose, and trehalose at different concentrations (5-500 mM) were used to compare their protective effects on LP activity . The activity of lyophilized and native LP enzyme was evaluated using the procedure of Schindler with slight modifications**.** The antibacterial activity of the lyophilized enzyme against *Pseudomonas aeroginosa, Escherichia coli, and Staphylococcus aureus* was also investigated using the antimicrobial effectiveness test. Trehalose at concentration of 500 mM was the most effective cryoprotectant in protecting the enzyme activity. It preserved LP activity for 40 days, while the native enzyme lost its activity after 6 days. Combinations of disaccharides resulted in an increment in the stability of the enzyme, compared to the native enzyme. Combination of 200 mM trehalose and 200 mM sucrose were found most effective cryoprotectant in freeze-drying of LP. The lyophilized LP decreased the growth rate of *Ps.aeroginosa, E.coli*, and *S.aureus* between up to 30.8% in 10^6 cfu/ml and 53.3% in 10^5 cfu/ml . Antimicrobial efficacy of LP was more pronounced when 10^5 cfu/ml was used as compared to 10^6 cfu/ml.

Keywords: Lactoperoxidase; Enzyme activity; Lyophilization; Antibacterial properties; Sugars

INTRODUCTION

Lactoperoxidase (EC.1.11.1.7) (LP) is a member of the peroxidase family and a component of bovine and human milk. Also, it is found in salivary and lachrymal glands of mammals. LP content of bovine milk is higher than that of other mammals' milk (1). Bovine LP is a glycoprotein consisting of a single peptide chain with a molecular weight of about 78.4 KDa (2). LP provides protection against a variety of susceptible microorganisms. The enzyme catalyzes the conversion of the thiocyanate (SCN^-) to hypothiocyanate ($OSCN^-$) in the presence of hydrogen peroxide (H_2O_2). Hypothiocyanate is the main intermediary oxidation product of thiocyanate and it is responsible for the antibacterial property of the LP enzyme. The LP with SCN^- and $OSCN^-$ is called LP system (LP-S). LP-S is a naturally occurring antibacterial system in milk (3). LP-S can be applied as a preservative agent in fish, meat, fruits and vegetables, cosmetics and associated products, ophthalmic solutions and as well in the development of drugs, antiviral agents and dental pastes (4). LP is one of the most abundant bovine milk enzymes, which enters to whey in the process of cheese production. It also has some anti carcinogenic and antimicrobial properties so it can be used as a treatment in some diseases like colon and mammary tumors and *Helicobacter pylori* infections (5).

The stability of LP enzyme has been investigated by different researchers. Al-Baarri and coworkers have recently shown that LP will lose its catalyzing activity within 4 weeks, 3 weeks and 2 weeks at -20, 4 and 25 °C, respectively. The LP activity in all different forms of whey including freeze-dried whey, non-dialyzed and dialyzed whey was also studied at different temperatures (6). In

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another study the effect of ionic interactions and the importance of these interactions on the stabilization of LP has been investigated (7). Cationic surfactant, benzalkoniumchloride, was found to be efficient in preserving the enzymatic activity of LP for over 10 days, while the native enzyme completely lost its activity within 3−4 days (8). Another study established that solid-phase LP (lactoperoxidase coupled to sepharose) can be further stabilized by cross-linking with glutarylaldehyde to reduce leakage of the enzyme from the sorbent (9).

The primary application of LP-S is the preservation of raw milk quality. The shelf-life of raw milk can be significantly extended by addition of LP-S (10). In these studies though the stability of the enzyme has been enhanced but the duration of the stability has not been extended to an acceptable limit. Therefore, stability of the enzyme for a longer period of time is of prime importance and demand further studies.

Lyophilization is one of the most common technique for preparation and stabilization of biological such as proteins, plasma, viruses and cell lines (11). The process, more commonly known as freeze-drying, involves elimination of water from a frozen material under vacuum by sublimation.

Freeze-drying is mainly used to remove water or other solvents from sensitive products with no damage. Consequently the lyophilized substance could be easily preserved at suitable temperatures and pressures in a permanently storable state and also can be reconstituted simply by addition of a suitable solvent like water. If the freeze-drying operation is performed correctly, the process will protect most of the initial biological activity of the substance in the dry state.

Different additives such as sugar-based excipients (i.e. sucrose, trehalose) are frequently appended to the lyophilized enzyme to protect the protein from different stresses during processing and to preserve its stability during storage (12). For instance, addition of trehalose or maltose to phosphofructokinase enzyme solution during lyophilization enhanced the catalyzing activity of the enzyme up to 80% (13).

In many cases a disaccharide or even a mixture of two different disaccharides appear to be most effective in stabilizing the enzyme during freeze-drying procedure. These cases include the protection of chymotrypsinogen in the presence of 300 mM sucrose (14), complete protection of four restriction enzymes by 15% trehalose (15) and stabilization of recombinant human serum albumin (16). Steven and colleages and colleagues demonstrated that the extent of stabilization of protein during lyophilization method depends on both the kind of the disaccharide and the disaccharide concentration (17).

Lactoperoxidase plays an important role in the protection of milk against a broad range of bacteria. The most recommended industrial application of the LP is in the dairy industry for the preservation of milk during storage or transit. The involvement of LP in the suppression of microbial growth was first suggested by Finn (18). The LP system could have a bacteriostatic and/or bactericidal activity on a variety of microorganisms including bacteria, fungi and viruses. Therefore the LP can be used in protecting fish, meat and hygiene products (19).

For producing a storable type of a perishable protein solution like LP, the ideal way is to derive a dry product from aqueous solution. A solid-form enzyme can be easily stored, transported or applied. In the current study, we attempted to prepare combination of LP-S with sugar-based excipients in a dry powder form to enhance the stability of LP for an extended period of time while retaining its original structural integrity and activity.

The LP enzyme is abundant in raw milk but thiocyanate and hydrogen peroxide are present at low concentrations. When activated, the LP can generate hypothiocyanate (10).

Therefore, in the present study sodium thiocyanate was used as a source of thiocyanate (SCN^-) and sodium percarbonate as a source of hydrogen peroxide **to** produce the main antimicrobial agent.

To determine if the LP functioning appropriately in inhibiting the growth of microorganisms in milk, three bacteria including *Pseudomonas aeroginosa,*

Escherichia coli and *Staphylococcus aureus* were used. These three organisms hae been specified to be better indicators of preservative effectiveness according to the general chapter 51 of the United States Pharmacopeia (20). The LP-S exerts both bacteriostatic and bactericidal activities against *S. aereus*. It is a bactericidal agent against enteric pathogens like *E.coli*. Catalase-positive organisms such as *Ps. aeroginosa* are not only inhibited by the LP-S but also, depending on the medium conditions, may be killed (2).

MATERIALS AND METHODS

Materials

Unpasteurized bovine milk was obtained from the National Diary Research Institute (Isfahan, Iran). 2,2' azinobis(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), diethylaminoethyl-cellulose (DEAE-Cellulose), sodium thiocyanate and sodium percarbonate were purchased from Sigma Chemical Co (USA). Other chemicals such as sodium phosphate, sodium chloride, sucrose, maltose, lactose, trehalose, hydrogen peroxide ,sodium acetate, hydrogen chloride and sodium hydroxide were from Sigma Chemical Co (USA). Pure cultures of *E. coli* (PTCC 1338), *S. aureus* (PTCC1337) and *Ps.aeruginosa* (PTCC1074) were supplied by the Persian Type Culture Collection (PTCC, Isfahan, Iran). All the chemicals and reagents used were of analytical grade.

Purification of the lactoperoxidase

Rennet whey

Bovine milk was centrifuged (International Equipment Co. Needkam HTS., Mass. USA) at 5000 × g at room temperature (25 ºC) for 20 min to remove fat to obtain skim milk. The skim milk was warmed upto 37 ºC and rennet (Sigma Company, St. Louis, MO, USA) was then added in the proportion of 0.1 g/l and kept for 1 h.

The rennet whey was initially separated by filtration using Whatman No.1 filter paper (W & R Balston, England) and subsequently by centrifugation (Laborzentrifugen, Sigma, Heraus Sepatech Suprafuge 22, Hanau, Germany) at 10000 × g for 20 min at 4 ºC for removing the remaining fat and insoluble proteins.

Diethylaminoethyl anion exchange chromatography

Diethylaminoethyl-cellulose (DEAE-cellulose) was prepared for anion exchange chromatography. The resin with a capacity of 1.0 μ/g (dry) was obtained from Sigma Corp (St. Louis, MO, USA). The resin was suspended in 100 ml of 0.1 M NaOH containing 0.5 M NaCl for chromatography. It was poured into a Buchner funnel (volume: 150 ml) and washed with 100 ml of the above mentioned solution.

This step was repeated by 0.5 M NaCl, 0.1 M HCl containing 0.5 M NaCl and finally with distilled water until the effluent pH was equal to or greater than 5. The resin was then suspended in 100 ml of 1 M NaCl and the slurry pH was adjusted to 7-8 with NaOH solution. Finally, it was filtered and suspended in 100 ml of sodium phosphate buffer and filtered again. Then it was removed from the funnel and was re-suspended in 250 ml of 0.05 M sodium phosphate buffer at pH 6.5. The pH of a small portion was measured to ensure it is within 0.15 pH units of the 0.05 M buffer at pH 6.5. The resin was packed into a 11.5 × 2.8 cm column and the buffer solution was drained by gravity flow.

Rennet whey was loaded onto the column, which had already been equilibrated with 0.05 M sodium phosphate buffer at pH 6.5 (according to the Sigma Guide for regeneration of DEAE-Cellulose). The column-bounded enzyme was washed with 250 ml of 0.05 M sodium phosphate buffer. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 600 ml of 0.05 M sodium phosphate buffer at pH 6.5. After elution from the column, various fractions were combined for dialysis. The enzyme solution was dialyzed overnight against 100 volumes of the same buffer at 4 ºC (21).

Freeze drying process

Fifteen ml enzyme solutions of LP (0.03 mg/ml) containing excipients at concentrations 5 to 500 mM was dispensed into glass lyophilization vials and frozen at -70 ºC for 24

h. The frozen samples were transferred to a freeze-dryer (Freeze-dryer, Alfa 2-4LDplus, Christ Co, Germany) in which temperature was set at -80 ºC and the vacuum pressure of 0.001 mBar was applied. After lyophilization the vials were immediately filled with dry nitrogen gas and sealed with rubber stoppers. Spectrophotometric analysis and LP activity measurements of freeze-dried samples containing fixed amount of LP and different quantities of sugars were carried out using the procedure explained in subsequent section.

Determination of lactoperoxidase activity

LP activity was determined by the procedure of Schindler with slight modifications (22). This method is based on the oxidation of ABTS as a chromogen substrate with hydrogen peroxide. The bluish compound gives a maximum absorbance at 412 nm. 100 µl of 10 mM ABTS in sodium acetate buffer (0.1 M, pH 5.2, 20 ºC) was mixed with 10 mg of lyophilized LP powder (with different concentrations of sugar) and 700 µl of sodium acetate buffer (0.1 M, pH 5.2). The reaction was initiated by adding 100 µl of H_2O_2 solution (5 mM). All reagents were stored and used at 22 ºC. The measurement of absorbances were recorded at 15 s and again at 75 s. Measurements were carried out against the blank containing ABTS and enzyme solution only. The rate of absorbance per min was then calculated (the extinction coefficient of the ABTS oxidation product at 412 nm is 32.4 × 10-3l/mol.cm) (23).

Antibacterial studies

Maintenance of test organisms

Pure cultures of *E. coli*, *S. aureus*, and *Ps. aeruginosa* obtained from the Persian Type Culture Collection (PTCC) and stored at 4 ºC as lyophilized ampoule. Stock cultures were prepared by transferring a loopful from the original cultures onto nutrient agar plates. The cultures were then incubated at 37 ºC for 24 h and stored at 4 ºC .

Activation of the lactoperoxidase system

Lyophilized powder of LP enzyme has been activated by addition of sodium thiocyanate as a source of thiocyanate (SCN^-) and sodium percarbonate as a source of hydrogen peroxide (24). The optimized concentration for activating LP in unpasteurized milk for sodium thiocyanate and sodium percarbonate is 16 mg/l and 30 mg/l, respectively. In the present study, after some preliminary experiments, the best antimicrobial effect was achieved by adding 0.9 mg sodium percarbonate and 1.2 mg sodium thiocyanate to 100 mg of lyophilized LP.

Inoculation of milk samples with the pathogens

The growth rates of three microorganisms including *E. coli*, *S. aureus*, and *Ps. aeruginosa* were assayed in two groups in the presence or absence of activated LP.

Nine ml of Muller-Hinton broth was transfered into screw-capped test tubes and sterilized in an autoclave for 15 min at 121 ºC. The suspensions of each pathogen were prepared by transferring fresh colonies, already grown on the surface of agar plates, into test tubes using sterile plastic loops. Optical density of 0.3 at 580 nm wavelength equates 10^8 cfu/ml. Then the suspensions of 10^5 and 10^6 cfu/ml were prepared by the serial dilution method. In all cases, tubes were inoculated with 0.1 ml of 10^5 cfu/ml or 10^6 cfu/ml of the appropriate bacteria strains. 1 ml of lyophilized and activated LP was added to bacterial suspensions. The tubes were incubated at 37 ºC for 20 h and their absorbances were read at 580 nm by the use of a spectrophotometer (Spectronic 70, Bausch and Lomb Co, Germany).

RESULTS

In the present work, different disaccharides were evaluated for their protective properties on the LP activity during lyophilization. The activity of the native LP was 1.35 ± 0.08 unit/ml. In the absence of sugars only 5% of the original activity of the freeze-dried LP was retained after 2 days while after lyophilization with carbohydrates its activity retained for at least 15 days.

The sugar-base excipients used in this study included sucrose, trehalose, maltose and lactose. LP activity was measured immediately after freeze-drying and several days later.

Though samples were kept at 4 ºC, stability measurements were carried out until the activity of the original enzyme decreased to less than 30%.

Figs.1-4 show the effect of different concentrations of sucrose, trehalose, maltose and lactose on the stability of LP, respectively. As shown in these figures, although percent retained activities of LP enzyme were significantly greater at 50 mM concentrations of al disaccharide tested as compared to 20 mM concentrations of various disaccharides, similar enzyme activities were observed for all disaccharides at each concentration.

While at concentrations 100, 300 and 500 mM, the percentage of recovered activity increased significantly up to about 90%, at certain levels increasing the concentration eventually reached a constant limit of stabilization during freeze-drying.

For instance, sucrose (Fig. 1) at 300 mM and 500 mM concentrations showed similar effect on enzyme activity but increasing the concentrations from 100 to 300 mM improved the recovered activity considerably. Trehalose (Fig. 2) was found to be the most effective sugar in protecting the enzyme activity and at 500 mM concentration, 91 % of the original activity was recovered in the first day of lyophilization lasting for more than 41 days. Maltose (Fig. 3) and lactose (Fig. 4) showed similar effects on LP activitities. These disaccharides showed good stability at 100 and 300 mM concentrations on the first day of lyophilization but the activities were decreased profoundly after few days, whereas trehalose and sucrose at concentration of 100 mM could retain the LP activity for more than 35 days.

The microbiological effect of LP-S on the *E.coli* growth rate is shown in Fig. 5. Absorbances of samples in the presence and absence of LP-S at 580 nm after inoculation during 20 h are shown in this figure. Table 1 shows the percent reduction in the absorbances at 6 h time point in LP and non-LP containing tubes to show the effect of LP in delaying the growth rate of three microorganisms. It shows that the overall effect of LP in 10^5 cfu/ml suspension is greater than 10^6 cfu/ml one. As a result, the lower the initial inoculums, the higher the enzyme activity would be.

Percent reduction in OD which is an indicative of the enzyme activity against microorganism was significantly different amongst different microorganism at both 10^5 and 10^6 cfu/ml suspensions. LP-S was found to be very effective against *Ps.aeroginosa* because it could delay the growth rate by 100% at 6 h once 10^6 cfu/ml suspension was used.

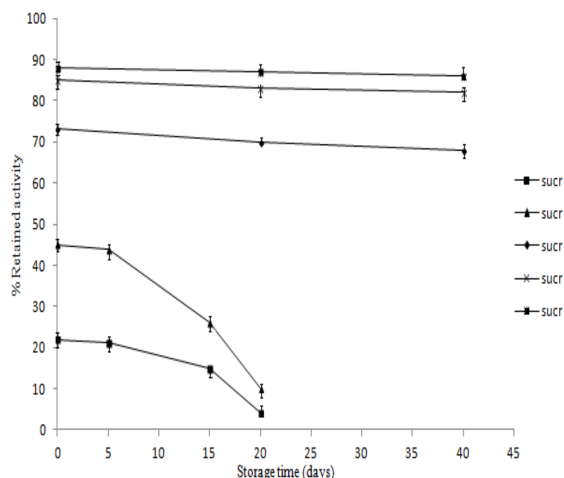

Fig. 1. The effect of different concentrations of sucrose on the stability of lactoperoxidase during 40 days after freeze-drying (n=3, mean ± SD).

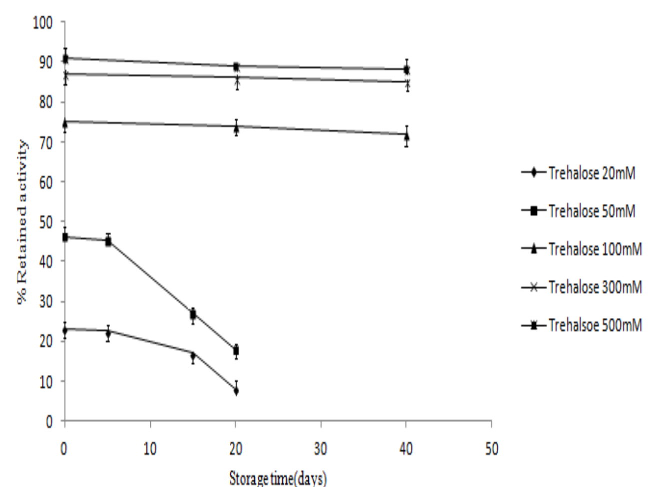

Fig. 2. The effect of different concentrations of trehalose on the stability of lactoperoxidase during 40 days after freeze-drying (n=3, mean ± SD).

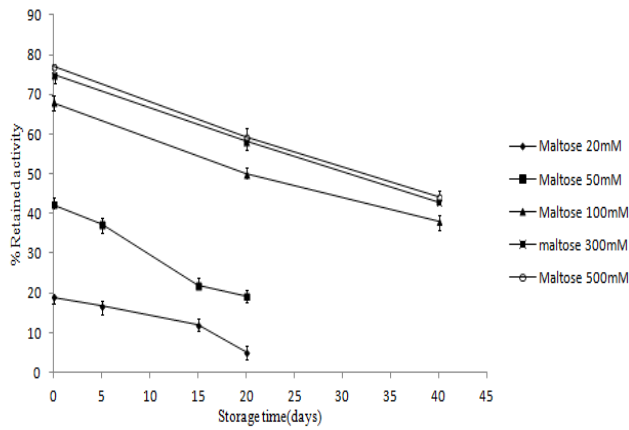

Fig. 3. The effect of different concentrations of maltose on the stability of lactoperoxidase during 40 days after freeze-drying (n=3, mean ± SD).

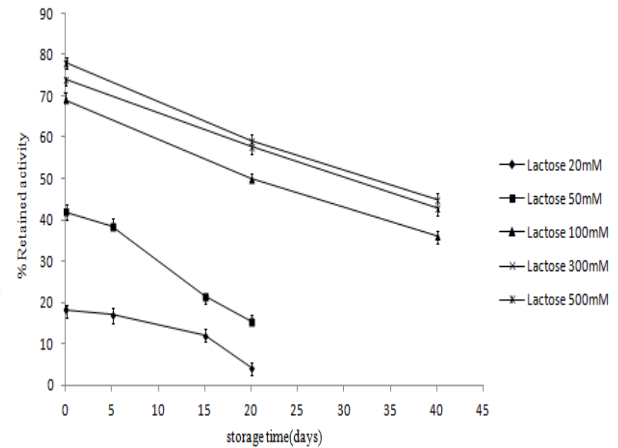

Fig. 4. The effect of different concentrations of lactose on the stability of lactoperoxidase during 40 days after freeze-drying (n=3, mean ± SD).

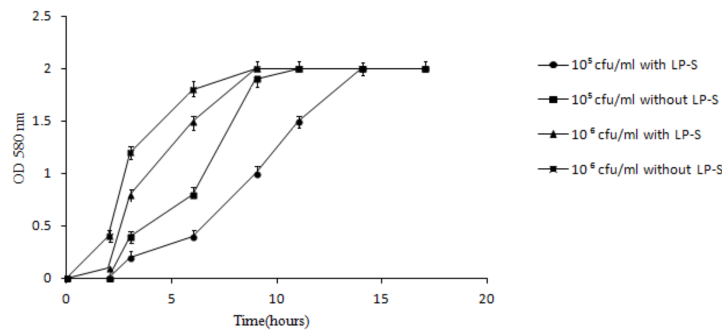

Fig. 5. The effect of the lactoperoxidase system against *E.coli* in Muller-Hinton broth.

Table 1: Activity of lactoperoxidase against three microorganisms at 6 h.

Bacteria	Viable count (cfu/ml) at 6 h	OD Without LPS (nm)	OD With LPS (nm)	Percent Reduction
S.aureus	**10^5**	0.30	0.00	100%
	10^6	1.00	0.78	22.0%
E.coli	**10^5**	0.80	0.40	50.0%
	10^6	1.80	1.50	16.7%
Ps.aeroginosa	**10^5**	1.00	0.50	50.0%
	10^6	1.30	0.90	30.8%

DISCUSSION

In this work, we studied the protection of LP during lyophilization with some sugars. Sugars are frequently applied as non-specific protein stabilizers in aqueous solutions during the lyophilization process. The mechanism by which these disaccharides stabilize the native structure of proteins seems to involve the direct interaction between the carbohydrate molecule and the protein through the establishment of hydrogen bonds. During the protein hydration, water is substituted with sugar and in the dry state the protein molecule becomes embedded in a sugar-glass matrix which preserves its activity and/or structure (25). On the other hand, even after successful lyophilization, a long term storage stability still is very limited, especially at high temperatures (26). Thus, in the present study all samples were kept at **4 ºC** to achieve their highest stability.

Among disaccharides, sucrose and trehalose appeared to be a preferable lyoprotectant for stabilizing the LP enzyme. They were selected because they are frequently used as an

excipient to stabilize proteins during drying and storage (25). Trehalose and sucrose are known as two suitable stabilizing disaccharides for therapeutic proteins because they can protect proteins during both freezing and dehydration. They are non-reducing sugars and tend to remain amorphous during lyophilization. These two disaccharides which are the preferred excipients for inhibiting lyophilization-induced protein unfolding, also provide a glassy matrix with acceptably high glass transition temperature values (27). The glass transition temperature of an amorphous pharmaceutical solid like sucrose can affect its viscoelastic property. A lyoprotectant with high glass transition temperature has an anti plasticizer effect which increases the stability of a lyophilized product. On the other hand, water molecules act as a potent plasticizer in the process of the lyophilization which lowers the stability of a lyophilized product (28). In comparison to sucrose, trehalose seems to be more protective, probably because of its higher glass transition temperature and thus for formulations containing trehalose it may be easier to obtain an appropriate cake structure with an economical lyophilization (27). Trehalose has some other properties which are required for a suitable stabilizer during freeze-drying such as lower hygroscopicity, an absence of internal hydrogen bonds, which allows more flexible formation of hydrogen bonds with the dried LP, and very low chemical reactivity (25).

Lower concentrations of sugars may or may not have any significant effect. For example, at concentrations of 5 to 100 mM, neither trehalose nor glucose could protect lactate dehydrogenase nor phosphofructokinase to a significant level during lyophilization (29). High concentrations of sugars are often necessary for lyoprotection (30). According to our results, lower concentrations of disaccharides couldn't protect LP to a significant level during freeze-drying. It seems that if the ratio of excipient to protein is not sufficiently high, adequate stabilization of the protein during freezing and dehydration will not take place.

In fact protection level afforded by different sugars can be either similar or significantly different due to the formulation, concentration, physical properties of the stabilizer, and its compatibility with the protein. At low concentrations, disaccharides couldn’t show a significant level of protection. They showed similar effects at concentrations between 5-60 mM but at higher concentrations (100-500 mM) each disaccharide showed its own ability in protecting the LP’s stability. Thus, at higher concentrations there are significant differences among sugars in promoting the stability of LP. In general the level of sugar needed for optimal protection during lyophilisation is dictated by that required for the inhibition of unfolding during dehydration. The stresses of freeze drying cause protein unfolding, and the formulation must be designed to inhibit unfolding at each step (27).

On the other hand, increasing the sugar concentration above certain level eventually reaches a limit of stabilization during freeze-drying. In the current study, at sugar concentrations of more than 300 mM, the remained activity of LP didn't change significantly. Possibly it is because disaccharides crystallize at high concentrations and this reaction prevent them from requisite hydrogen bonding to the dried protein (29).

At higher concentrations, sucrose and trehalose achieved better results compared to maltose or lactose. Possibly this is because lactose and maltose are reducing disaccharides and although they may effectively inhibit protein unfolding during the lyophilization cycle, during storage they have the propensity to degrade proteins via the Maillyard reaction between carbonyls groups of the sugar and free amino groups on the protein (25).

For assessing the antimicrobial effect of lyophilized LP, three different strains of bacteria were used in this study because they are the most common pathogens in food industries. Many researches indicate the antimicrobial effect of LP on different bacteria (31,32). Different groups of bacteria showed a varying degrees of sensitivity to the LP. LP could delay the growth rate of all 3 microorganisms but it was more effective against *S.aureus* at 6 h after inoculation. The effect of LP against *E.coli* and *Ps.aeroginosa* were almost the same (33)..

CONCLUSION

Addition of trehalose and sucrose at concentrations greater than 100 mM could preserve the activity of LP during and up to 40 days after lyophilization. The lyophilized product could delay the growth rate of *E.coli, Ps.aeroginosa* and *S.aureus*.

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REFERENCES

1. Sisecioglu M, Kirecci E, Cankaga M, Ozdemir H, Gulcin I, Atasever A. The prohibitive effect of lactoperoxidase system on some pathogen, fungi and bacteria. Afr J Pharm Pharacol. 2010;4:671-677.
2. Kussendrager KD, Von Hooijdink CM. Lactoperoxidase: Physico-chemical properties, Occurrence, Mechanism of action and applications. Br J Nutr. 2000;84:S19-S25.
3. Bjorck L, Claesson O, Schulthess W. The lactoperoxidase/thiocyanate/hydrogen peroxides system as a temporary preservative for raw milk in developing countries. Milchwissenschaft. 1979;34:726-729.
4. Ponce P. Lactoperoxidase system under tropical conditions: use, advantages and limitations in conservation of raw milk and potential applications. Rev Salud Anim. 2010;32:146-154.
5. Madureira AR, Pereira CI, Gomes AMP, Pintado ME, Malcata FX. Bovine whey proteins–Overview on their main biological properties. Food Res Int. 2007;40:1197-1211.
6. Ahmad Nimatullah AB, Masahiro O, Shigeru HK. Application of lactoperoxidase system using bovine whey and the effect of storage condition on lactoperoxidase activity. Int J Dairy Sci. 2011;6: 72-78.
7. Boscolo B, Leal SS, Salgueiro CA, Ghibaudi EM, Gomes CM. The prominent conformational plasticity of lactoperoxidase: A chemical and pH stability analysis. Biochim Biophys Acta. 2009;1794:1041-1048.
8. Marcozei G, Di Domenico C, Spreti N. Effects of surfactants on the stabilization of the bovine lactoperoxidase activity. Biotechnol Prog. 1998;14:653-656.
9. Kowal R, G Parsons R. Stabilization of proteins immobilized on Sepharose from leakage by glutaraldehyde cross linking. Anal Biochem. 1980:102:72-76.
10. Barret NE, Grandison AS, Lewis MJ. Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. J Dairy Res. 1999;66:73-80.
11. Jalali M, Abedi D, Varshosaz J, Najjarzadeh M, Mirlohi M, Tavakoli N. Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. Res Pharm Sci. 2012;7:31-36.
12 Li Y, Williams TD, Topp EM. Effects of excipients on protein conformation in lyophilized solids by hydrogen/deuterium exchange mass spectrometry. Pharm Res. 2008;25:259-267.
13. Carpenter JF, Crowe LM, Crowe JH. Stabilization of phosphofructokinase with sugars during freeze-drying: Characterization of enhanced protection in the presence of divalent cations. Biochim Biophys Acta. 1987;923:109-115.
14. Allison SD, Dong A,Carpenter JF. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing drying and rehydration. Biophys J. 1996;71:2022-2032.
15. Colaco C, Sen S, Thangavelu M, Pinder S, Roser B. Extraordinary stability of enzymes dried in trehalose. Biotechnology (N Y). 1992;10:1007-1011.
16. Yongmoon H, Byung-Seok J, Sang-Bong L, Yeowon S, Jee-Won J, Jue-Hee L. Effects of sugar additives on protein stability of recombinant human serum albumin during lyophilization and storage. Archives of Pharmacal Res. 2007;30:1124-1131.
17. Steven JP, Nicole T, Tsutomu A, John FC. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys J. 1993;65:661-671.
18. Finn SH. The bactericidal property of milk. Br J Exp Pathol. 1924;5:211-280.
19. Seifu E, Buys EM, Donkin EF. Significance of the lactoperoxidase system in the dairy industry and its potential application: A review. Trends Food Sci Tech. 2005;16:137-154.20. Yablonski JL. Fundamental concepts of preservation .Bull Parenter Drug Assoc. 1972;26:220-225.
21. Mohamad H, Rosenthal N, Rahman Y. Production, purification and characterization of thermostable lipase enzyme from a thermophilic Bacillus sp. J Med Res Ins. 2005;26:149-154.
22. Sisecioglu M, Cankaya M, Ozdemir H. Effects of some vitamins on lactoperoxidase activity. Int J Vitam Nutr Res. 2009;79:188-194.
23. Kumar R, L Bhatia K. Standardization of method for lactoperoxidase assay in milk. Lait. 1999;79:269-274.
24. Aboelbashar Musa Z, Hamid IAO. Effect of lactoperoxidase activation on the keeping quality of raw milk kept at refrigeration temperature. Am J Res Commun. 2013;1:1-9.
25. Wei W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm. 2000;203: 1–60.

26. Lorendana D, Nicoleta S, Silivius S, Gabriela R. Thermal inactivation of lactoperoxidase in goat, sheep and bovine milk–A comparative kinetic and thermodynamic study. J Food Eng. 2012;113:47-52.
27. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol. 2002;13:109-133.
28. Hancock BC, George Z. The Relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids. Pharm Res. 1994;11:471-477.
29. Carpenter Jf, Prestrelski SJ, Arakawa T. Seperation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. Arch Biochem Biophys. 1993;303:456-464.
30. Carpenter JF, Crowe JH. An infrared spectro-scopic study of the interactions of carbohydrates with dried proteins. Biochemistry. 1989;28:3916-3922.
31. Björck L, Rosén C, Marshall V, Reiter B. Antibacterial activity of the lactoperoxidase system in milk against *pseudomonas* and other gram negative bacteria. Appl Microbiol. 1975;30:199-240.
32. Eyassu S, Buys EM, Donkin EF, Petzer IM. Antibacterial activity of the lactoperoxidase system against food-borne pathogens in Saanen and South African Indigenous goat milk. Food Control. 2004;15:447–452.
33. Kangumba JG, Venter EH, Coetzer JA. The effect of activation of the lactoperoxidase system and souring on certain potential human pathogens in cows' milk. J S Afr Vet Assoc. 1997;68:130-136.