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STABILITY, STORAGE AND ACTIVITIES OF FREEZE DRIED HUMAN PARAOXONASE-1 LOADED SOLID LIPID NANOPARTICLES

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ABSTRACT

Human serum paraoxonase-1 (PON1) is a calcium dependent interfacial activated membrane protein associated with high density lipoproteins (HDL) play significant role in fundamental biological processes. This study the storage, stability of PON1 and determine the feasibility of formulating PON1 loaded solid lipid nanoparticles for develop a surrogate for the HDL associated PON1 in the form of solid lipid nanoformulations. Studies were made on the preparation of h-PON1(human Paraoxonase-1) loaded solid lipids nanoformulations with palmitic acid and all activities of PON-1 like lactonase, aryl esterase and paraoxonase were performed and PON1 did not lose its activity in varios steps of nanoparticle preparation. Further most important cryoprotective agents (excipients) like sugars and Polyols were used to standardized for storage and stability of freeze dried human PON1 at different time intervals as well as temperature conditions. Nanopartiiculated palmitic acid based PON1 showed highest stability with trehalose.

KEY WORDS

Human serum paraoxonase, stability, solid lipid nanoparticles.

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INTRODUCTION

Serum paraoxonase 1 (PON1) is a Ca2+-dependent mammalian enzyme, synthesized in the liver present in the plasma, and have broad range of hydrolytic activities that can be grouped under three categories: lactonase, aryl esterase and phosphotriesterase.[1,2,3,4,5] The lactonase activity of PON1 is responsible for the anti-oxidant properties and it has been significant connection with anti-atherogenic properties against macrophage foam cell formation, it modulates atherosclerotic lesion development,[6] attenuation of cholesterol and oxidized lipid influx, inhibition of macrophage cholesterol biosynthesis and stimulation of macrophage cholesterol efflux[7,8,9,10,11,12] while its phosphotriesterase activity imparts an important role to the enzyme in natural defense against various organophosphates (OPs) intoxication, including nerve gases[13,14,15] However, recent reports have suggested that PON1 and the other PONs are in fact lactonases, catalyzing both hydrolysis and formation of a variety of lactones[16,17,18] The structure-reactivity studies have also established that the native activity of PON1 is lactonase,[19,20] while other activities, e.g. arylesterase and paraoxonase are now being considered as merely promiscuous activities of PON1 which are not shared by other family members (e.g. PON2 and PON3).

PON1 has potential antioxidant properties as demonstrated in several studies and playing very crucial role in various disease conditions in human beings, but due to their short half-life, in stability and delivery problems, the complete therapeutic potential may not be possible with PON1 peptides.[21] Novel drug delivery

systems for interfacially activated membrane proteins are still not yet resolved.[21,22] The present study demonstrated preparation of HDL mimic palmiticacid based PON1 loaded solid lipid nanoparticle, understanding the activity of PON1 when formulated as solid lipid nanoparticles and paid attention on freeze drying of purified human PON1, The particle size of the h-PON1 loaded solid lipid nanoparticles and their combinations were measured by using SEM, AFM and TEM, their images were showed to be similar size of nanoparticle and were found to be spherical in shape, nanorange from all the images in all instances. further it was studied the preservation of storage and stability of freeze dried human PON1 by utilizing various cryoprotective agents, that are sugars like, dextrose, glucose, glycerol, maltose, trehalose and polyols like, mannitol, sorbitol. Briefly, freeze-dried form of the human PON1 was incubated at -4, 4, 15, 25 and 35°C temperature conditions for 4 weeks and arylesterase activity was measured in every one week of intervals. Nanoparticulated PON1 showed highest stability with trehalose compare to other excipients.

Materials: Cholesterol, dextrose, ethyl acetate, glucose, glycerol, maltose, mannitol, Poly vinyl alcohol. palmitic acid, tripalmitin, sodium lauryl sulphate, sorbitol, trehalose, calcium chloride, ethylene diamine tetra acetic acid (EDTA), trizma hydrochloride (Tris-HCl), M-cresol purple indicator, tergitol NP-10 nonionic, paraoxon-ethyl pestanal, γ- nonanoic lactone, imidazole, bicine buffer, phenyl acetate, protease inhibitor cocktail, sodium chloride, sodium dodecyl sulfate (SDS) and all the other chemicals used were purchased from sigma aldrich and were of analytical grade.

METHODOLOGY

Freeze drying of purified human PON1 (h-PON1)

Purified human PON1 was freeze dried by using Scanvac cool safe freeze dryer. Freezer was turned on (Scanvac cool safe) 3 hours before use in order to achieve -110^{0}C. Weigh and measured volume of sample with excipients (1% Trehalose 1% maltose) and was in RB (Round bottom flask). Then sample was frozen by direct submersion in a low temperature bath or freezing chamber using methanol (-110^{0}C). The pre-frozen product was attached to the manifold. All the rubber valves of manifold were closed. After the sample was adequately frozen in the condenser at a temperature of -110^{0}C vacuum (0.1-0.6 m bar) was created in the drying chamber using vacuumed pump. It was continued until the entire frozen matrix appeared dry/powdered form. Then freeze drying process was stopped by closing the vacuum valve or rubber valve and removing the RB flask from the adaptor.

Preparation of h-PON1 loaded solid lipid nanoparticles

PON1 loaded nanoparticles were prepared by the emulsion-diffusion-evaporation method with modifications. PON1 was dispersed in glycerine, which is used with a view to stabilize the enzyme. The aqueous phase is generally water containing a stabilizer. In this experiment, pH 8.0 buffer with 1mM $CaCl_2$, 0.01% tergitol was used. The buffer was prepared by adding 1mM $CaCl_2$, 0.01% tergitol, and 10% glycerine to Tris HCl 50 mM (Trizma hydrochloride) reagent grade. The prepared buffer was used to solubilise 1% PVA which acts as the stabilizer. Freeze dried PON1, was added to 0.3 ml glycerine and stirred for 5 min. This solution was added to 2 ml ethyl acetate solution containing 50 mg palmitic acid. The organic phase was stirred using a magnetic stirrer for at least 15 min. In a separate vial 50 mg PVA was dissolved in 5 ml buffer solution. While homogenization, using tissue homogenizer, the solution containing PON1 and lipids was added in a drop wise manner to 5ml of 1% PVA in buffer solution. Homogenization was carried out at 15,000 rpm for 2 min. Later on, the formed emulsion was added to 20 ml buffer solution on the magnetic stirrer, stirring at 800 rpm. To facilitate complete diffusion followed by evaporation of the organic solvent stirring was continued for at least 15 h. Then, the nanoparticles were centrifuged at 15,000 g for 5 min and the pellet was washed with the buffer solution and the supernatant, which contains unbound stabilizer, was discarded. The pellet was redispersed in required amount of buffer solution for characterization.

Characterization of nanoparticles

Atomic force microscopy (AFM)

The surface morphology of solid lipid nanoparticles was analyzed using AFM Park system Model: XE-70 that was attached to a Nikon eclipse TE 2000-S microscope. The system was run by nanoscope software. The nanoparticles suspension was placed on a silicon wafer with the help of a micro pipette and allowed to dry in air. The microscope was vibration damped and measurements were made using commercial pyramidal Si3N4 tip (Park system). The cantilever used for scanning has a length 325 µm and width 26 µm with a

nominal force constant 0.1 N/m. For all the nanoparticles, the scan size were 5 µm with scan rate of 1 Hz. Set point 13 -15.01 nm the images were height images obtained in the basis of Non-Contact mode. AFM studies were done in Dept. of Physics, Osmania University[21].

Scanning Electron Microscope (SEM)

In this study, images from ZEISS scanning electron microscope (SEM) from Department of physics, Osmania university, for surface topography studies, microstructure, and chemistry of metallic and nonmetallic specimens at magnifications from 30.00 KX, with a resolution limit < 10nm (down to ~ 1nm) and a depth of focus up to several µm (at magnifications ~ 10, 000 X). EHT.20.00KV and all images are measured below 200 nm. After removing the excess stabilizer in the sample by repeated centrifugations (15,000 rpm/5 min) and h-PON1 loaded solid lipid nano particles are resuspended in water, a few drops of the dispersion were placed on a slab and dried under vacuum at room temperature. A Sputter Coater® JFC-1100 (JEOL, Tokyo, Japan) was used to coat the dried samples with copper (~20 nm thickness), placing them onto stubs. After coating, the sample was mounted with a conductive "bridge" (e.g. carbon / copper tapes, or silver paint) connected from the top surface of the sample to the sample holder. Finally, the samples were observed under a ZEISS scanning electron microscope (SEM).

Stability studies of purified h-PON1

Effect of the various excipients on the stability of human PON1 was studied by measuring the arylesterase activity of PON1. In this study sugars like dextrose, glucose, glycerol, maltose, trehalose and polyols like mannitol, sorbitol were used. Briefly, after freeze-dried form of the enzyme was incubated at -4, 4, 15, 25 and 35°C temperature conditions for 4 weeks for check its stability. Arylesterase activity was measured in every one-week interval by adding enzyme solution (10µl) to 0.5 ml of activity buffer 50 mM Tris buffer, pH 8.0, containing 1 mM $CaCl_2$ solution and 1 mM phenyl acetate, and the rate of generation of phenol was monitored at 270 nm. The reaction was initiated by the addition of enzyme, and the increase in absorbance at 270 nm recorded. The activity of the enzyme without excipients at '0' day was considered as 100 %.

RESULT AND DISCUSSION

Activity measurements of h-PON1 during various steps of solid lipid nanoparticles (SLN) preparation

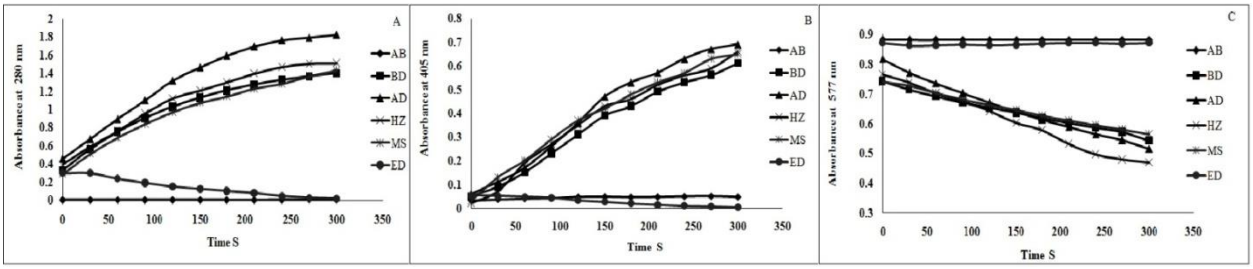

Figure 1: A: Phenyl acetate activity[22] of PON1 in various steps of SLN preparation. AB-Activity buffer; BD-Before freeze drying; AD-After freeze drying at -110°C with 0.1 % trehalose and 0.1% maltose; HZ-After homogenization at 5000 rpm/2 min; MS- Final step of nanoparticle process after overnight magnetic stirrer; ED-PON 1 with EDTA; B: Paraoxonase activity[22] of PON1 in various steps of SLN preparation. AB- activity buffer; BD-before freeze drying; AD-After Freeze drying at -110 °C with 0.1 % trehalose and 0.1% maltose; HZ-After homogenization at 5000 rpm/2 min; MS- Final step of nanoparticle process after overnight magnetic stirrer; ED-PON 1 with EDTA; C: Lactonase activity[22] of PON1in various steps of SLN preparation. AB-Activity buffer; BD-Before freeze drying; AD-After Freeze drying at -110°C with 0.1 % trehalose and 0.1% maltose; HZ-After homogenization at 5000 rpm/2 min; MS-Final step of nanoparticle process after overnight magnetic stirrer; ED-PON 1 with EDTA.

Activity of PON1 has been measured during various steps of the solid lipid nanoparticle (SLN) preparation process. After purification the enzyme has been freeze dried until the nanoparticle preparation process has been started. The activity of the enzyme with respect to paraoxonase, arylesterase and lactonase has been studied before freeze drying and after freeze drying. There was no difference in activities of the enzyme before and after freeze drying (Fig. 1A, 1B & 1C). The activities were also tested from the samples collected

during various steps of the nanoparticle preparation process viz, after homogenization and after overnight stirring step for the removal of organic solvent from the nanoparticle preparation. All the results indicate that the enzyme PON1 retained its all the three activities during these process (Fig. 1A, 1B & 1C).

Preparation and characterization of h-PON1 loaded SLN by AFM (Atomic force microscopy) and SEM (Scanning Electron Microscopy):

PON1 solid lipid nanoparticles have been successfully prepared using various lipids and their combinations. The choice of a particular method for encapsulation of a substance in a colloidal carrier is most commonly determined by the solubility characteristics of the active ingredient as well as the lipid. Pharmaceutical compounds are soluble in either aqueous or non-aqueous solvents, which facilitate incorporation of these compounds into the nanoparticles when the emulsification technique is adopted. A modified emulsion-diffusion-evaporation process was optimized to obtain PON1 loaded nanoparticles and most importantly to get active enzyme loaded in to the nanoparticles.[27] Several modifications were carried out in the preparation process like controlling the temperature while emulsification at 4°C, reducing the homogenization speed from 15,000 to 5,000 rpm and using different types of surfactants like didodecyl dimethyl ammonium bromide (DMAB) and PVA. However, none of these modifications could preserve the enzyme activity. It was understood from the literature that the pH, ionic strength and solvents have definite roles in the stabilization of PON1 in nanoparticles. Therefore, considering these facts, a suitable buffer was used instead of water in various steps of the preparation process of nanoparticles. This strategy resulted in nanoparticles without complete loss of the enzyme activity, which was confirmed by analyzing the enzyme present in the supernatant. The literature suggests the use of double emulsion process for preparation of most of the enzyme loaded nanoparticles. However, in single emulsification process, with the use of a co-solvent, suitable conditions like pH and ionic strength for a specific enzyme will prevent the loss of enzyme activity. The present method provides extended conditions for the enzyme to remain stable[28,31,32,33].

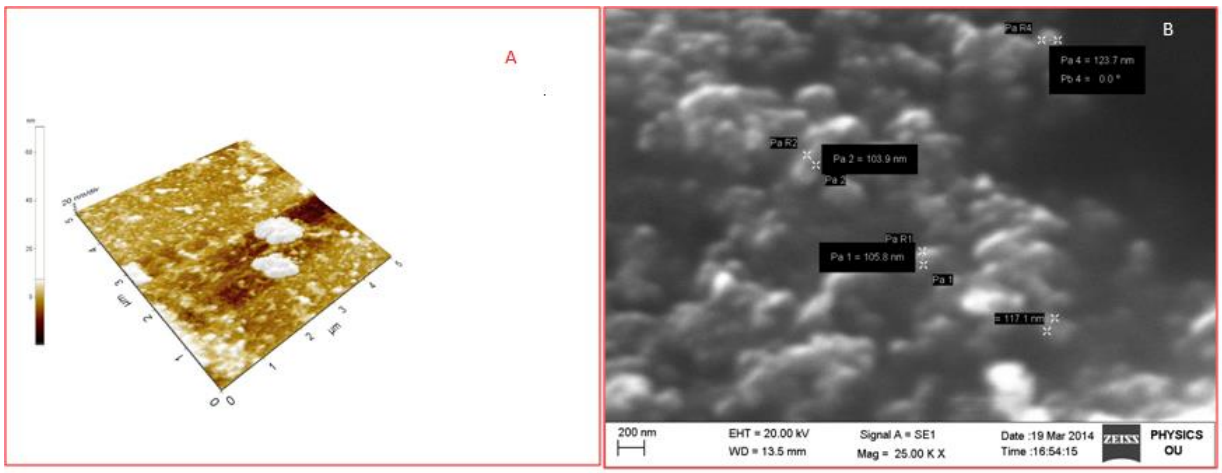

Fig: 2 A. AFM. B. SEM images of h-PON1 loaded palmitic acid solid lipid nanoparticle

The particle size of the PON1 loaded solid lipid nanoparticles prepared from palmitic acid combinations were listed in table 1. The AFM and SEM, images showed similar size of nanoparticle were found to be spherical from all the images in all instances (Figures 2.A. AFM. B. SEM images)

Table 1: Particle sizes of the PON1 containing solid lipid nanoparticles prepared by using palmitic acid

Types of nanoparticles	Size (nm)
PON1 palmitic acid nanoparticles (PON1 PA)	254 ±20

Stability studies of h-PON1

Till now; there are no detailed studies that have been carried out to find conditions that ensure continued storage of the purified PON1 without loss of its activities. To increase the shelf life, pharmaceutically important proteins are usually stored as liquid or

freeze-dried formulations[24]. However, under these storage conditions at different temperature or during the formulation process, proteins experience a variety of stress conditions that adversely affect their stability/bioactivity. Considering the above facts after successful completion of purification of protein we went for stability studies at different temperature conditions. Our main purpose of this study is to improve the storage and stability conditions of purified PON1. For this study, we have selected few most important cryoprotective agents (excipients) that are sugars like sucrose, glucose, glycerol, maltose, trehalose and polyols like mannitol, sorbitol and standardized their different concentrations for improving the stability of PON1 and 0.5 M concentrations of exipients was finally decided[24,25]. Briefly, after freeze-dried form of the human PON1 was incubated at various temperatures such as -4°C, 4°C, 15°C, 25°C and 35°C with different concentration of above mentioned excipients for 30 days, it was measured the arylesterase activity at every one week interval but in case of temperature studies it was measured the activity at every 10 days interval for both studies the aryl esterase activity[22] by adding enzyme solution (10 µl) to 0.5 ml of activity buffer 50 mM Tris buffer, pH 8.0, containing 1 mM $CaCl_2$ solution and 1 mM phenyl acetate, and the rate of generation of phenol was monitored at 280 nm. The reaction was initiated by the addition of enzyme, and the increase in absorbance at 280 nm recorded. The activity of the enzyme with excipients at 0 day was taken as 100 %.

Storage and stability profile of h-PON1 with various excipients in (0- 4 weeks) 0-28 days week.

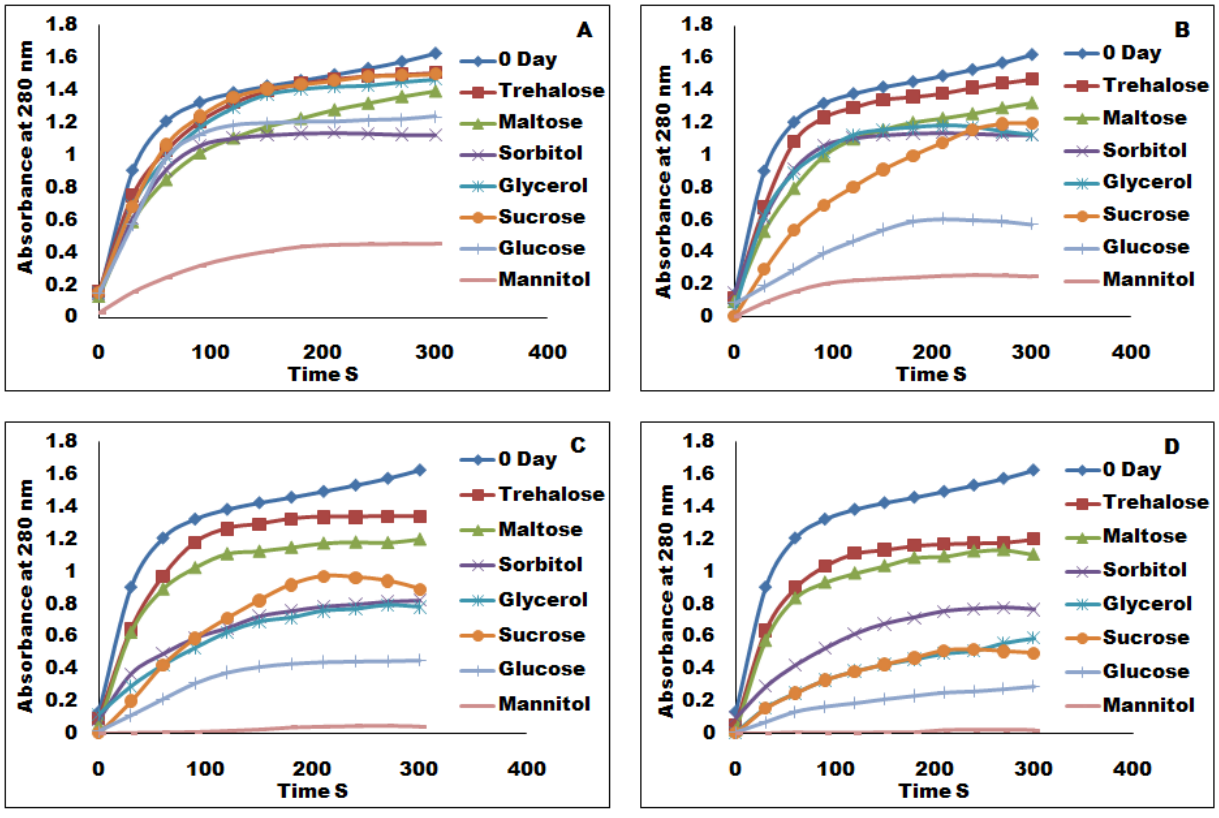

Figure 2 A: Storege Stability profile of purified h-PON1 with different excipients (0.5M) during A.first week; B. Second week; C. Third week; D. Fourth week by measuring arylesterase activity.

Storage and stability profile of h-PON1with trehalose at -4°C for 30 days

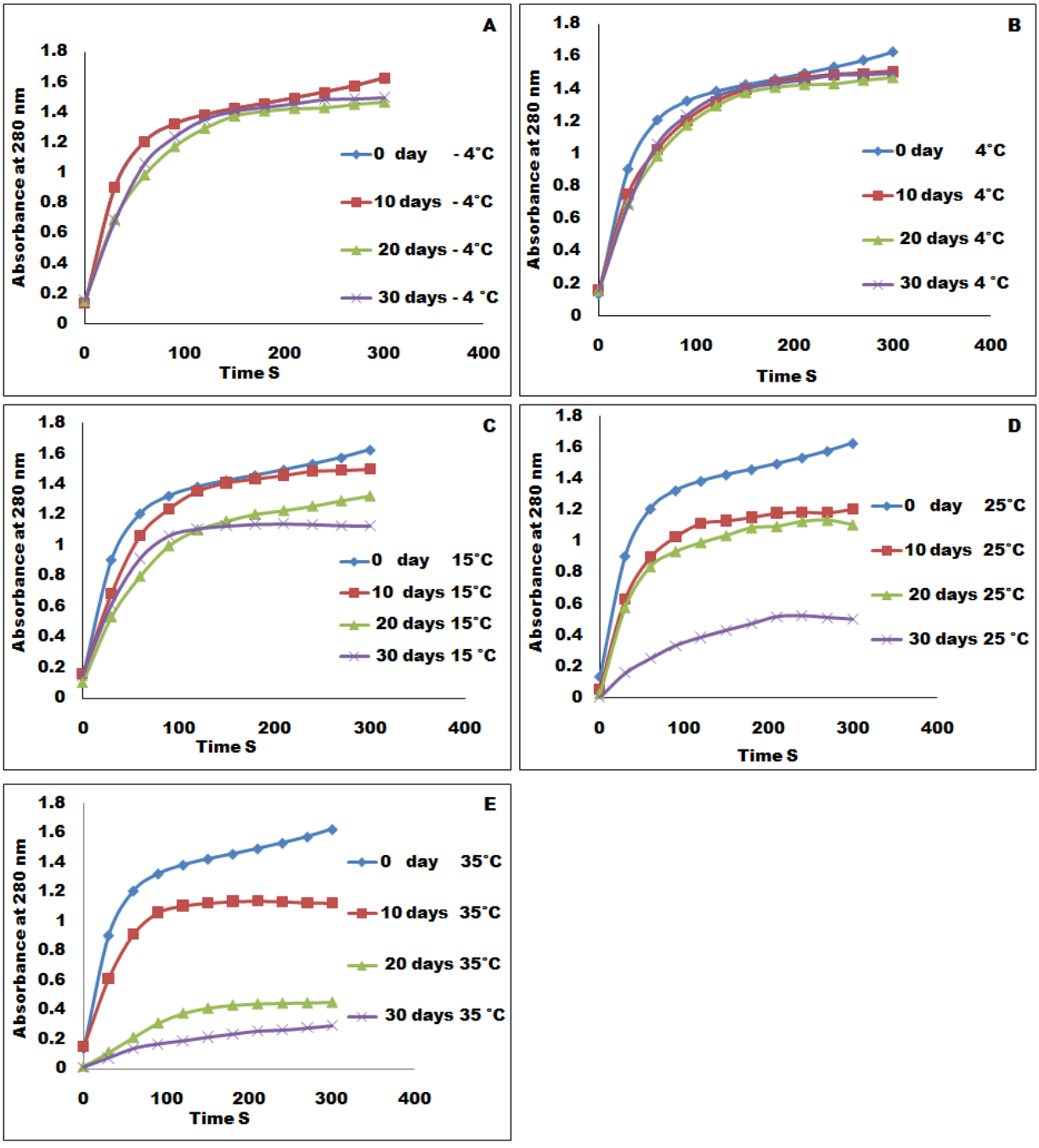

Figure 3: Storage and Stability studies of purified h-PON1 with 0.1% trehalose (0.5M) and 0.1% maltose (0.5M) at Fig: A. -4°C; B. 4°C; C.15°C. D. 25°C; E. 35°C by measuring arylesterase activity.

Utilizing several excipients for increasing the shelf life of the proteins in the formulation technology is the most advanced process in protein drug delivery[28]. However, sugars, polyols, amino acids, polymers, and certain salts are used for formulation development process[24,25]. The stabilizing effects of the excipients differ from protein to protein and also depend on other parameters such as the storage, pH, and the temperature, the concentration of the excipients, as well as the nature and the interaction of other components of the formulation. In this study, PON 1 activity was monitored for 30 days at an interval of one week with 0 day taken as a standard (100%) activity and with different excipients. Briefly, freeze dried PON1 10

µl was taken for stability studies with 0.1% and 0.5M concentration and aryl esterase activity was monitored for 3 minutes. It was standardized the activity of purified protein at different temperatures with various excipients but we observed -4°C, and 4°C PON1 has stable activity with trehalose and maltose for 30 days. But as temperature increases PON1 loses its activity. In present study it was observed that at 15°C, 25°C, 35°C PON1 showed decrease activity with excipients. During first week PON1 has showing significant stable activity in trehalose, sucrose, glycerol, at 4°C almost equal to 0 days sample (Fig.2A). PON1 with maltose, sorbitol, glucose also shown good considerable aryl esterase activity in first week and PON1 in mannitol has shown some activity but it is very less than other excipients (Fig. 2A). During second week PON1 activity in trehalose was highest, when compared to other excipients, which was equal to 0 days activity (Fig.2B).

PON1 in maltose also showed good activity almost near to trehalose, PON1 in glycerol and sorbitol exhibited good activity near to maltose, sucrose second week of PON1 activity initially PON1 showed less activity in sucrose but after one and half minute of the reaction PON1 activity is raised and its almost equal to glycerol and sorbitol, but the activity is less in sucrose, when compared to trehalose, maltose. PON1 in glucose also had significantly decreased activity. PON1 activity in mannitol is very less during first week (Fig. 2B). As per this observation, in third week study (Fig. 2C), PON1 in trehalose had highest activity and without appreciable loss, followed by PON1 in maltose which is almost near to trehalose. PON1 in sorbitol and glycerol showed its activity but it is comparatively less than other excipients like trehalose, maltose. PON1 in sucrose maintained stable activity as showed earlier in second week (Fig.2C). Finally, fourth week study, PON1 showed good activity with trehalose and maltose but as compared to initial weeks PON1 activity is significantly reduced in trehalose and maltose after fourth week (Fig.2D). After fourth week PON1 in sorbitol has activity half less than trehalose and maltose. PON1 in glycerol, sucrose and glucose had some activity but it is not significantly considerable when compared with 0 day. PON1 in mannitol almost had its activity declined (Fig. 2D). PON1 0.1% trehalose (0.5M) concentration had been continuously showing activity without appreciable loss up to third week. After fourth week, PON1 activity in trehalose also significantly reduced. PON1 in mannitol also exhibited good activity almost equal to trehalose but after fourth week PON1 in mannitol also significantly decreased its activity. PON1 in trehalose and maltose recorded significantly stable arylesterase activity. PON1 activity for better storage and stability PON1 activity with different excipients at various temperature conditions such as -4°C, 4°C,15°C, 25°C, 35°C was taken up in 0.5M, 0.1% trehalose and 0.5M ,0.1% maltose. In our observation PON1 in 0.5M, 0.1% trehalose and 0.5M, 0.1% maltose at -4°C and 4°C revealed highest and significant activity. PON1 activity is significantly stable at above conditions for 30 days (Fig.3A and 3B). At 15°C temperature PON1 retain its activity up to 10 days without appreciable loss equaling to 0 days. Till 30 days activity is lesser than the initial 10 days (Fig.3C).

At 25°C temperature, PON1 had continuous activity up to 20 days but when compare to other temperature conditions like, -4°C, 4°C,15°C its activity progressively decreased (Fig. 3D). Finally, at 35°C PON1 showed some activity up to 10 day but by 30Th day its activity significantly declined. Few studies in literature proposed that optimum temperature for PON1 is in the range of 30-45°C [29] but in this study, it was observed that when incubated at 15-35°C it significantly decreased its activity with various excipients. It was assumed that optimum temperature for PON1 is 4°C with few excipients like trehalose, maltose for one month. When compared both the above excipients trehalose is showing maximum stable activity with other excipients. PON1 shows good stability with sucrose almost equal to trehalose up to one week at 4°C and in another observation with sorbitol and glycerol also exhibits good activity but little bit lesser than trehalose and maltose.

CONCLUSIONS

Purified freeze-dried h-PON1 did not lose its activity in various steps of nanoparticle preparations**.** The particle size of the h-PON1 loaded solid lipid nanoparticles prepared with palmitic acid. The AFM and SEM, images showed similar size of nanoparticle were found to be spherical from all the images in all instances. Stability and storage condition of PON1was assessed by monitoring arylesterase activity and it is concluded that PON1 maintained its activity without loss for 30 days at - 4°C, 4°C with 0.1% trehalose (0.5M) and 0.1% maltose (0.5M). PON1 also maintained its activity without appreciable loss for 10 days at 15°C with 0.1% trehalose (0.5M) and 0.1% maltose (0.5M), at 25°C, 35°C PON1

loses its activity. Nanoparticulate PON1 (PON1+process) shows highest lactonase, arylesterase and paraoxonase activities of PON1 compared to normal PON1.

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