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Lumbrokinase for degradation and reduction of amyloid fibrils associated with amyloidosis

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ABSTRACT

Amyloidosis is a group of diseases caused by the accumulation of insoluble protein aggregates in different parts of the body. Repeated subcutaneous injection of insulin hormones in diabetic patients leads to localized amyloidosis that is found to be cytotoxic. Thus, agents that can dissociate these aggregates are critically needed. In the present study, insulin amyloid dissociation was demonstrated by the treatment of an enzyme lumbrokinase (LK) isolated from earthworm. Thioflavin T (ThT) fluorescence, solution turbidity, particle size analysis, FTIR, CD, atomic force microscopy and cell viability assay were employed to support the dissociation of insulin amyloid *in vitro*. The small animal optical imaging was used to explore the dissociation of amyloid fibrils *in vivo* using zebrafish model. The activity of LK towards amyloid dissociation was compared with the standard amyloid fibril degrading agent nattokinase (NK). Our results indicated that LK can be a probable fibril degrading agent for the dissociation of amyloids.

Introduction

Amyloids are proteinaceous aggregates having a predominant β-sheet structure, and the formation of amyloid plaque is the key pathological feature of various amyloid diseases. The transition of native soluble proteins into insoluble protease resistant fibrils are the common symptoms of Alzheimer's disease, Parkinson disease, prion-associated encephalopathy and type II diabetes (Höppener et al., 2000; Stefani and Dobson, 2003; Fändrich et al., 2009; Jiménez et al., 2002). The aggregated state of this protein could be permeable to cell membranes after attachment and could induce cellular dysfunction and apoptosis (Stefani and Dobson, 2003). Thus, the denaturation of this amyloid aggregates could lead to a therapeutic approach for the treatment of amyloid diseases. Insulin hormone had been routinely administered subcutaneously for an antidiabetic medication. Repeated insulin injection could result in localized amyloidosis (Shikama et al., 2010) and causes cytotoxicity *in vitro* (Yoshihara et al., 2016). Due to the structural similarity of insulin amyloid to the other category of amyloidogenic proteins such as Alzheimer's β and prion protein, insulin can be considered as a model amyloid protein for studying the denaturation of amyloid aggregates (Yoshihara et al., 2016).

Lumbrokinase (LK), is a serine protease that has fibrinolytic activity but its potential for the degradation of insulin amyloid has not been explored yet (Nakajima et al., 1993; Mihara et al., 1991). Nattokinase is another serine protease that (NK, also known as subtilisin NAT) (EC 3.4.21.62) is extracellularly produced by *Bacillus subtilis* natto possessing fibrinolytic activity (Dabbagh et al., 2014). LK is a collection of six proteolytic isozymes (*EC* 3.4.21) and can dissolve intravascular blood clots. Compared to other thrombolytic medications, earthworm LK is a cost effective source, highly stable and can be administered orally (Phan et al., 2011). In the present study, we have isolated the enzyme LK from the earthworm and compared its effect on insulin amyloid degradation with the known amyloid denaturing enzyme, NK. Other than fibrinolytic activity, LK also exhibits antimicrobial activity (Mathur et al., 2010). Several quinones have also been reported to degrade insulin fibrils, but the effect of LK on insulin amyloid degradation has not

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been explored (Gong et al., 2014). Although NK has been patented for reducing and degrading Alzheimer's β amyloid (Hsu et al., 2009), no such report exists studying the effect of LK on amyloid degradation. Thus, the study to isolate a stable enzyme for amyloid degradation is warranted. In the present study we have explored LK mediated degradation of insulin amyloids *in vitro* using various physicochemical techniques like turbidity, fluorescence, dynamic light scattering, infrared (IR) spectroscopy, circular dichroism (CD) analysis and atomic force microscopy (AFM). The *in vivo* degradation of amyloids was also studied using zebrafish model and small animal imaging system.

Experimental

Materials

Live Earthworms (*Lumbricus terrestris)* were collected from agricultural fields of Kanchipuram District, Thiruvidandhai, Tamil Nadu 603112 (India). Huminsulin 30/70 40 IU/ml (Eli Lilly and Company (India) Limited), NK plus Astaxanthin and Coq10 (Hawaiian Herbals, USA); Thioflavin T (ThT) and Sephadex G-75 from Sigma Aldrich, Ammonium sulphate, dialysis membrane, DEAE cellulose-52, DMEM, Antibiotic Solution (penicillin and streptomycin in 0.9% saline) (HIMEDIA-A003), Trypsin, Fetal Bovine Serum (FBS) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), acrylamide, bis-acrylamide, TEMED, ammonium persulfate from HIMEDIA and other chemicals were purchased locally. All the experiments were done following the guidelines of Institutional Ethical Committee, CHRI after getting the approval (Proposal No. 14/IHEC/3-16).

Extraction of LK from earthworm

LK was extracted from earthworm following the protocol of Li et al. (2015) with little modification (Li et al., 2015). Fresh earthworms were kept in water for a day to clear the intestinal dirt and then refrigerated overnight. 100 g of frozen earthworms was suspended in 100 ml saline and homogenized at room temperature. The mixture was stirred for 10 h and then filtered with gauze. After centrifugation at 3000 rpm for 30 min at 4 °C, the supernatant was aspirated. The saturation of ammonium sulfate was prepared according to the table, and certain amount of ammonium sulfate was added to make saturation 20S, and then allowed to stand overnight at 4 °C. The protein pellets thus obtained by ammonium sulfate saturation was resolubilized with phosphate buffer (PBS, 10 mM, pH = 7.4) and dialyzed overnight at 4 °C. The dialyzed solution was kept in the refrigerator and purified next day by sephadex G-75 gel filtration chromatography followed by DEAE cellulose-52 ion exchange chromatography. The eluent was stored at −20 °C for further use. The final yield of the pure enzyme was 37% and the protein concentration was 0.4025 mg/ml. The purified LK was analyzed using polyacrylamide gel electrophoresis (SDS PAGE) according to Ghosh et al. (2012). This solution was used for further amyloid degradation studies and taken as the source of LK.

Preparation of NK

NK plus Astaxanthin and Coq10 (Hawaiian Herbals, USA) capsule were used for the preparation of stock solution of NK. The working solution of NK was 2.78 mg/ml which was further used for the amyloid degradation studies.

In vitro *fibrinolytic activity*

To normalize the enzyme activity of NK and LK, *in vitro* fibrinolytic activity was assayed according to Metkar et al. (2017). Briefly, blood clot formation was allowed to form in sterile preweighed tubes which were further degraded by different volumes of the enzymes LK and NK respectively. After degradation of clots the fluid was aspirated and the tubes with remaining clots were weighed again. Water was used as a negative control as it does not cause any clot lysis. The percentage of clot degradation was calculated by the formula given below:

$$\%\ \text{clot } \textit{deg}\text{radation} = \frac{\text{weight ofclot remaining after } \textit{deg}\text{radation}}{\text{weight of clot taken}} \times 100$$

The test was repeated three times.

Degradation of insulin amyloid fibrils by NK and LK

Insulin amyloid formation was induced in a similar way as done by Lee et al. (2007) and Metkar et al. (2017). Insulin forms amyloids if incubated at 65 °C but the degradation study was conducted at 40 °C after the formation of amyloids as it is relevant to conduct the amyloid degradation study at physiological temperature (Hsu et al., 2009). Thus, Huminsulin 30/70 40 IU/ml was incubated at 65 °C to form insulin aggregates and the amount of fibrillation at designated time (2 h, 4 h, 6 h, 9 h, 12 h, and 24 h) was monitored. The fibrinolytic activity of LK was found to be 1.25 fold higher than NK. Thus, for every 10 μl of LK, 12.5 μl of NK was utilized for further experiments with equal activity. Briefly, at each designated time, 12.5 μl NK or 10 μl LK was added to 179 μl of pregrown insulin amyloid samples respectively and incubated at 40 °C for 1 h to allow enzymatic degradation reaction. After incubation, the temporal fibril degradation status was compared with insulin fibrils formed by turbidity assay, thioflavin-T (ThT) fluorescence and dynamic light scattering (DLS), atomic force microscopy (AFM) and FT-IR spectroscopy.

ThT fluorescence assay

ThT gives fluorescence on binding with amyloids and is the key molecule to monitor amyloid formation (Girigoswami et al., 2008). For each sample (control and enzyme treated) 200 μl ThT (20 μM in PBS, pH = 7.2) was added to 30 μl insulin amyloid (1.388 mg/ml). 20 μl of this solution was diluted with 3 ml of distilled water to measure the fluorescence monitoring emission from 440 to 600 nm after exciting at 412 nm using FP-8300 Jasco Spectrofluorimeter. The final concentration of ThT was 0.115 μM and that of insulin amyloid was 1.2 μg/ml. The reaction was conducted and data was recorded at 40 °C.

Turbidity assay

The absorbance at 600 nm is extensively used to quantify insoluble protein aggregates (fibrils) (Lee et al., 2007). The aggregation of insulin amyloid and its dissociation mediated by NK and LK at various time intervals were estimated by turbidity assay using Shimadzu UV-1800 UV–vis double beam spectrophotometer.

Dynamic light scattering (DLS) analysis

The size of insulin fibrils in control as well as NK and LK treated samples at different time intervals was monitored by DLS using a Malvern nano ZS90 zeta sizer (Gong et al., 2014; Metkar et al., 2017; Kavya et al., 2013; Liu et al., 2010). The samples (10 μl) were added in 1 ml PBS, mixed properly and equilibrated for 1 min. The approximate size of amyloid fibrils was obtained from the hydrodynamic diameter estimated from DLS data.

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