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Formulation of abalone hemocyanin with high antiviral activity and stability

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ABSTRACT

Hemocyanin has been shown to have potential antiviral activity against herpes simplex virus type-1. However, current liquid formulations have short shelf life and high risk of bacterial contamination. The aim of our study was to develop a stable functional formulation. Analytical techniques (nano-differential scanning calorimetry and spectroscopy) and biological assays (cytotoxicity and plaque reduction) were employed to measure the effect of sugar addition on the physical properties and shelf life of the solid formulated hemocyanin. Sucrose improved thermal stability significantly by both increasing the aggregation onset temperature (70 °C to >78 °C) and enhancing the activation energy (18%). Lyophilisation without trehalose caused degradation and unfolding of the α-helices of hemocyanin. However, the addition of an optimal proportion of trehalose:protein (5:1 by weight) prevented the degradation and unfolding during lyophilisation, hence maintained the protein solubility. The estimated ED_{50} values of the formulated solid (0.43 ± 0.1) and liquid samples (0.37 ± 0.06) were similar in magnitude, and were significantly lower than the respective controls; thus, confirming enhanced antiviral activity of the formulation. Formulated compounds were stable for six months at 5 °C storage. The enhanced shelf life and stable antiviral activity of the formulation offers its significant potential as effective therapeutic agent in future clinical applications.

1. Introduction

Hemocyanins are giant copper-containing glycoproteins dispersed in the hemolymph of most molluscs and arthropods. Their primary function is uptake, transporting and releasing oxygen in the desired tissues (Vanholde and Miller, 1982). However, the antigenic and immunogenic nature of these proteins has made them interesting targets in the field of biomedical and clinical research (Harris and Markl, 1999).

Different species of molluscan hemocyanins have been contemplated in diverse biomedical and clinical applications. For example, *keyhole limpet* hemocyanin (KLH) was studied for immunotherapy of bladder cancer (Lammers et al., 2012). KLH was also used in conjugate vaccines, to carry small antigens (haptens) such as mono-oligosaccharides and polysaccharide (Holmberg et al., 2000; Gilewski et al., 2007), glycopeptides, glycoproteins (Gilewski et al., 2000; Betting et al., 2008) and gangliosides (Helling et al., 1994, 1995; Livingston et al., 1994a,b; Dickler et al., 1999; Chapman et al., 2000). In addition, KLH contains oligosaccharide epitopes cross-reacting with glycoconjugates of *Schistosoma mansoni* and thereby can be applied for diagnosis of infections caused by *S. mansoni* (Alvesbrito et al., 1992; Thors and Linder, 2003). Apart from KLH, other hemocyanins such as *Rapana venosa* (RvH), *Helix lucorum* (HlH) and *Helix vulgaris* (HvH) hemocyanins have been shown to be immunogenic and are potentially applicable as effective carriers (Dolashka-Angelova et al., 2008; Dolashka et al., 2011). *In vitro* studies demonstrated that KLH exhibits anticancer properties by itself (Riggs et al., 2002, 2005; McFadden et al., 2007). The antiviral activity of hemocyanins has recently drawn the attention of many researchers (Dang et al., 2011a, 2011b, 2012; Nesterova et al., 2011; Zagorodnya et al., 2011). For example, the hemolymph of abalone *Haliotis rubra* (Dang et al., 2012) and abalone *Haiotis lae-*

Abbreviations: HSV-1, herpes simplex virus type 1; AH, abalone hemocyanin; KLH, keyhole limpet hemocyanin; RvH, *Rapana venusa* hemocyanin; HlH, *Helix lucorum* hemocyanin; HvH, *Helix vulgaris* hemocyanin; BN-PAGE, blue-native polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; MW, molecular weight; TA, thermal analysis; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; CD, circular dichroism; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; GFP, green fluorescent protein; WST-1, water soluble tetrazolium salt; OD, optical density; MOI, multiplicity of infection; LD_{50}, concentration of a compound at which 50% of cell are viable; ED_{50}, concentration of a compound giving rise to 50% reduction in the number of plaques.

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vigata (Dang et al., 2011a, 2011b) has antiviral activity against herpes simplex virus type-1 (HSV-1). RvH (Nesterova et al., 2011) and HlH (Zagorodnya et al., 2011) were active against Epstein–Barr virus. Moreover, *Rapana thomasiana* hemocyanin blocked infections caused by HSV-1 and HSV-2 (Genova-Kalou et al., 2008).

In spite of wide applications of hemocyanins in experimental immunology and clinical medicine, little is known about their stability and much less studies have been devoted to design a formulation to prolong the shelf life of these glycoproteins. Previous studies have demonstrated that, hemocyanin is thermally stable due to its copper active site and disulphide bridges (Georgieva et al., 2004). The melting temperature of these glycoproteins ranged from 68 to 79 °C (Georgieva et al., 1998). However, limited information is available about the stability of hemocyanin in aqueous solution. Proteins in solution usually have a short shelf life, which is a limiting factor in characterisation, development and utilisation of these products. Preparation of a dry solid form using lyophilisation generally increases the shelf life of proteins (Dong et al., 1995; Wang, 2000; Han et al., 2007). The freezing and drying (dehydration) processes during lyophilisation generate a variety of stresses, which can cause unacceptable denaturation and aggregation of the protein (Wang, 2000). Therefore, it is necessary to formulate the proteins using a stabiliser to protect them from denaturation during freezing (cryo-protection) and dehydration (lyo-protection). Sugars and polyols, and more specifically disaccharides such as trehalose, sucrose, maltose and lactose are effective cryo- and lyo-protectants (Prestrelski et al., 1993; Dong et al., 1995; Wang, 2000).

The aim of this study was to examine the effect of formulation with sucrose and trehalose on the stability of abalone hemocyanin (AH). The feasibility of stabilising the AH protein with disaccharides as excipients and obtaining a stable dry powder formulation were investigated. Finally, the biological activities of the optimal formulations were assessed. Analytical methods such as nano-DSC, solubility measurement, gel electrophoresis, and spectroscopic techniques have been used to determine the optimum weight ratio and type of sugar to prepare a stable dry powder.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney (Vero) cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 5% Fetal Bovine Serum (FBS) (Sigma) and 1% penicillin/streptomycin (Life Technologies). vUL37-GFP (green fluorescent protein)-labelled HSV-1 (strain 17) was kindly provided by Frazer Rixon (MRC Virology Unit, Institute of Virology, United Kingdom). Virus titer was calculated using plaque assay on Vero cells.

2.2. Preparation of hemocyanin

Sera were collected from *H. rubra* under sterile conditions. The sera was first centrifuged (Biofuge stratus, Thermo Fisher Scientific) at 4000g for 15 min to remove blood cells and debris, then sterilised using vacuum driven filtration (0.22 μm, Millipore) to avoid degradation of the samples and stored at 5 °C for future use. For *in vitro* tests, freeze dried samples were reconstituted in distilled water. In the case of lyophilised AH without trehalose, the solution was cloudy and clumps of AH powder were visible. Therefore, the mixture was placed in a water bath at 37 °C for a few minutes until a clear solution was obtained. All samples were purified using Amicon Ultra-4 Centrifugal Filter Units (100 kDa, Millipore), and then syringe filtered (0.22 μm).

The FD-XY or L-XY abbreviations are used in the manuscript to simplify citation of different formulation of hemocyanin. FD stands for freeze dried sample, L refers to liquid sample, X denotes type of sugar used in formulation (i.e.: sucrose (S) and trehalose (T)). Y refers to weight ratio of sugar:hemocyanin. As an example FD-S5 is used for freeze dried sample formulated with 5:1 weight ratio of sucrose.

2.3. Determination of protein content

Protein content was determined using Qubit assay according to the procedure provided by Invitrogen. Firstly, working solution was prepared by 200 times dilution of protein reagent in a buffer. Three pre-diluted Bovine Serum Albumin (BSA) standards (Invitrogen) were used for the calibration of instrument (Qubit® 2.0 Fluorometer, Invitrogen). Protein samples were diluted with working solution 20 times in 0.5-mL optical-grade real-time PCR tubes and incubated at ambient temperature for 15 min (preferably in the dark). The Fluorometer measures the concentrations of protein samples using the absorbance values.

2.4. Transmission electron microscopy

Glow discharge treated 300 mesh carbon support films (ProSciTech) were negatively stained using a 5% (w/v) ammonium molybdite solution containing 1% (w/v) trehalose to stabilise the protein. The films were placed consecutively on two droplets, one containing sample and the next staining solution. Placing the film on three consecutive droplets of water washed off the precipitate of the negative stain. The films were kept for one day prior to imaging to allow enough time for drying. TEM imaging was performed using JEM 1400 (Joel, Japan) electron microscope at 100 kV.

2.5. Blue-native gel electrophoresis

All reagents for electrophoresis were purchased from Invitrogen. BN-PAGE was performed using pre-casted 3–12% acrylamide gradient gels according to the user guides for NativePAGE Novex Bis-Tris Gel Systems provided by Invitrogen. Gels were run at constant volts (250 V) for 2 ½ hours and staining was performed using Coomassie staining kit.

2.6. Differential scanning calorimetry

Samples were tested with a Thermal Analysis (TA) nanoDSC instrument at a scan rate of 1 K/min against deionised water standards. The scan rate of 1 K/min is commonly used for DSC analysis (Idakieva et al., 2005). Baseline scans were run through repeat scanning of the water solvent until identical baselines were recorded. AH sample was diluted in distilled water to 1 mg/ml and mixed with sucrose to make L-S1, L-S10 and L-S100 samples. The test was repeated in triplicate for each sample.

Reversibility of the denaturation was tested by the repeated scanning of samples. If suspected of irreversibility, heating was stopped at the onset of denaturation before repeat scanning to confirm this. Irreversible denaturation was analysed through the method of Sanchezruiz (1992). This is characterised by the rate constant of denaturation as a barrier to the unfolding reaction (Eq. (1)):

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (1)$$

where k is rate constant; E_a is activation energy; R is universal gas constant. The rate constant of the denaturation is calculated according to Eq. (2).