Evaluation of thermo-stability of bluetongue virus recombinant VP7 antigen in indirect ELISA

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Abstract This study shows the thermo-stability of lyophilized and purified recombinant VP7 bluetongue virus (BTV) protein in the presence of two sugar stabilizers (trehalose and mannitol) at different temperature. Truncated VP7 protein purified by nickel affinity column was lyophilized in the presence of trehalose and mannitol at 60 mM final concentration and then exposed to different temperature like 4, 25, 37 and 45 °C for various periods like 5 months, 7 weeks, 7 days and 48 h, respectively. After thermal treatment, the reactivity of the protein was evaluated in indirect ELISA. At 4 and 25 °C, the protein was stable up to 5 months and 7 weeks, respectively, irrespective of stabilizers used. At 37 °C, it was stable up to 3 days with both the stabilizers, after which it lost its stability and reactivity. At 45 °C, the protein was stable up to 30 and 24 h with trehalose and mannitol stabilizers, respectively. Both stabilizers found suitable for stability of the protein. However, trehalose appeared to have better stabilizing effect, particularly at higher temperatures than the mannitol. Trehalose could be used as stabilizer for freeze-drying the recombinant VP7 protein if an indirect ELISA kit based on the purified rVP7 protein is supplied to different laboratories of the country for detection of BTV antibody in sheep.

Keywords Bluetongue virus · Mannitol · Recombinant VP7 · Lyophilization · Thermo-stability · Trehalose

Introduction

Bluetongue (BT) is an infectious, non-contagious, arthropod-borne viral disease primarily of sheep caused by the bluetongue virus (BTV), which is the prototype species of the genus *Orbivirus* in the family *Reoviridae* [2]. The virus can infect all domestic and wild ruminants and is transmitted between hosts by certain species of biting midges (*Culicoides* species), which are most abundant and active in hot and humid climates. BT is enzootic in India, and frequent outbreaks have been reported since its detection in 1964 [23]. A total of 21 serotypes have now been reported to be present in India as evidenced by virus isolation and/or antibody detection [29].

Traditionally, laboratory confirmation of BTV is done by intravenous egg inoculation followed by passages in mammalian cells. Virus isolation is tedious and may take up to 5 weeks for completion. Consequently, alternative methods of virus detection have been sought, which include immunoelectron microscopy, sandwich ELISA, reverse transcription polymerase chain reaction (RT-PCR) and real time RT-PCR [15, 17, 19, 26, 30]. The real time RT-PCR is now-a-days the method most commonly used for direct detection of BTV. Competitive ELISA or indirect ELISA is used for detection of BTV-specific antibodies in sera [1, 20]. These assays could be used to screen large number of clinical or experimental samples in a very short time during sero-epidemiological campaign. All these techniques, individually or in various combinations, have been applied for diagnosis and detection of BTV in cell cultures, eggs, insect vectors and ruminants infected naturally or experimentally.

A number of ELISAs have been developed to detect BTV group-specific antibodies, which utilizes cell-associated viral antigen or partially purified virus antigen or rVP7

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antigen [1, 8, 14, 16, 18]. Use of rVP7 as antigen in ELISA, either in indirect or competitive format, has several advantages over whole-virus antigens. Compared to recombinant antigen, purification of equal amount of virus is much time-taking, laborious and expensive [32]. Moreover, recombinant antigens are stable with minimum batch-to-batch variation and lack infectivity that makes them suitable reagents for a wide distribution in ELISA kit format. Recently, in our laboratory, VP7 of BTV-23 has been expressed in prokaryotic system and the purified recombinant VP7 (rVP7) was found to have good reactivity with all the 24 BTV serotype-specific sera [20]. Using this rVP7, an indirect ELISA was developed for detection of BTV antibody in sera and the assay was validated in various field diagnostic laboratories. To use the rVP7 antigen in ELISA kit format and supply the kits to different laboratories in a tropical country like India, it is essential that the recombinant antigen should be thermo-stable enough to produce satisfactory reactivity after exposure to high temperature. Sugars stabilize the proteins against drying and dehydration stresses due to high temperature [22]. Various non-protein stabilizers namely, trehalose, mannitol, sucrose, glycine etc. have been used for thermo stabilization of different vaccines, proteins or antigens [21]. In the present study, the BTV rVP7 was lyophilized with two stabilizers (trehalose and mannitol) separately then exposed to different temperatures and the reactivity of the exposed protein was evaluated by indirect ELISA.

Materials and methods

Expression vector and bacterial host

Truncated VP7 gene (nucleotide 390–939) of BTV-23 (Dehradun isolate) was cloned in pET 32a vector and expressed as histidine-tagged fusion protein in *Escherichia coli* (strain BL21 (DE3) pLysS) cells [20]. The fusion protein (~17.8 kDa) was at the N-terminal of the truncated VP7 (~19.9 kDa) and the predicted molecular weight of the fusion rVP7 was 37.7 kDa. However on SDS-PAGE, the protein was obtained as a 36 kDa band. The expressed region of VP7 (amino acid 130–313) contained most of the antigenic determinants.

Stabilizers

Two chemical stabilizers, namely, trehalose dihydrate and D-mannitol were used for lyophilization of purified rVP7 protein. The stock solutions of each stabilizer were prepared in de-ionized water, sterilized through 0.2 µm membrane filter and added to protein solution at a final concentration of 60 mM [6].

Sera and conjugates

Sheep hyperimmune serum (HIS) against BTV-23 and normal sheep serum were used in the ELISA as positive and negative controls, respectively. Donkey anti-sheep IgG conjugated with HRPO (Sigma, St. Louis, MO, USA) was used as the secondary antibody in ELISA.

Extraction and purification of rVP7 by nickel affinity chromatography

BL-21 strain of *E. coli* expressing truncated VP7 protein (clone pET-VP7) was revived from glycerol stock for bulk production of the recombinant protein. 5 ml of LB broth containing ampicillin (50 µg/ml) was inoculated with 50 µl of glycerol stock and incubated at 37 °C for 16 h (over night) in an orbital shaker at 220 rpm. This over-night culture was inoculated to 100 ml of fresh LB broth containing ampicillin (50 µg/ml) in a flask at a ratio of 1:100 (inoculum:medium). The flask was incubated at 37 °C until cells reached mid-log phase ($OD_{600} = 0.6$, approximately 2.5–3 h of incubation) and then induced by adding IPTG (Promega, Madison, USA) to a final concentration of 1 mM, and incubation was continued at 30 °C. Culture was harvested at 8 h post induction by centrifugation at $2,500 \times g$ for 15 min. The cell pellet was re-suspended in 15 ml of lysis buffer (6 M guanidine hydrochloride, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl, pH 7.8) and rocked slowly for 15 min at room temperature to assure thorough cell lysis. The cell suspension, thus obtained, was sonicated on ice by three 5-s bursts at high intensity with a 10-s cooling period between each burst. The cell lysate was then centrifuged at $10,000 \times g$ at 4 °C for 15 min and the supernatant was mixed thoroughly with 4 ml of nickel-resin (Ni-NTA His.Bind Resin, Novagen, Madison, WI, USA), which was pre-equilibrated with the binding buffer (8 M urea, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl, pH 7.8). The mixture was centrifuged at $1,200 \times g$ for 10 min and the supernatant was removed while the resin precipitate was packed in a column and washed with a large volume of washing buffer (20 mM imidazole, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl, pH 7.8). A second washing was done with buffer containing 40 mM imidazole and the protein was eluted with elution buffer (500 mM imidazole, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl, pH 7.8). The eluted protein was collected in fresh tubes and imidazole was removed by ultra filtration through Amicon 30,000 MWCO ultra filters (Millipore, Billerica, MA, USA). The purity of the rVP7 was checked by SDS-PAGE and protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, USA).

Lyophilization of purified rVP7

Purified rVP7 was lyophilized with 60 mM each of Trehalose and Mannitol (final concentration) and without any stabilizer in a freeze-dryer (Martin Christ, GmbH, Osterode, Germany). 0.2 ml protein ($\sim 2.4 \mu\text{g}$ rVP7) in phosphate buffer (pH 7.8: 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl) was dispensed in sterile 2 ml-capacity glass vials and partially sealed with vented rubber stoppers. The vials were then slowly frozen at -70°C (at 4°C for 30 min followed by at -20°C for 30 min, and then at -70°C for 1 h) to avoid sudden exposure to very low temperature, as higher freezing rate may cause formation of insoluble aggregates of protein [9]. Frozen vials were then placed in the freeze-drying machine and drying was done at -60°C condenser temperature and at 0.120 mbar vacuum. The vials were sealed under vacuum after completion of drying which took at least 24 h and stored at -20°C until further use.

Thermo-stability testing of lyophilized rVP7

Sufficient number of vials was prepared containing lyophilized rVP7 protein in the presence or absence of stabilizer. Four groups were made, namely, Group I (rVP7 lyophilized with Trehalose), Group II (rVP7 lyophilized with Mannitol), Group III (rVP7 lyophilized with no stabilizer) and Group IV (non-lyophilized i.e., rVP7 in phosphate buffer, pH 7.8: 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 500 mM NaCl). Vials of each group were exposed at 4°C for 5 months, 25°C for 7 weeks, 37°C for 7 days and 45°C for 48 h and sampling was done monthly, weekly, daily and 6-hourly intervals respectively from these thermal conditions. After taking the reading on 0 day exposure, sufficient number of vials of each group was exposed at different temperatures. Vials were collected in such a manner that at the end of the total period of exposure, all vials were available at a time for testing. This helped to reduce plate-to-plate and time-to-time variations. Exposed samples were reconstituted with 1 ml of sterile phosphate buffer saline (PBS) and tested in ELISA.

Indirect ELISA for evaluation of reactivity of exposed rVP7

ELISA was performed according to the procedure described earlier [20]. Briefly, the reconstituted or protein in liquid state was added ($50 \mu\text{l}/\text{well}$, containing approximately 120 ng rVP7) on to ELISA plates (MaxiSorp, Nunc A/S, Roskilde, Denmark) and incubated at 37°C for 1 h on a plate shaker, and washed three times with PBST (0.03 % Tween 20 in PBS). The unbound area was blocked with $100 \mu\text{l}$ of blocking buffer (3 % skimmed milk powder

and 2 % gelatin in PBS) and incubated for 1 h at 37°C on a plate shaker. Washing was done as described above. Sheep HIS to BTV was added to the wells and incubated at 37°C for 1 h on a plate shaker. Following the incubation and washing, donkey anti-sheep horseradish peroxidase (HRP) conjugate was added at 1:6,000 dilutions. After incubation and washing, *o*-phenylenediamine (OPD) substrate solution was added and incubated at 37°C for 15 min in dark. The reaction was stopped by adding $50 \mu\text{l}$ of stopping solution (1 M H_2SO_4) and the absorbance in the form of optical density (OD) was measured at 492 nm in an ELISA reader. A value twice (or more) the mean OD value of the negative antigen control was considered as positive (i.e. positive to negative (P/N) ratio ≥ 2). Regression curve analysis was performed for each temperature condition using P/N ratio values and period of protein exposure at each temperature. The data on the reactivity of both the groups (Group I-trehalose and Group II-mannitol) at different thermal conditions in ELISA were subjected to statistical analysis using student's *t* test [28] to find out level of significance.

Results

Expression and purification of rVP7 protein

The culture was harvested at 8 h post induction which was the optimum time for maximal expression of rVP7 as ascertained in earlier experiments [20]. A thick band, corresponding to 36-kDa-protein marker, was observed in dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of sample collected at 8 h post induction. The recombinant VP7 was histidine-tagged at its N-terminal end. So, the purification was essentially done by affinity chromatography through Nickel chelated resin column, which yielded a single clear band of 36 kDa size (Fig. 1). Virtually, there were no other contaminating proteins or impurities in the preparation. Five different batches of bacterial cultures were produced and the yield of purified rVP7 from different batches, as measured by BCA protein assay kit, varied from 2.5 to $3.1 \mu\text{g}/\text{ml}$ of culture with an average of $\sim 2.8 \mu\text{g}/\text{ml}$.

Thermo-stability of lyophilized rVP7

Indirect ELISA was performed to determine the reactivity of lyophilized rVP7 after exposure at 4, 25, 37 and 45°C for different time intervals. The P/N ratios thus obtained were subjected to regression curve analysis. The degradation values obtained for different groups of lyophilized rVP7 protein after exposure at different temperatures were depicted in Table 1.

Fig. 1 SDS-PAGE analysis of recombinant VP7 of BTV 23. A thick band of rVP7 (approximately 36 kDa size) was observed at 8 h post induction (*lane 3*) whereas no such signal in 0 day induction control (*lane 2*). The rVP7 was purified by nickel affinity column which yielded high purity recombinant protein (*lane 4*). *Lane 1* contains protein molecular weight marker (Fermentas, Vilnius, Lithuania; PAGE Ruler Pre Stained protein ladder # SM0671)

Stability at 4 °C

At this temperature, rVP7 was stable during the observation period of 5 months in the presence of both the stabilizers i.e., trehalose and mannitol. Up to 1 month, there was no change in the reactivity compared to 0-day reading. The reactivity got slightly reduced later; however, this exposure did not affect much stability of the protein. The non-lyophilized samples (Group IV) were stable up to 4 months at this temperature. The P/N ration of this group was comparable to that of stabilized groups initially up to 2 months, thereafter, the ratio drastically declined and stability was lost after 4 months. The Group III (lyophilized without stabilizer) was stable up to 5 months, but the P/N ratio was always below the stabilized groups (Fig. 2). At this temperature, the stability of Group III was longer than that of the liquid sample (Group IV). Further, it is found that mannitol performed better than trehalose at 4 °C (t_{10} , 0.329, $p = 0.02$).

Stability at 25 °C

The rVP7 of Group I and II was found stable for 7 weeks at this temperature, however, rVP7 of Group III and IV was stable for four and 3 weeks respectively (Fig. 3). Trehalose appeared to have performed better than mannitol (t_{10} , 0.22, $p = 0.10$) as evident by the P/N ratio. The P/N ratio of the liquid sample (Group IV) was always less as compared to that of Group III. There was a drastic effect of temperature on Group IV as evident by the sharp decline of P/N ratio after exposure of 1 week.

Stability at 37 °C

The rVP7 of Group I and II was stable for 3 days at this temperature and thereafter, stability declined sharply evidenced by reactivity in ELISA (Fig. 4). There was no significant difference between trehalose and mannitol as the stabilizing effects were concerned, although trehalose appeared to have better effects. The Group III and IV samples were stable at this temperature only for 1 day.

Stability at 45 °C

At this temperature, rVP7 of all groups lost the stability much earlier as compared to lower temperatures. Group I and Group II samples were stable for 30 and 24 h respectively (Fig. 5). Stability of Group III and IV samples was 6 and 12 h respectively. There was drastic decline in the P/N ratio of all the groups after 6 h of exposure. On statistical analysis, trehalose was found to be efficient with significant difference (t_{10} , 0.22, $p = 0.10$) compared to mannitol.

Discussion

Trehalose, a non-reducing disaccharide, is one of the best stabilizers which protect proteins against drying and dehydration by hydrogen bonding to the dried protein by serving as water substitute [5]. It has high thermostability and a wide range of pH-stability. Because of its non-reducing property, this saccharide does not show Maillard reaction with amino compounds such as amino acids or proteins [10]. Mannitol is also used for stabilizing the protein. It provides stability to protein by acting against protein aggregation, one of the degradation mechanisms; thereby protects the conformational stability and specific activity of the protein [27]. Both the stabilizers were used at 60 mM final concentration [13] for lyophilization in the present study. The duration of freeze-drying of the purified rVP7 was 24 h only since the volume and total amount of the protein was less (2 µg in 100 µl). This duration was sufficient for complete drying of low volume with less amount of protein.

At 37 °C, both trehalose and mannitol provided stability to rVP7 for 3 days, and at 45 °C it was 30 and 24 h respectively. Trehalose has been found to be very effective in stabilization of proteins during lyophilization [13]. In addition to thermodynamic stability to proteins; trehalose also helps in retention of activity of protein during storage at high temperature [12]. Trehalose provides stability to protein at higher temperatures due to hydrogen bonding property. It replaces water and occupies the places around protein. Thus, a protective shield is provided around the protein. Whereas, mannitol forms soluble aggregates with

Table 1 Comparison of degradation values of purified recombinant VP7 protein in different groups exposed at various temperatures

Temperature (°C) (time period)	Different groups freeze dried (FD)	0-day exposure reactivity (OD value in ELISA)	Sample size (N)	Regression equation	R ² value
4 (monthly up to 5 months)	Trehalose FD	0.55	3	$y = -0.1451x + 3.613$	0.732
	Mannitol FD	0.480	3	$y = -0.0717x + 3.231$	0.971
	Non-stabilized FD	0.510	3	$y = -0.0101x + 2.990$	0.777
	Liquid protein non-FD	0.680	3	$y = -0.3491x + 3.683$	0.984
25 (weekly up to 7 weeks)	Trehalose FD	0.55	3	$y = -0.1274x + 3.536$	0.853
	Mannitol FD	0.480	3	$y = -0.1000x + 3.283$	0.880
	Non-stabilized FD	0.510	3	$y = -0.2081x + 2.968$	0.885
	Liquid protein non-FD	0.680	3	$y = -0.2254x + 3.030$	0.671
37 (daily up to 7 days)	Trehalose FD	0.55	3	$y = -0.3520x + 3.779$	0.871
	Mannitol FD	0.480	3	$y = -0.3120x + 3.528$	0.867
	Non-stabilized FD	0.510	3	$y = -0.1530x + 2.393$	0.592
	Liquid protein non-FD	0.680	3	$y = -0.2400x + 2.850$	0.601
45 (hourly up to 48 h)	Trehalose FD	0.55	3	$y = -0.0260x + 2.910$	0.670
	Mannitol FD	0.480	3	$y = -0.0180x + 2.603$	0.502
	Non-stabilized FD	0.510	3	$y = -0.0150x + 2.344$	0.448
	Liquid protein non-FD	0.680	3	$y = -0.0300x + 2.817$	0.544

protein in freeze-dried state, indicating decreased stability upon storage at higher temperatures. The addition of sodium phosphate buffer to polyol (e.g. mannitol) mixture lowers the crystalline formation upon freeze-drying and thereby formation of aggregates in the product will also reduce [7]. The improved performance of mannitol at higher temperatures may be due to the presence of sodium phosphate used during elution of rVP7. The samples in Group III and IV have shown sharp decline of reactivity after 24 h of exposure at 37 °C compared to the initial reactivity. The lower reactivity in Group III is probably due to the paucity of water molecules to provide hydrogen bonding with the protein. Heat denaturation and loss of biological activity has been linked to the breakup of the 2-D-spanning water network around the protein (due to increasing hydrogen bond breakage with higher temperature [4]. As a result, the secondary structure of the protein was hampered and thereby, the activity of the protein was also lost. The liquid rVP7 (Group IV) lost the stability more quickly than other groups.

It is a general practice in the laboratory to store the freeze-dried biologicals at lower temperature, usually −20 °C. Stability of freeze-dried rVP7 protein was studied after storing at −20 °C. The stored samples were tested bi-monthly to check the reactivity up to 10 months to assess the long-term storage stability. In this study, mannitol appeared to provide better stabilizing effects on protein than trehalose at least for the first 4 months. Since the stabilizing effects of mannitol are attributed to its single amorphous form with the protein and it will remain in such form during drying. It stabilizes the protein as long as in

dried state with protein i.e. during storage [25]. The stability of the protein with both the stabilizers was up to 10 months. However, the stability may be longer as we did not study it beyond 10 months time.

It is also a general practice in the laboratory to use small aliquot of samples and keep the remaining quantity stored at −20 °C for a long time. In this view, we also studied the stability of rVP7 freeze-dried protein (with stabilizers) after reconstitution and storage at −20 °C. We observed that, trehalose-stabilized protein remained stable up to 120 days after reconstitution, whereas it was only 80 days in case of mannitol-stabilized protein. Once reconstituted, the mannitol will turn into crystalline form in aqueous solution [3]. Mannitol was comparatively not suitable for exposure at high temperature as described earlier and for storage in frozen form after reconstitution. In general, the thermostability of protein depends on its electrostatic forces of charged amino acids within the protein and its hydrophobicity nature [31] or hydrophobic cluster [24]. The recombinant VP7 protein in this study may be considered as moderately thermo stable due to hydrophobic nature of protein although, it contains very less number of charged amino acids [11].

In our study, we demonstrated that the rVP7 protein was stable at 45, 37, 25 and 4 °C for 30 h, 3 days, 7 weeks and 5 months, respectively with trehalose stabilizer as freeze dried form. Trehalose stabilized protein could be transported without any cold chain to the place of testing within 3 days even the temperature is as high as 37 °C without any loss of activity. It is appeared to be a better stabilizer than mannitol at higher temperature. After transport of

Fig. 2 **a** Thermo-stability of rVP7 protein of different groups exposed at 4 °C; **b** ratio of positive to negative (P/N) serum reactivity in Indirect ELISA

Fig. 3 **a** Thermo-stability of rVP7 protein of different groups exposed at 25 °C; **b** ratio of positive to negative (P/N) serum reactivity in Indirect ELISA

Fig. 4 **a** Thermo-stability of rVP7 protein of different groups exposed at 37 °C; **b** ratio of positive to negative (P/N) serum reactivity in Indirect ELISA

Fig. 5 **a** Thermo-stability of rVP7 protein of different groups exposed at 45 °C; **b** ratio of positive to negative (P/N) serum reactivity in Indirect ELISA

protein, it should be stored at -20°C of deep freezer or freezer section of refrigerator available in the diagnostic centers/research laboratories. Trehalose could be used as stabilizer for freeze-drying the rVP7 protein intended to supply as antigen in an indirect ELISA kit to different diagnostic laboratories of the country for detection of BT virus in sheep.

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