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Bluetongue virus viral protein 7 stability in the presence of glycerol and sodium chloride

Purpose: The *Orbivirus* Bluetongue virus (BTV) is an economically significant disease that affects mainly wild and domestic ruminants. BTV is most often seen symptomatically in sheep, but is easily carried by goats, cattle, and wild ruminants. To date there are several problems with the vaccines currently available for BTV, and one of the most promising candidates to increase vaccine efficacy is a protein-based vaccine, for which viral protein 7 (VP7) is a great candidate to be included in it. In order to further these studies, the stability of BTV VP7 in common vaccine additives needs to be investigated.

Materials and Methods: Recombinant BTV VP7 was expressed in a bacterial cell system and purified before being analysed using spectroscopic techniques including far-ultraviolet (UV) circular dichroism and intrinsic tryptophan fluorescence. BTV was analysed in a number of different buffer conditions.

Results: We report here that BTV VP7 maintains its native secondary structure until at least 52°C and native-like tertiary structure to at least 80°C. Far-UV circular dichroism and intrinsic tryptophan fluorescence emission spectra indicate significant secondary and tertiary structure remaining even at 90°C, respectively. Six M guanidinium chloride is able to unfold BTV VP7 while 8 M urea could not.

Conclusion: Twenty percent glycerol and 300 mM sodium chloride appear to have a protective effect on BTV VP7's structure, as significantly more structure is seen at 90°C when compared to BTV VP7 without the addition of these chemicals. Both glycerol and sodium chloride are common vaccine additives.

Keywords: Bluetongue virus, Viral protein 7, Glycerol, Sodium chloride, Stability

Introduction

Bluetongue virus (BTV) belongs to the genus *Orbivirus* from the family *Reoviridae*. Twenty-seven serotypes of BTV have been identified to date [1,2]. BTV affects many domestic and wild ruminants [3]. The disease is symptomatically seen most often in sheep, occasionally in goats, and rarely in cattle and wild ruminants [4]. A number of different symptoms are seen in different infected animals including depression, fever, nasal discharge, facial oedema, and general muscle weakness [3-5]. BTV has become common throughout the world and is particularly important economically, not only due to the direct loss of livestock through fatalities, but also the indirect loss due to banning livestock movement to prevent the spread of infection [6]. BTV is carried and spread by blood-feeding *Culicoides* midges, which act as a vector and transmit the dis-

ease when feeding [7]. The spread of the disease is therefore highly reliant on the vector and outbreaks are seen to be seasonal [5].

BTV has a distinctive structure of an outer capsid that surrounds an inner capsid core that itself is composed of two protein layers [8]. The core encloses 10 strands of dsRNA. The viral particle contains seven structural proteins named viral protein (VP)1 through 7 and four non-structural proteins named NS1 through 4 [9]. The inner capsid or core is composed of the two major proteins VP3 and VP7 and three minor ones VP1, VP4, and VP6 and the outer capsid is composed of VP2 and VP5 [9,10]. The protein VP7 of the BTV, consists of 349 amino acids and is encoded by dsRNA genome segment 7, which is 1154–1156 base pairs long and contains only a single open reading frame [11].

Each monomer subunit consists of two distinct domains where the domain that forms the outer surface of the core, is the smaller domain and it consists of the central third of the polypeptide chain (residues 121–149), which is folded into a beta-sheet. The second domain is larger and consists of the residues 1–120 and 250–349. This domain is composed of alpha-helices and contains long extending loops [12]. Each viral particle contains 780 copies of the VP7 protein, which arranges into 260 trimers with a T=13 quasi-equivalent lattice [8,13].

Virus antigen, inactivated viruses and attenuated live virus vaccines have all been developed against BTV [14,15]. There is however two major problems with the current vaccines in use, firstly that it is not currently possible to distinguish between a vaccinated animal and an infected one which is of course important for the ability to move livestock (DIVA-differentiating infected from vaccinated animals) and secondly BTV is not immunologically simple, as in it exists in 27 serotypes that are distinct from each other and the vaccines given are serotype specific, that is they only guard against a specific serotype [1,2]. One additional issue which affects the majority of vaccines, including the BTV vaccines, is that vaccines are so sensitive to temperature, that is, that if they are not kept between 2°C and 8°C, there is a decrease in the effectiveness of the vaccine when it is administered [16].

Protein-based vaccines are the most promising to overcome all these shortfalls [17]. VP7 has a sequence that is highly conserved across the different serotypes [11] and is the major serogroup-reactive antigen [18]. VP7 is therefore an excellent candidate for cross-serotype protection. In addition to this effective, reliable and fast diagnostic tools are very im-

portant in controlling BTV. There are various serological and virological procedures for diagnosis. The BTV VP7 protein is the favourite for the development of group-specific serological assays because of it having a high sequence similarity between all serotypes [11,17].

In this paper we report on the effect of sodium chloride and glycerol on BTV VP7, as well as the effect of these additives on the stability of BTV VP7 when exposed to thermal stress. As sodium chloride and glycerol are common vaccine additives, the work done in the project could help with the future development of diagnostic tools and vaccines that could potentially have a more comprehensive protection and be biologically simpler.

Materials and Methods

Materials

The pET28a plasmid containing the cDNA that encodes the BTV VP7 consensus sequence was purchased from Genscript Corp. (Shanghai, China) and was generated by inserting the codon-optimized cDNA segment between the NdeI and XhoI restriction sites. Kanamycin and IPTG were purchased from Melford (Ipswich, UK). DNase I was purchased from Roche Diagnostics (Mannheim, Germany). Sodium dodecyl sulphate (SDS)-molecular weight marker was purchased from Thermo Scientific (Waltham, MA, USA). Ni-Sepharose resin was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). High purity urea was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

Expression, solubilisation and purification of bluetongue virus viral protein 7

BTV VP7 was expressed, solubilized and purified using the methods defined in NiCo21 (DE3) *Escherichia coli* cells [19]. The protein samples were visualized and quantified using a reducing SDS-PAGE (polyacrylamide gel electrophoresis) with 15% separating gel and 4% stacking gel. In order to study BTV VP7 in a number of different conditions, purified BTV VP7 was dialysed into a number of different buffers: namely 50 mM sodium phosphate, pH 6 with 0.02% sodium azide, and the addition of (1) 20 mM sodium chloride (0 M urea), (2) 2 M urea, (3) 8 M urea, (4) 6 M guanidinium chloride, (5) 20% glycerol, and (6) 300 mM sodium chloride with 5 M urea. Dialysis was performed using 10,000 molecular weight cut-off snake skin dialysis tubing, with buffer volume being 10 times

the sample volume. For each dialysis three buffer changes were used 4, 6, and 12 hours apart, respectively.

Spectroscopy

All measurements were conducted using an Applied Photophysics Chirascan Plus instrument (Applied Photophysics Ltd., Leatherhead, UK) with Photophysics Pro-Data software (ver. 4.2.8; Applied Photophysics Ltd.), equipped with a Peltier temperature controller. Buffer contributions were subtracted for all measurements. At least three repeats were made for each sample and averaged. In addition, experimental conditions were repeated at least twice to confirm reproducibility.

A concentration of 1.7 μL of BTV VP7 was used for all experiments unless otherwise stated. The concentration of protein was determined using Beer Lamberts Law with the molar extinction coefficient $40,910 \text{ M}^{-1} \cdot \text{cm}^{-1}$ calculated using the formula described by Perkins [20]. Absorbance spectra were recorded from 360 to 220 nm for purified VP7 in a quartz cuvette with a path length of 10 mm.

Far-ultraviolet (UV) circular dichroism (CD) spectra measurements were performed between 250 nm and 190 nm, depending on sample, using a 1-mm quartz cuvette with a step size between measurements of 1 nm, bandwidth of 1 nm, and scan speed of 2 nm/s. Data obtained was converted to mean residue ellipticity by using the following formula $[\theta] = 100 (\text{signal}) / \text{Cnl}$, where C is the concentration of protein in mM, n is the number of amino acid residues in the polypeptide chain, and l is the path length in cm. Turbidity (dynode voltage) plots were recorded for each spectra.

Fluorescence spectra were performed using a 10-mm quartz cuvette, a bandwidth of 1 nm, step size of 2 nm, and spectra was recorded between 500 nm and 275 nm, with an excitation wavelength of 295 nm. BTV VP7 contains five tryptophan residues, namely Trp119, Trp141, Trp188, Trp255, and Trp278. Residue Trp278 is completely buried in the folded protein. Trp141, Trp188, and Trp225 are in the beta-sandwich domain while Trp119 is present in the alpha-helical domain.

Thermal denaturation of bluetongue virus viral protein 7

Far-UV CD and fluorescence measurements were collected as described in "Spectroscopy" section, whilst heating the protein sample from 20°C to 90°C. A spectrum was recorded at each degree increase in temperature and a final spectrum once the protein was cooled back to 20°C. A spectrum was then recorded at 20°C, 24 hours and 7 days after thermal

stress to check if recovery was time delayed.

Chemical denaturation of bluetongue virus viral protein 7

BTV VP7 was tested in the presence of guanidinium chloride. Guanidinium chloride was prepared as described by Pace [21] in 1986 using the necessary buffer as the solvent. The pH was adjusted as needed before being filtered with a 0.4- μm filter.

Stock solution concentrations were confirmed using an Atago R5000 Refractometer (Tokyo, Japan), using refractive indices described by Pace [21] and Nozaki [22]. Samples were incubated in buffers containing the desired denaturant concentration for at least 3 hours prior to measurement. Guanidinium chloride recovery studies were undertaken by diluting the protein back down from 6 to 1 M by a rate of decrease of 1 M each time. Protein samples were incubated for 1 hour before spectra were recorded. Spectra were recorded at 22°C as described in "Spectroscopy" section.

Results

Secondary structure

Far-UV CD spectrometry was used to predict the secondary structure of purified VP7 in the presence of 20% glycerol, 20 mM sodium chloride (0 M urea), 2 M urea, 8 M urea, and 6 M guanidinium chloride (Fig. 1A). The spectra could only be recorded to 210 nm as the noise to signal ratio became too high below this wavelength, due to the presence of certain chemicals in the buffers, as detected by turbidity (dynode voltage) (Fig. 1B).

BTV VP7 has been reported to contain both alpha-helices and beta-sheets with a higher alpha-helical content. Using the data collected from the spectra for BTV VP7 the K2D3 tool of DichroWeb was used to estimate the percentage alpha-helices and beta-strands for BTV VP7 under the various conditions tested [23]. The predicted values can be seen in Table 1 as well as the percentages of alpha-helices and beta-strands for BTV VP7 obtained from a secondary structure server, namely 2Struc [24], for the PDB file 1BVP generated from crystallography studies by Grimes et al. [12].

BTV VP7 was shown to contain secondary structure even in the presence of 8 M, with 0 M, 2 M, and 8 M urea showing similar spectra and similar percentage predictions by K2D3 tool of DichroWeb [23] for both alpha-helices and beta-strands to one another as well as to the predicted percentages by 2Struc secondary structure server [24] for BTV VP7 from Grimes et al. [12].

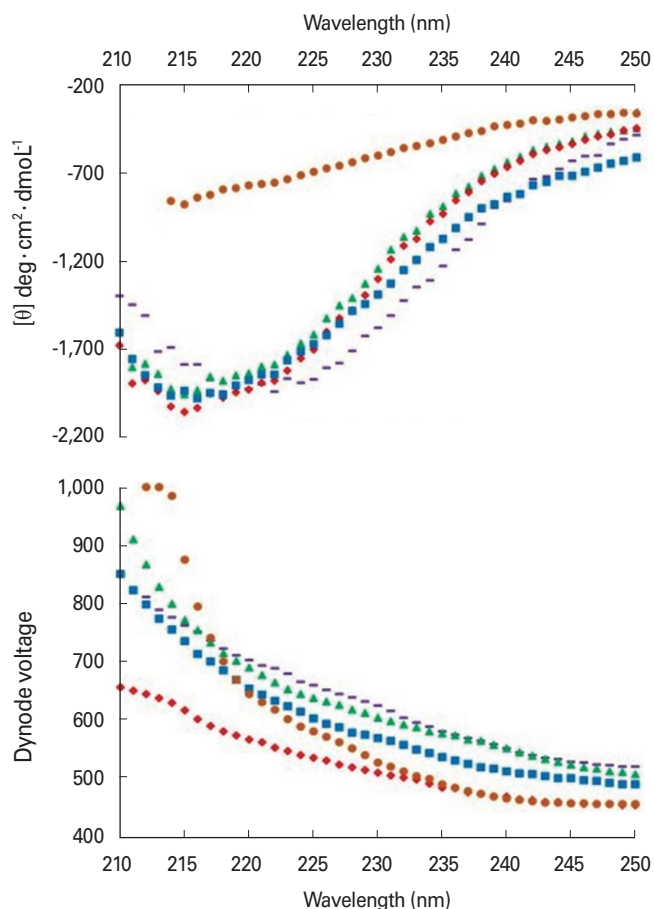


Fig. 1. Far-ultraviolet CD spectra of BTV VP7. (A) CD spectra of BTV VP7 and (B) Turbidity (dynode voltage) plots, obtained between 250 and 210 nm for BTV VP7 in 50 mM sodium phosphate, pH 6 with 0.02% sodium azide with 20 mM sodium chloride (0 M urea) (green triangles), 2 M urea (blue squares), 8 M urea (red diamonds), 6 M guanidinium chloride (orange circles), and 20% glycerol (purple dashes). BTV, Bluetongue virus; VP7, viral protein 7; CD, circular dichroism.

BTV VP7 in the presence of 20% glycerol shows a slightly higher alpha-helical content; however, when K2D3 tool of DichroWeb was used [23], it predicted an alpha-helical content of 38.19% and a beta-strand content of 16.21% (Table 1), which is within a reasonably small structural change or instrumental error. BTV VP7 in the presence of 6 M guanidinium chloride shows spectra more characteristic of a denatured protein, as seen in Fig. 1A, with a spectrum depicting a more positive ellipticity than samples without guanidinium chloride [25].

Tertiary structure

Intrinsic tryptophan fluorescence emission spectra collected confirmed that BTV VP7 has a native like tertiary structure even in the presence of 8 M urea (Fig. 2A), with BTV VP7 in 2

Table 1. Predication of alpha-helical and beta-strand protein content for BTV VP7

Software	Sample	% alpha-helices	% beta-strands
2Struc	1BVP monomer	36.38	17.19
	1BVP trimer	36.38	18.62
K2D2	0 M urea	36.10	18.92
	2 M urea	35.90	18.11
	8 M urea	36.22	17.20
	20% glycerol	38.19	16.21

2Struc secondary structure server [24] predicated alpha-helical and beta-strand content of BTV VP7 monomer and trimer from PDB file 1BVP from Grimes et al. [12] and K2D2 DichroWeb [23] predicted alpha-helical and beta-strand values using far-ultraviolet circular dichroism spectra (Fig. 3) of BTV VP7 with 0 M, 2 M, and 8 M urea as well as 20% glycerol. BTV, Bluetongue virus; VP7, viral protein 7.

M urea, 8 M urea, and 5 M urea with 300 mM sodium chloride all showing a similar tertiary structure, with a peak at approximately 341 nm, which is consistent with BTV VP7 in 0 M and 5 M urea (without the presence of any of the additives evaluated in this study) seen in Russell and Gildenhuis [19]. Increased fluorescence is seen at 295 nm for BTV VP7 in 2 M urea, when compared to 8 M urea and 5 M urea with 300 mM sodium chloride, with the lowest fluorescence at 295 nm seen in 6 M guanidinium chloride (Fig. 2B).

Increased fluorescence intensity around the excitation wavelength (295 nm in our case), often called light scatter, is indicative of the presence of protein aggregation. Therefore, it appears that urea does not disrupt the proteins structure but instead prevents the formation of larger oligomers or aggregates. BTV VP7 in the presence of 6 M guanidinium chloride shows a more characteristic spectra of an unfolded protein with a peak at 355 nm (Fig. 2A). This is consistent with the tertiary structure of a protein that is denatured, as the altered emission results from tryptophan residues moving from a hydrophobic to a hydrophilic environment as they become exposed as the protein denatures [26,27].

Thermal denaturation

The secondary structure of BTV VP7 in 50 mM sodium phosphate, pH 6 with 0.02% sodium azide and 20% glycerol was investigated by monitoring far-UV CD spectra as the protein sample was heated from 20°C to 90°C. The signal to noise ratio was best at 218 nm and 222 nm. BTV VP7 in 20% glycerol demonstrated secondary structural changes that are consistent with a protein that is denatured as the 218 nm and 222 nm ellipticity values increased when the temperature was in-

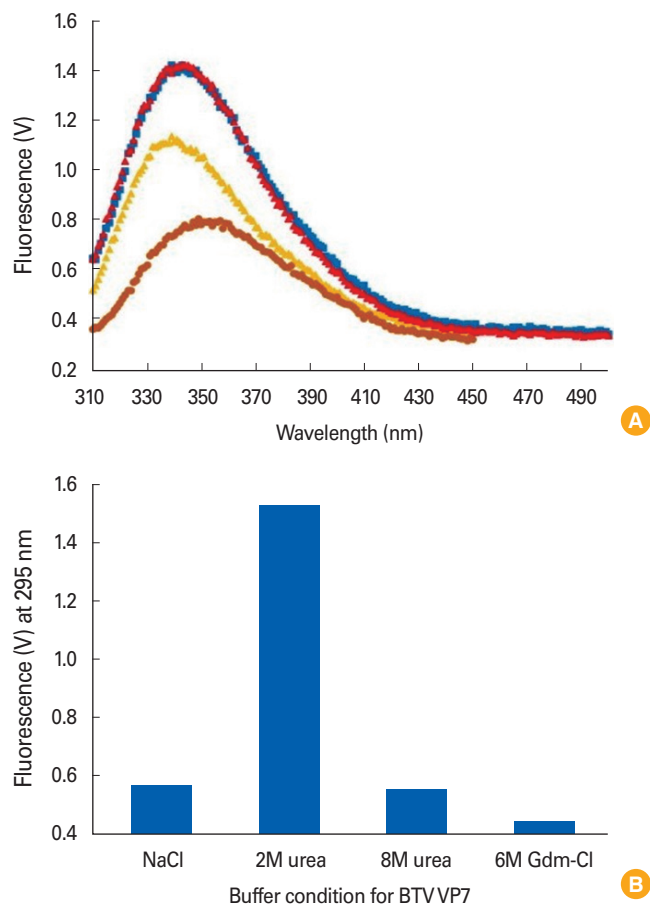


Fig. 2. Fluorescence emission spectra of BTVP7. (A) Fluorescence emission spectra obtained from 500 to 275 nm and (B) 295 nm fluorescence values for BTVP7 in 50 mM sodium phosphate, pH 6 with 0.02% sodium azide at 2 M urea (blue squares), 8 M urea (red diamonds), 6 M guanidinium chloride (orange circles), and 5 M urea with 300 mM sodium chloride (yellow triangles). BTVP, Bluetongue virus; VP7, viral protein 7.

creased, shifting towards the characteristic spectra of a random coil with a positive peak at 212 nm and a trough at 195 nm [28]. A Far-UV CD spectrum for thermal denaturation of BTVP7 in 50 mM sodium phosphate buffer, pH 6 with 5 M urea, 300 mM sodium chloride, and 0.02% sodium azide, was not obtainable as the noise interference was too high.

BTVP7 in the presence of 20% glycerol showed a transition with significant loss in secondary structure from approximately 52°C (Fig. 3A). However, when looking at the far UV spectrum at 20°C and 90°C for BTVP7 in 20% glycerol (Fig. 3B), there is a 34% and 41% loss of ellipticity at 218 nm and 222 nm, respectively. When compared to BTVP7 in 5 M urea which showed approximately a 70% loss of ellipticity at both 218 nm and 222 nm [19], this indicates significantly more secondary structure remaining at 90°C for BTVP7 in

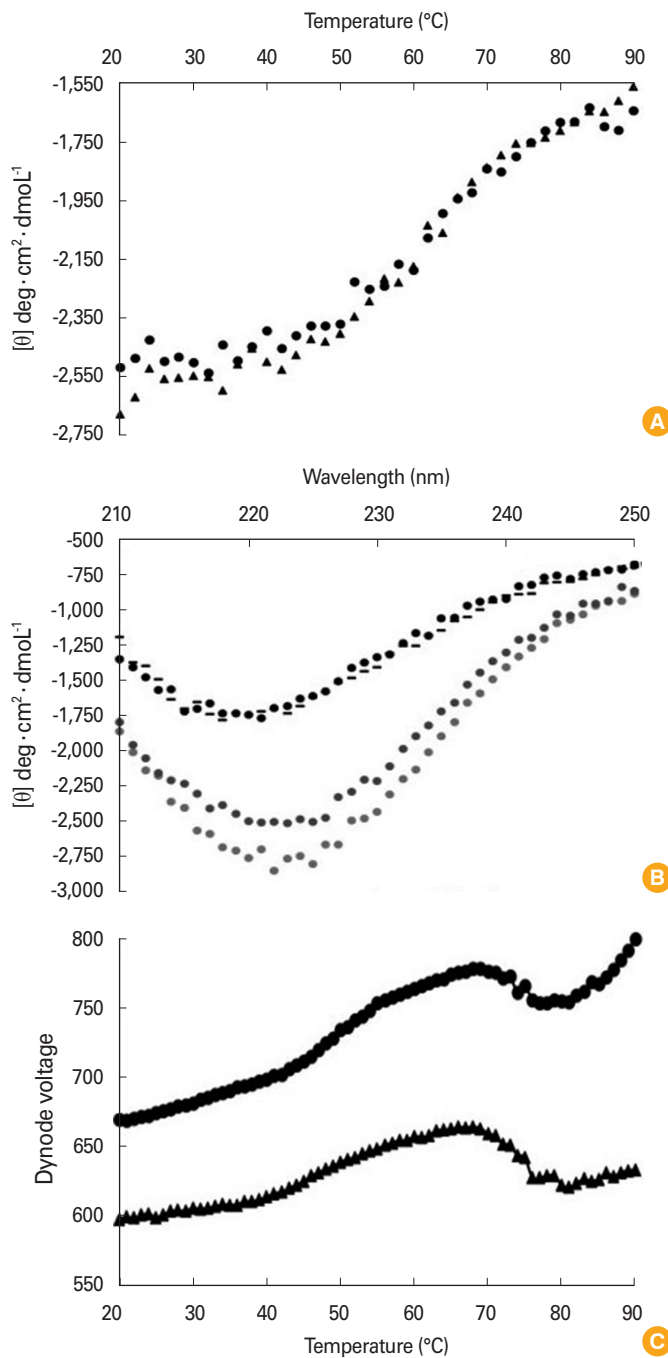


Fig. 3. Far-UV circular dichroism monitoring of thermal unfolding of BTVP7. (A) Heat unfolding curves using far-UV circular dichroism values at 218 nm (circles) and 222 nm (triangles) wavelengths between 20°C and 90°C for BTVP7 in 50 mM sodium phosphate, pH 6 with 20% glycerol, and 0.02% sodium azide. (B) Far-UV spectra for BTVP7 at 20°C (light grey), 55°C (dark grey), and 90°C (black), protein recovered after heating shown in black dashes (-). (C) Turbidity (dynode voltage) plots BTVP7 at 218 nm (circles) 222 nm (triangles). BTVP, Bluetongue virus; VP7, viral protein 7; UV, ultraviolet.

20% glycerol. Fig. 3C shows a turbidity (dynode voltage) plot which can be indicative of protein aggregation occurring as

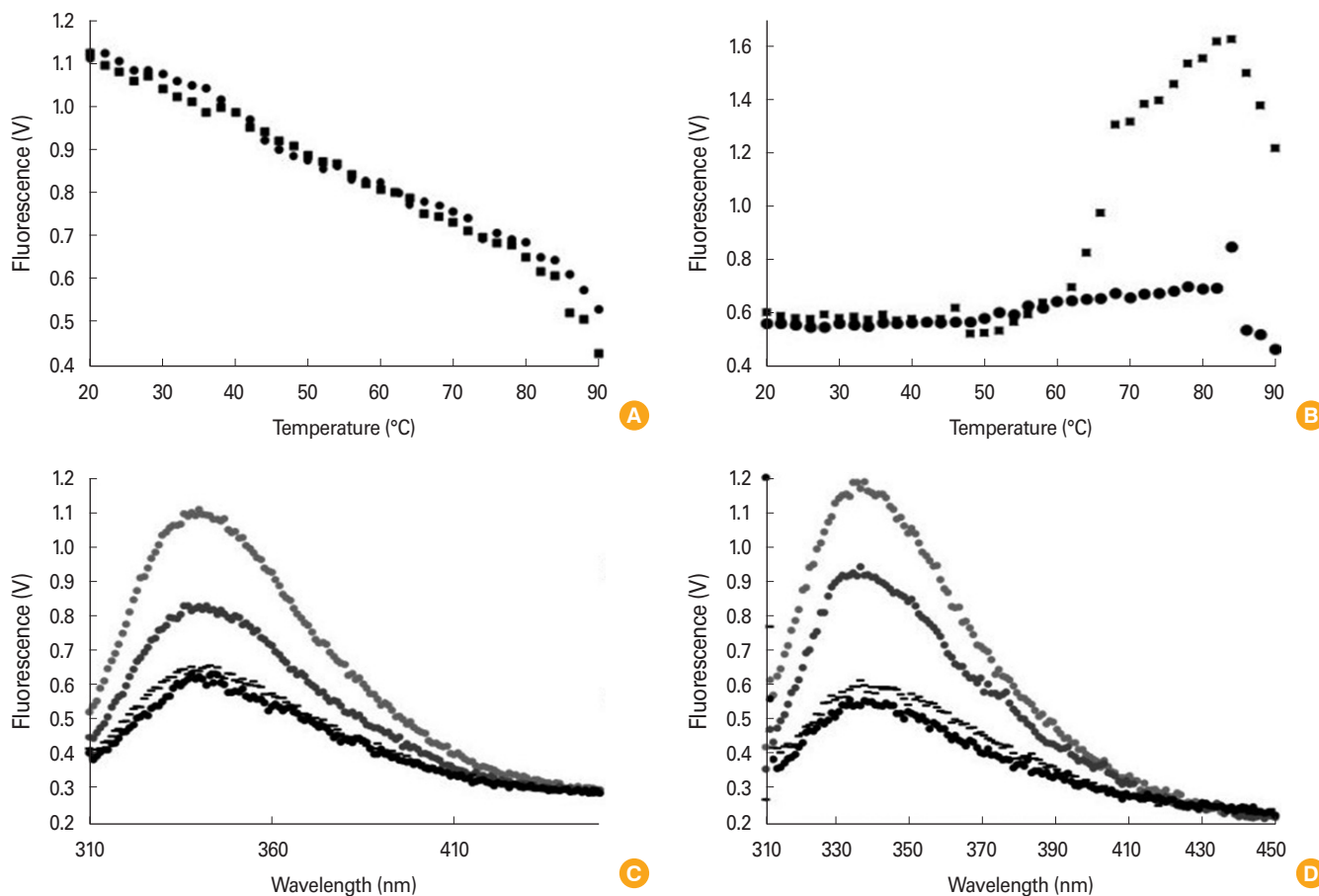


Fig. 4. Fluorescence monitored thermal unfolding of BTV VP7. (A) Heat unfolding curve for fluorescence emission readings from 20°C to 90°C at 339 nm for BTV VP7 in 50 mM sodium phosphate, pH 6 with 0.02% sodium azide and 20% glycerol (squares), and 341 nm for 5 M urea with 300 mM sodium chloride (circles). (B) Emission at 295 nm from 20°C to 90°C or BTV VP7 in 50 mM sodium phosphate, pH 6 with 0.02% sodium azide and 20% glycerol (squares), and 341 nm for 5 M urea with 300 mM sodium chloride (circles). Spectrum for between 450 nm and 310 nm for 20°C (light grey), 55°C (dark grey), and 90°C (black) and the protein refolding after heating shown by dashed lines (-) for (C) 5 M urea and 300 mM sodium chloride and (D) 20% glycerol. BTV, Bluetongue virus; VP7, viral protein 7.

the protein unfolds with increasing temperature [29].

The drop in readings between 70°C and 80°C could be due to precipitation of protein aggregates before a further spike in aggregation formation between 80°C and 90°C. The instrument limitation of being unable to record data from a sample that is being boiled, resulted in the inability to record the full transition.

Tertiary structure can be probed using intrinsic fluorescence, as the aromatic amino acid, tryptophan, is sensitive to the polarity of the amino acid's immediate environment [27]. The fluorescence spectrum changes when the tryptophan residue moves from the hydrophobic environment of a folded protein to the hydrophilic environment of an unfolded protein [26,27]. The emission for several proteins that contain tryptophan residues show a reduction in fluorescence intensity and a red shift in λ_{max} when unfolded. Fluorescence mon-

itored denaturation of BTV VP7 with heat denaturation shows a decrease in fluorescence intensity, for both BTV VP7 in 20% glycerol and 5 M urea with 300 mM sodium chloride (Fig. 4A).

Both samples show a decline in fluorescence intensity and a slight shift in λ_{max} to a longer wavelength with an increase in temperature. For BTV VP7 in 20% glycerol the λ_{max} shifted from 339 nm to approximately 343 nm and BTV VP7 in 5 M urea with 300 mM sodium chloride showed a λ_{max} shift from 341 to 345 nm (Fig. 4B, C). The heat denaturation curves for BTV VP7 in 20% glycerol at 339 nm and 5 M urea with 300 mM sodium chloride at 341 nm shows that there are only slight structural changes until approximately 85°C, after which there are more prominent structural changes (Fig. 4A).

The instrument limitation of being unable to record data from a sample that is being boiled, again resulted in the in-

ability to record the full denaturation transition. At 90°C the emission maximum for BTV VP7, 20% glycerol and 5 M urea with 300 mM sodium chloride was only, 343 nm and 345 nm, respectively, suggesting that complete exposure of tryptophans to an aqueous medium does not occur at this temperature [27].

Fluorescence intensity at 295 nm (excitation wavelength) was investigated as it is indicative of the presence of aggregates in the sample. Spectra recorded at 295 nm between 20°C and 90°C (Fig. 4B) shows a significant increase in fluorescence intensity with an increase in temperature for BTV VP7 in the presence of 20% glycerol, suggesting an increase in aggregation as the protein is heated. For the spectra for BTV VP7 in 5 M urea and 300 mM sodium chloride (Fig. 4B), there is a much smaller increase in fluorescence intensity with an increase in temperature, indicative of a lot less aggregation occurring for this sample in comparison to glycerol, this again could be due to urea's ability to prevent the formation of larger oligomers and/or aggregates. The drop in readings from 85°C in both samples could be due to precipitation of protein aggregates.

The fluorescence intensity decreased by 40% and 44% for BTV VP7 in the presence of 20% glycerol (without urea) and 300 mM sodium chloride (with 5 M urea), which is significantly lower than BTV VP7 in 5 M urea which showed an approximate 85% decrease in fluorescent intensity at 341 nm [19]. Along with smaller λ_{\max} shift, this shows an increase in tertiary structure stability when BTV VP7 is in glycerol or sodium chloride. Both of which are known substances for increasing protein stability and the preserved structure in these conditions is indicative of their protective function in proteins [30,31].

The fluorescence and far-UV CD spectra of the cooled protein sample was not identical to those for the protein before being heated to 90°C indicating incomplete recovery, even 7 days after thermal stress (dashed line in a Fig. 3C and Fig. 4C, D). The percentage recovery values for BTV VP7 in 20% glycerol at 218 nm was 63.7% and at 222 nm was 58.5% and 56.1% recovery when looking at the fluorescence data collected. The recovery percentages obtained for BTV VP7 in 5 M urea with 350 mM sodium chloride was 53.1% when using fluorescence data. As stated above far-UV CD data for BTV VP7 in 5 M urea with 350 mM sodium chloride could not be collected due to chloride ions causing interference at these wavelengths.

The most common cause of irreversible denaturation of heated protein samples is aggregation [29]. The turbidity

(dynode voltage) recorded during far-UV CD experiments and scatter at 295 nm in fluorescence can be used as an indicator of the level of aggregate formation as the protein is denatured via increasing heat. Thermal denaturation of BTV VP7 showed an increase in aggregation depicted by an increase in turbidity with the increase in temperature (Fig. 3C) and an increase in fluorescence at 295 nm (Fig. 4B). Therefore, the likely cause of the irreversibility of thermal denaturation of BTV VP7 is the heat-induced aggregation. The irreversibility of thermal denaturation of BTV VP7 prevents further thermodynamic analysis.

Chemical denaturation

Fluorescence and far-UV CD spectra were recorded for purified BTV VP7 in 50 mM sodium phosphate dibasic, pH 6, with 0.02% (weight/volume) sodium azide, and 6 M guanidinium chloride (Fig. 5A, B). The sample was then diluted down in 1 M guanidinium chloride increments to check at which concentration the protein regained native structure. For purified BTV VP7 the presence of guanidinium chloride resulted in the far-UV CD spectra only being recorded to approximately 214 nm as the noise to signal ratio was too high below this wavelength, as detected by turbidity (dynode voltage) (Fig. 1B). For far-UV CD, there is a substantial loss of secondary structure in 6 M guanidinium chloride (Fig. 5A). As seen in Fig. 5B, at 6 M guanidinium chloride there is a distinct peak at 355 nm, suggesting an unfolded protein. After diluting to 1 M guanidinium chloride the peak returns to 342 nm, suggesting the protein has regained native like tertiary structure.

Fig. 5C shows the turbidity (dynode voltage) at 218 nm and 222 nm for BTV VP7 in the presence of 1 to 6 M guanidinium chloride, showing an increase in turbidity with an increase in guanidinium chloride concentration which is consistent with an increase in chloride ions in the sample. Fig. 5D shows the fluorescence intensity values at 295 nm for BTV VP7 in the presence of 1 to 6 M guanidinium chloride, showing an increase in fluorescence intensity with a decrease in guanidinium chloride concentration suggesting an increase in protein precipitation or aggregation that occurs during renaturation when not in the presence of urea or glycerol.

Fig. 5E shows λ_{\max} fluorescence values and mean residue ellipticity values at 218 nm and 222 nm for samples from 0 to 6 M guanidinium chloride, showing that 3–6 M guanidinium chloride disrupts the renaturation of BTV VP7 to form native secondary and tertiary structure. The ellipticity at 222 nm and 218 nm was used to evaluate the recovery of secondary

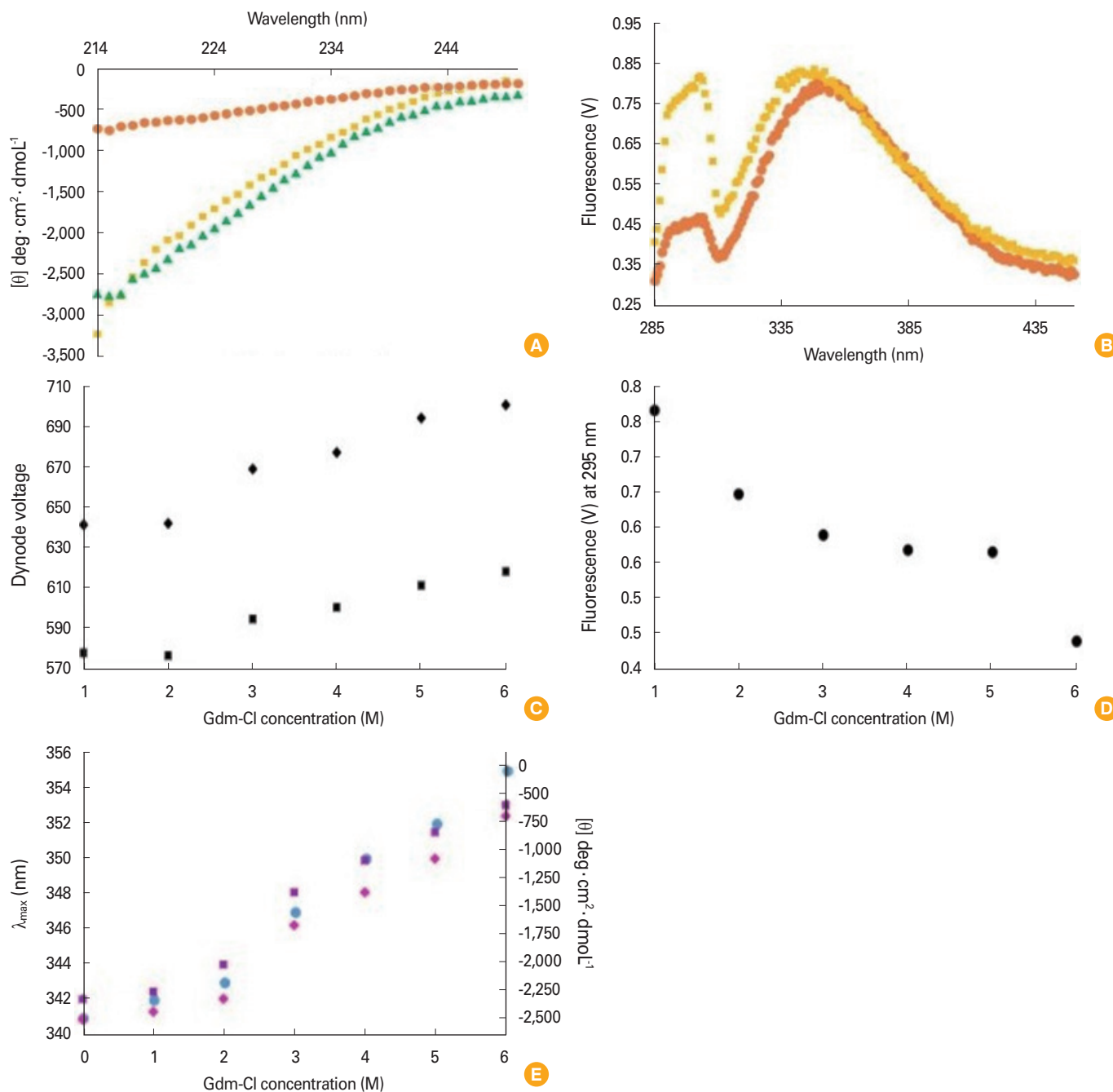


Fig. 5. BTV VP7 in presence of guanidinium chloride. Far-UV CD spectra between 250 nm and 214 nm for BTV VP7 in 50 mM sodium phosphate dibasic, pH 6 with 0.02% (w/v) sodium azide (green triangles) at 1.9 μM, with 6 M guanidinium chloride (orange circles) at 1.9 μM, and 1 M guanidinium chloride (yellow squares) at 1 μM. (B) Fluorescence emission spectrum between 450 nm and 285 nm for purified BTV VP7 (not concentration corrected) with 6 M guanidinium chloride (1.9 μM) (squares), and when allowed to refold in 1 M guanidinium chloride (1.0 μM) (triangles). (C) Turbidity (dynode voltage) plots BTV VP7 at various guanidinium chloride at 218 nm (diamonds) 222 nm (squares). (D) Spectrum for fluorescent emission at 295 nm for various guanidinium chloride concentrations. (E) Shows λ_{max} fluorescence values (blue circles), far-UV CD 218 nm values (pink diamonds), and 222 nm values (purple squares) at several guanidinium chloride concentrations. BTV, Bluetongue virus; VP7, viral protein 7; UV, ultraviolet; CD, circular dichroism.

structure as these are signature troughs in a protein containing both beta-strands and alpha-helices. Renatured BTV VP7 shows a 94% recovery at 218 nm and an 89% recovery at 222

nm (Fig. 5A). Thus, the equilibrium unfolding transition cannot be further analysed to determine thermodynamic parameters.

Due to dilutions the fluorescence intensity percentage recovery cannot be obtained as the signal is not linearly dependent on protein concentration.

Discussion

In this study, we tested the stability of BTV VP7 in a number of different conditions including in the presence of denaturants, stabilizers and when under thermal stress. From the results obtained, BTV VP7 appears to still contain a native-like secondary structure and a folded tertiary structure even in the presence of 8 M urea as seen in Figs. 1 and 2. Furthermore, the folded structure of BTV VP7 in 2 M urea, 8 M urea, and 5 M urea with 300 mM sodium chloride are similar, with highest aggregation seen in 2 M urea. Even 8 M urea therefore does not significantly alter BTV VP7's structure which has also been seen in Basak et al. [32] who reported that VP7 in *Orbiviruses* are able to retain their structures even in 8 M urea.

Instead urea appears to prevent the formation of larger oligomers or aggregates. Guanidinium chloride on the other hand was able to prevent full protein refolding to the native state from a concentration of 3 M as seen in Fig. 5. A concentration of 6 M was however still required in order to shift the fluorescence λ_{\max} to 355 nm, a characteristic of fully denatured protein. Therefore, significant concentrations of the strong denaturant guanidinium chloride are required to disrupt the proteins structure, further supporting the conclusion that BTV VP7 is highly stable.

Protein stability is particularly important in vaccine design as the conformation of a protein plays a large role in the immunogenic effectiveness of the antigens that the protein is presenting. This has been shown to be particularly true for African horse sickness VP7 which is structurally similar to BTV VP7 [33]. The correct protein conformation is significant for both conformational B cell epitopes and linear T and B cell epitopes as proteases can access the peptide backbone easily when the protein is unfolded [34]. The single intrinsic tryptophan fluorescence emission peak at 339 nm in 20% glycerol at 20°C is indicative of a tryptophan residues emission spectrum in hydrophobic surroundings, indicating the BTV VP7 is folded and contains native-like tertiary structure (Fig. 4D) [27].

Often the fluorescence intensity decreases, and λ_{\max} shifts to longer wavelengths when a protein denatures [27]. The beginning of these shifts is seen with BTV VP7 as it was heated from 20°C to 90°C, showing slight decline until approximately

80°C, after which a much sharper decline occurs. The λ_{\max} values at 90°C are 343 nm and 345 nm for BTV 20% glycerol and 300 mM sodium chloride with 5 M urea, respectively, and is suggestive that denaturation is not complete at 90°C and that significant tertiary structure remains, especially when compared to BTV VP7 denatured in 6 M guanidinium chloride (Fig. 2), which shows a shift in λ_{\max} to 355 nm, a characteristic of a fully denatured protein [26,27].

For BTV VP7 to be a viable candidate for use in a vaccine, it needs to be stabilized without the use of urea to prevent aggregation. BTV VP7 in the presence of 20% glycerol showed similar thermal stability to BTV VP7 in 5 M urea with significant loss in secondary structure occurring from approximately 52°C [19]. There was however significantly more secondary structure remaining at 90°C for BTV VP7 in 20% glycerol when compared to BTV VP7 in 5 M urea [19]. As well as a much higher percentage recovery with an estimated 60% recovery for secondary structure and 56% of tertiary structure in 20% glycerol compared to an approximate 30% recovery of secondary structure and approximate 50% tertiary structure recovery for 5 M urea seen in Russell and Gildenhuis [19]. Glycerol is a viscous osmolyte and is well known for increasing protein stability during both thermal and chemical denaturation [35].

The exact molecular mechanism of glycerol increasing protein stability is unknown; however, glycerol has been seen to alter the native structure of a protein to a more compacted state [31] (Fig. 4D), with slight shift in λ_{\max} from 341 to 339 nm and a slight increase in fluorescence intensity seen for the same protein concentration between BTV VP7 in 5 M urea with 300 mM sodium chloride (Fig. 4C) and 20% glycerol (Fig. 4D). Similarly, sodium chloride acts as a weak kosmotrope, as it contributes to the stability and structure of water-water interactions, creating favourable interactions that result in the stabilization of intermolecular interactions in proteins [30].

Kosmotropic co-solvents, such as sodium chloride, interact with the water molecules instead of the protein molecules themselves, resulting in preferential hydration much like glycerol [36].

VPs are increasing in importance as they are vital to improvements in the vaccine, treatment, and diagnostic development. One key problem in vaccine development and administration in general is the temperature sensitivity of vaccines, and the resulting decline in the effectiveness of the administered vaccine when not kept between 2°C and 8°C (in other words refrigerated) [16]. The significance of seeing little

secondary and tertiary structural changes in BTV VP7 until at least 52°C is that if BTV VP7 were to be used in a vaccine, the structure of BTV VP7 would remain stable without being refrigerated which could be vital for delivering the vaccines to the rural communities where the vaccines for BTV are the most needed.

Glycerol and sodium chloride are both common vaccine additives [37,38]; therefore, their presence in vaccines should not cause alarm. Furthermore, the apparent aggregation occurring upon heating could have little effect on the vaccine itself as studies have used aggregated protein in protein-based vaccines successfully before, with some studies showing an increase in immunogenicity towards aggregated proteins [39,40]. Therefore, even if the protein were to be heated above 52°C and cooled, this does not necessarily mean there would be a decrease in the effectiveness of the vaccine being administered.

In conclusion, BTV VP7 is stable in up to 8 M urea, with little secondary or tertiary structural changes seen between 0 M, 2 M, and 8 M urea; however, there is an increase in aggregation as urea concentration decreases; therefore, urea appears to prevent the formation of large oligomers and aggregates but does not denature the protein. On the other hand, guanidinium chloride is capable of denaturing BTV VP7 when at a concentration of 6 M. The addition of glycerol and sodium chloride to BTV VP7 appears to have a protective role in preserving the protein's structure, resulting in significantly more structure remaining at 90°C in comparison to samples that do not contain these additives.

Glycerol and sodium chloride are thought to increase protein stability through preferential hydration. This is significant as sodium chloride and glycerol could therefore be used as preservatives to increase the proteins stability in a potential VP-based vaccination for BTV.

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