

## Preparation of Envelope Glycoprotein V3 (E) Fraction Associated with Haemagglutinating Activity from Purified Japanese Encephalitis Virus by Triton X-100 Treatment

Ashok Kumar SRIVASTAVA and Akira IGARASHI

*Department of Virology,  
Institute of Tropical Medicine, Nagasaki University  
12-4, Sakamoto-machi, Nagasaki 852, Japan*

**Abstract:** Fractions containing envelope glycoprotein V3(E) was prepared from purified Japanese encephalitis (JE) virus by treating with neutral detergent, Triton X-100 (TX-100), followed by ultracentrifugation through sucrose gradients. When virion was mixed with 1.3 to 75 mg of TX-100/mg protein of the virus, the virion was disrupted and virus ELISA antigen formed a peak in the gradients sedimenting slower than the control virion. The higher ratio of TX-100 to the virus protein resulted in the slower sedimentation of the disrupted virus antigen. The ELISA antigen peak was associated with haemagglutinating activity (HA) and contained V3(E) of JE virus structural proteins as revealed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) followed by the Western blotting. Electron microscopy with negatively stained specimens showed that the ELISA antigen fraction prepared with high concentration of TX-100 contained unit particles between 7-8 nm forming aggregates between 30-40 nm, while the fraction prepared with low concentration of TX-100 contained unit particles of 10-15 nm forming larger aggregates about 240 nm. On the other hand, control virion showed spherical particles between 40-50 nm in diameter.

*Key words:* JE virus, Triton X-100, Haemagglutinin, ELISA antigen

### INTRODUCTION

Neutral detergents have been used to solubilize and isolate several membrane proteins (Helenius and Söderlund, 1973; Helenius and Simons, 1975; Tanford and Reynold, 1976; Simons *et al.*, 1978). Triton X-100 (TX-100) was proven to be one of the suitable solubilizer having mild effects on structural proteins that still retain their biological activities such as antigenicities. We have studied the effect of TX-100 on Japanese encephalitis (JE) virus in order to isolate its envelope glycoprotein, V3(E).

JE virus is a member of the family Flaviviridae with single-stranded RNA genome of 42S, and was also classified as mosquito-borne group B arbovirus (Friedman, 1968; Shapiro *et al.*, 1971). The virus structural proteins consist of membrane protein, V1(M)

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with estimated molecular weight (MW) of 7,700; core protein, V2 (C) with MW of 13,500; and V3 (E) with MW of 53,000 (Shapiro *et al.* 1971, 1973). Since V3 (E) exists on the outer surface of the virion, it plays important roles in the initial step of virus infection. The antibodies raised against isolated V3 (E) were shown to inhibit haemagglutination as well as infectivity of the flaviviruses (Trent *et al.*, 1976; Trent, 1977; Takegami *et al.*, 1982; Mathews and Roehring, 1984). Several monoclonal antibodies reacting with V3(E) of JE virus were shown to inhibit haemagglutination (HI) and/or also to neutralize (N) JE virus, although some anti-V3(E) monoclonal antibodies did not show these biological activities even reacting in the ELISA test (Kimura-Kuroda and Yasui, 1983; Kobayashi *et al.*, 1984; Kimura-Kuroda and Yasui, 1986).

Several reports have been published on the TX-100 treatment of alphaviruses of Togaviridae followed by ultracentrifugation through sucrose gradients to obtain envelope glycoproteins (Helenius and Söderlund, 1973; Helenius and Bonsdorff, 1976; Simons *et al.*, 1978). This paper describes similar approach to obtain the aggregates of the V3(E) of JE virus.

#### MATERIALS AND METHODS

*Cells:* *Aedes albopictus*, clone C6/36, cells (Igarashi, 1978) were grown at 28°C as mass culture in spinner bottles as described before (Srivastava *et al.*, 1987).

*Virus:* A wild strain of JE virus, JaOArS982 (Hori *et al.*, 1986), was inoculated to the cell culture. Virion was concentrated and purified from infected fluid by polyethylene glycol precipitation and ultracentrifugation through sucrose gradients in STE buffer (0.1M NaCl, 0.01M Tris-HCl, 0.001M EDTA, pH7.4) as described before (Srivastava *et al.*, 1987). Fractions of 0.6 ml volume were collected by an ISCO density gradient fractionator model 640 and peak fractions of OD<sub>254</sub> were used as purified virion.

*TX-100 treatment and sucrose gradient centrifugation:* One-half ml volume of constant concentration of the purified virion (0.276 mg/ml) was mixed with equal volumes of STE buffer containing varying concentration of TX-100, to make TX-100 to virion protein ratio of 1.3, 10, and 75. The mixtures were incubated at 20°C for 10 minutes, and the samples were loaded onto 15-50% sucrose gradients in STE and centrifuged at 40,000rpm for 20 hours at 20°C in an SW 50.1 rotor of a Beckman model L8M ultracentrifuge. Fractions of 0.4 ml volume were collected by an ISCO gradient fractionator, model 640.

*Sandwich ELISA to detect virus antigens:* The procedure was performed as described by Voller *et al.* (1976). Anti-flavivirus monoclonal mouse ascitic fluid (Srivastava *et al.* unpublished data) was used as catching antibody at 1:16,000 dilution. The IgG fraction prepared from the same monoclonal fluid was conjugated to horseradish peroxidase by Wilson and Nakane's method (1978) and was used as detecting antibody at 1:1,000 dilution.

*Haemagglutinating activity (HA) of JE virus and the HI test:* The tests were performed according to the method of Clarke and Casals (1958).

*SDS-PAGE:* Slab gel method (Studier, 1973) with discontinuous buffer system

(Laemmli, 1970) was used with 10% gel (acrylamide: bisacrylamide ratio of 30 : 0.8) in 1mm thickness. Specimens were solubilized under nonreducing condition in 0.125M Tris-HCl, pH 6.8, containing 0.5 mg/ml of iodoacetamide and 1% SDS by heating at 100°C for 1 minute. After electrophoresis, the protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R250 in 10% acetic acid and 30% methanol by diffusion, followed by destaining in 10% acetic acid and 30% methanol.

*Western blotting:* Proteins from the SDS-PAGE were electrophoretically transferred on nitrocellulose membrane according to Burnette (1981) and Naser and Miltenburger (1983) with some modifications. The transfer was performed in the buffer containing 0.025 M Tris, 0.125M glycine, 20% methanol, pH 8.3, at 4°C for overnight at 8-10 V/cm in an ETB-15 apparatus (Tohyoh Kagaku Sangyoh). The nitrocellulose membrane was briefly rinsed in deionized water and was inactivated by 3% Casine in PBS (phosphate buffered saline) containing 0.01%  $\text{NaN}_3$  for 45 minutes. The membrane was washed 3 times in PBS and reacted with anti-JE polyclonal antibodies at 1:1000 dilution in PBS containing 0.01%  $\text{NaN}_3$  at 37°C for 3 hours. The membrane was washed as above and reacted with peroxidase-conjugated anti-mouse IgG (Cappel laboratories, U.S.A.) at 1:1000 dilution in PBS at 37°C for 2 hours. The membrane was washed as above and the antigenically active protein bands were visualized by incubation with substrate solution of 0.03% 4-chloro-1-naphthol and 0.03%  $\text{H}_2\text{O}_2$  in PBS at room temperature for appropriate time. Anti-JE polyclonal mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus grown in suckling mouse brain.

*Electron microscopy:* Specimens were dialysed against 1% ammonium acetate, pH 7.0, at 4°C overnight, and then negatively stained with 4% uranyl acetate. The specimens were observed under JOEL Electron Microscope type JEM 100 B at direct magnification between 48,000 to 58,000.

*Chemicals:* Acrylamide, bisacrylamide, TX-100 and iodoacetamide were the products of Wako Pure Chemicals Industries Ltd. Japan. The horseradish peroxidase, type VI, was purchased from Sigma Chemicals Co. Ltd.

## RESULTS

*Disruption of JE virion by TX-100:* In the sucrose gradients, control virion without TX-100 treatment sedimented to the fraction 11 (Fig. 1A), while treatment by low concentration of TX-100 (0.25 mg/ml) appeared to have disrupted the virion and virus antigen sedimented to fraction 8 with relatively broad peak (Fig. 1B). When TX-100 concentration was increased to 1.9 mg/ml, the virus antigen also sedimented to fraction 8 with a sharper peak (Fig. 1C). While, treatment with higher concentration of TX-100 (14.25 mg/ml) resulted in the broader peak of virus antigen found in fraction 6 (Fig. 1D). A separate experiment using 2 mg/ml of TX-100 showed that the peak of the ELISA was associated with HA activity (Fig. 2), which was inhibited by the anti-flavivirus monoclonal antibody mentioned above.

Similar experiment was repeated using TX-100 at final concentration of 1 mg/ml. Each fraction was examined by the SDS-PAGE, and the result is shown in Fig. 3. Although control virion and the bottom fractions 13 and 12 contained V1(M) besides V3(E), the fractions between 4 to 11 containing large amount of V3(E) showed only a trace staining at the position of V1(M) coinciding to the dye front. However, these fractions were not stained by the Western blotting using anti-JE polyclonal mouse serum reacting with V1(M). In contrast, the bottom fraction 13 showed positive reaction for V1(M) with less reaction for V3(E) as shown in Fig. 4.

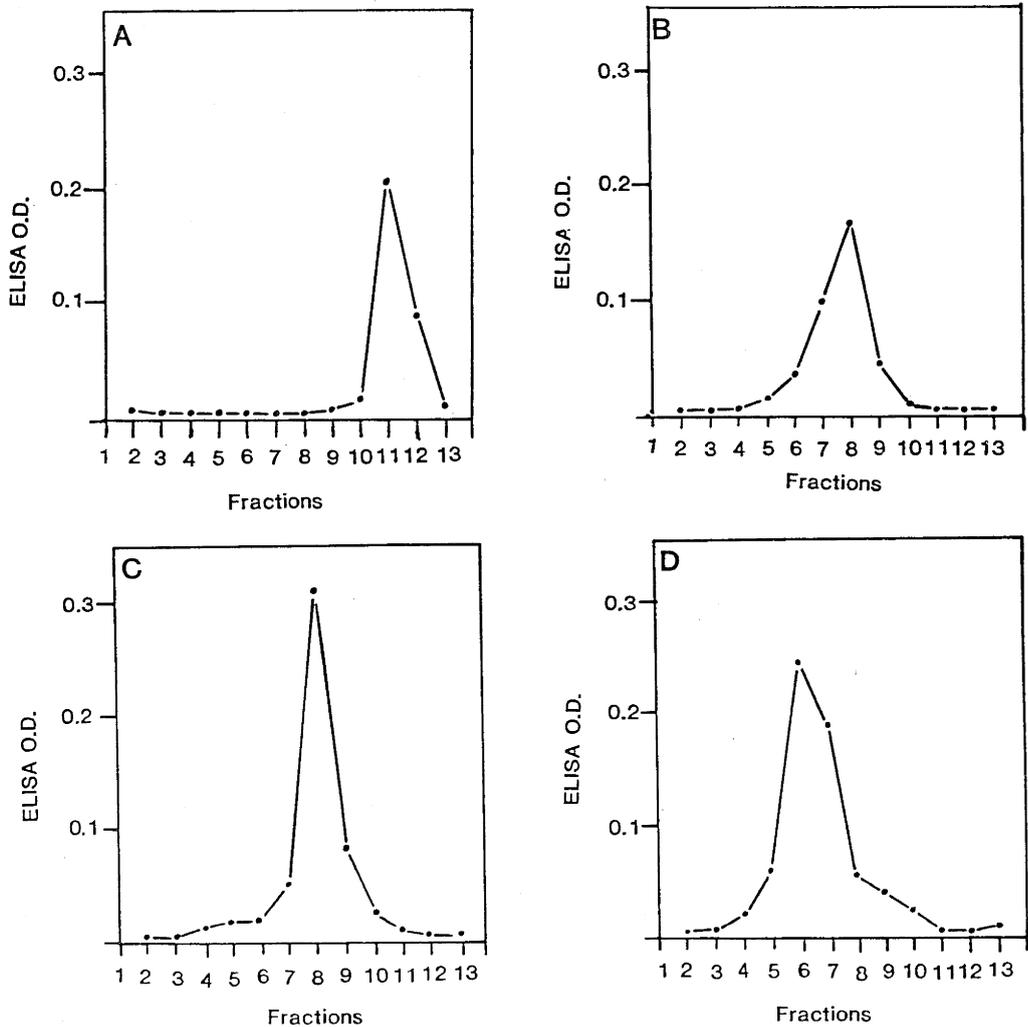


Fig. 1. Sedimentation of JE virus ELISA antigen in sucrose gradient centrifugation with or without TX-100 treatment. One-half ml of purified JE virus (0.276 mg/ml) was mixed with varying concentrations of TX-100 and incubated at 20°C for 10 minutes before centrifugation through 15-50% sucrose gradients in STE buffer. (A) Control without TX-100, (B) 0.25 mg/ml of TX-100, (C) 1.9 mg/ml of TX-100, (D) 14.25 mg/ml of TX-100.

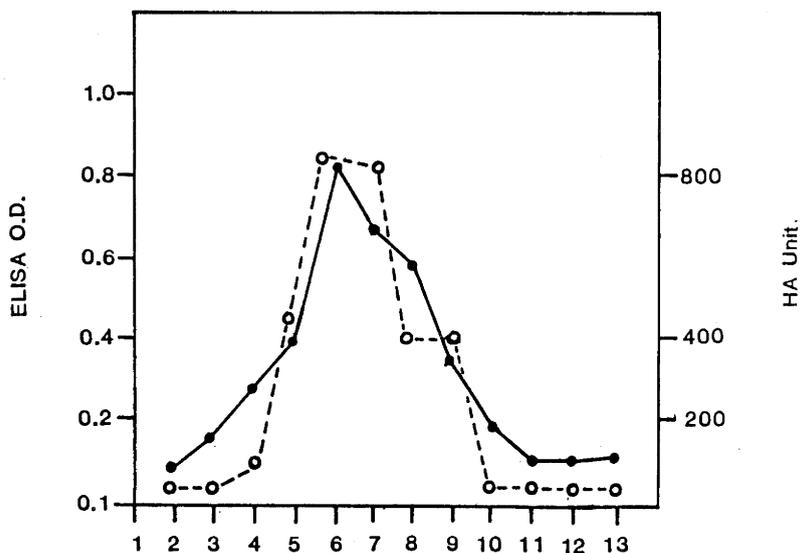


Fig. 2. Sedimentation of ELISA antigen and HA of JE virus treated with TX-100. Procedures are the same as described in the Materials and Methods as well as in the legend to Fig. 1, except that the TX-100 concentration was 2 mg/ml. For each fraction, ELISA OD (●—●), and HA (○---○) were measured.

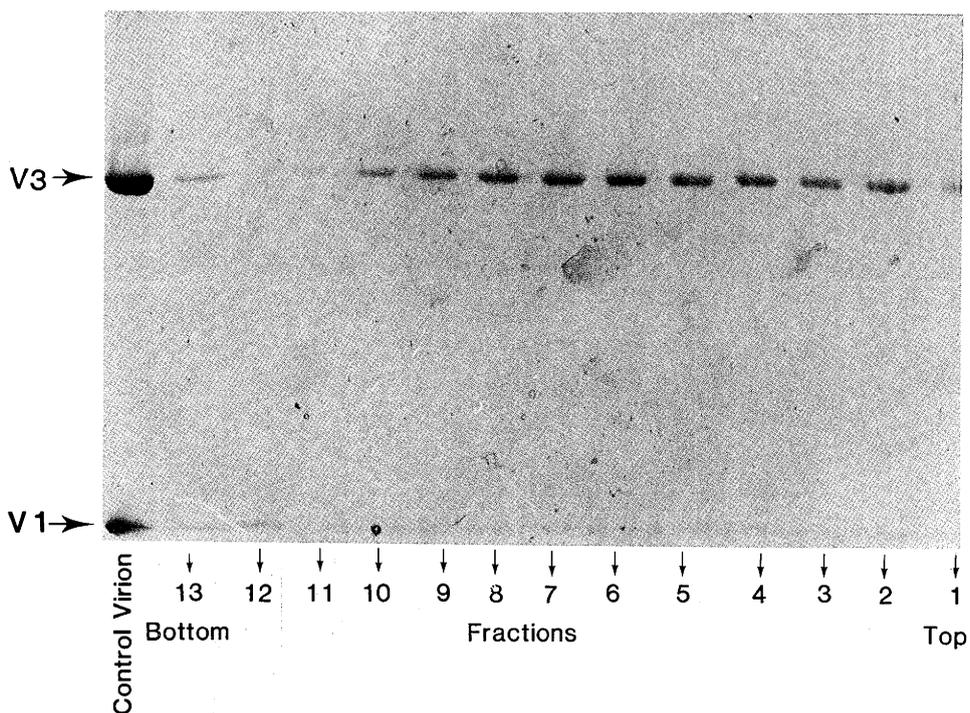


Fig. 3. Coomassie blue stained SDS-PAGE of JE virus before and after TX-100 treatment (1 mg/ml) followed by the sucrose gradient sedimentation as described in the legends to Figs. 1 and 2. Specimens were solubilized and analyzed as described in the Materials and Methods.

The peak fraction for control and fractions from TX-100-treated specimens were observed under an electron microscope as described in the Materials and Methods. The negatively stained control virion showed spherical enveloped particles with 40–45 nm in diameter (Fig. 5A) which agrees with already published results of Ota (1965), Kitano *et al.* (1974), Yoshinaka and Shiomi (1975) and Hayashi *et al.* (1978). On the other hand, specimens treated with low concentration of TX-100 (0.25 mg/ml) showed unit particles of 10–15 nm in diameter which formed aggregates of more than 240 nm (Fig. 5B). While, the specimen treated with high concentration of TX-100 (14.25 mg/ml) showed smaller unit particles around 8 nm in diameter sometimes forming aggregates between 30–40 nm (Fig. 5C).

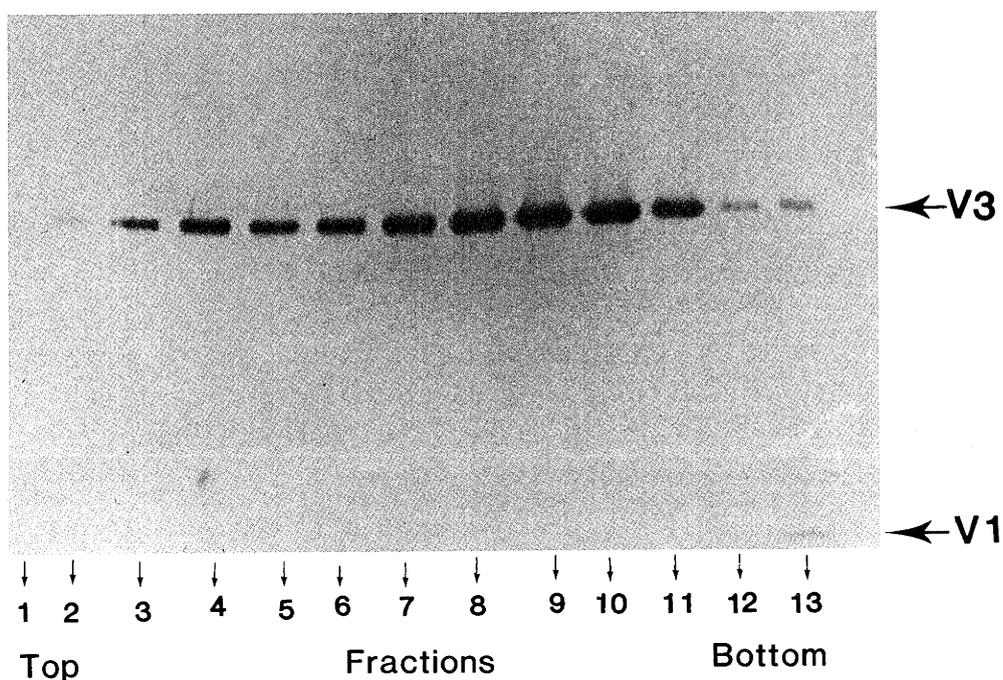


Fig. 4. Western blotting pattern of TX-100 treated purified JE virus fractionated by sucrose gradient sedimentation. Procedures were as described in Materials and Methods with TX-100 treatment of 1 mg/ml at final concentration.

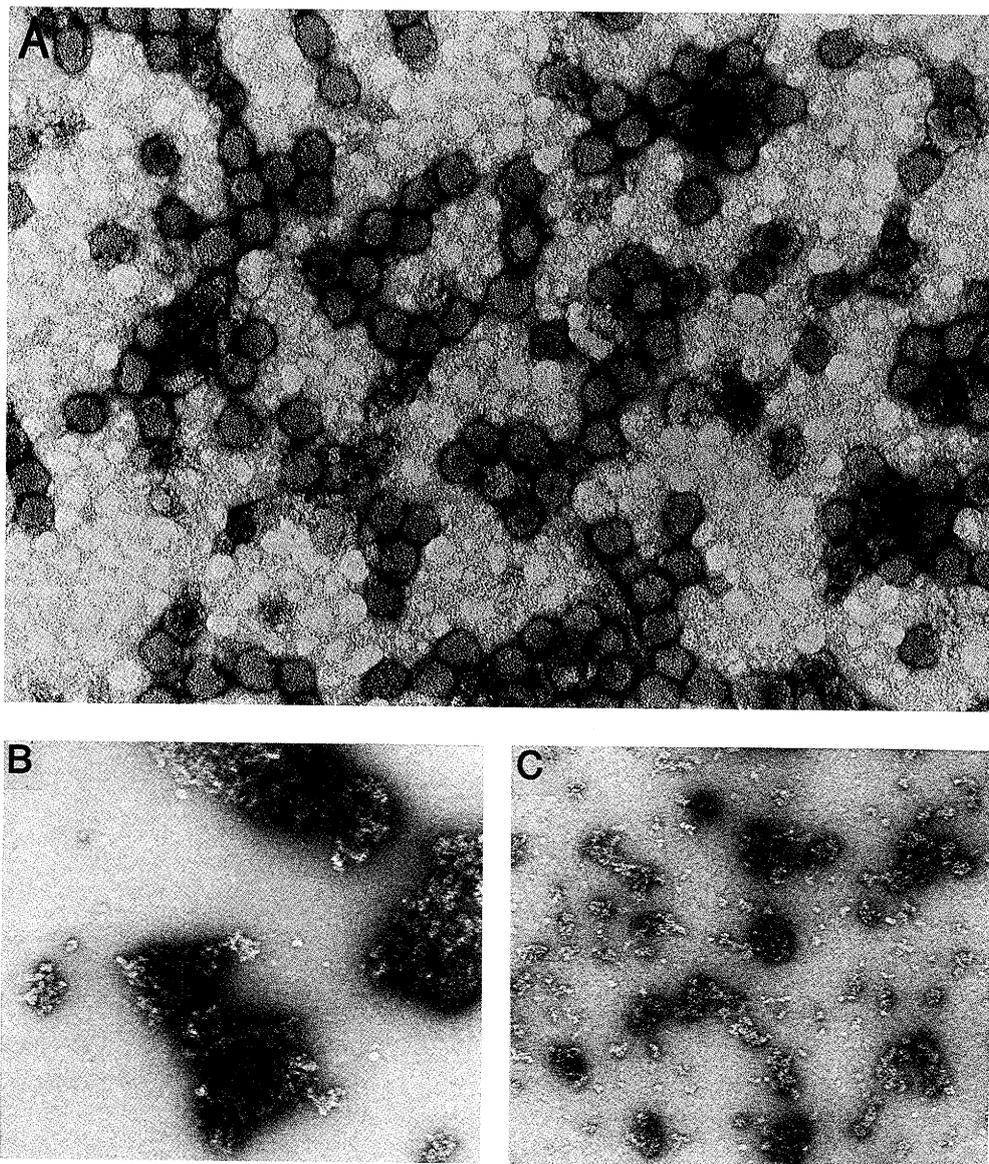


Fig. 5. Electron micrographs of negatively stained JE virus specimens. Control JE virion without TX-100, magnification:  $3 \times 48,000$  in (A). ELISA peaks of the sucrose gradient sedimentation of JE virion treated with 0.25 mg/ml of TX-100 in (B), or 14.25 mg/ml of TX-100 in (C), magnification:  $2.2 \times 58,000$ .

## DISCUSSION

Our data showed that the treatment of the purified JE virion with TX-100 between 0.25–14.25 mg/ml (ratio of TX-100 to virion protein between 1.3–75) resulted in the disruption of the virion. When this treatment was followed by the sedimentation through sucrose gradients without TX-100, the envelope unit structure appeared to have reaggregated retaining its antigenicities. The size of the aggregates and also the size of their unit structure seem to be larger, when lower concentration of TX-100 was used, compared with the treatment with higher concentration of TX-100. These reasonings were drawn from the sedimentation profile of the virus ELISA antigen in the sucrose gradients and electron micrographic observations. Helenius and Söderlund (1973) mentioned that the protein–lipid–detergent complex were gradually delipidated as the concentration of TX-100 was increased for Semliki Forest virus. With 5 mg/ml of TX-100, they observed uniform structure of 25 nm in average diameter, however, without appreciable degree of aggregation. In their paper, they mentioned that unpublished data of Simons *et al.* finally obtained lipid-free membrane protein as small homogeneous complexes binding large amounts of TX-100. These findings are quite different from our results with JE virus which showed aggregated structures and did not form homogeneous complexes even with much higher concentration of TX-100. Kitano *et al.* (1974) disrupted JE virion by another nonionic detergent (NP40), followed by ultracentrifugation. They found minute granulous particles and some rosette-like structures, possibly the aggregated surface projections, but neither virion nor fragmented envelope. By CsCl density gradient centrifugation of the supernatant, they found 2 peaks of HA activity. One at the top of the gradient contained amorphous substance, probably composed of virus envelope lipid and a part of HA<sub>n</sub>. Another fraction at a density of 1.256 g/cc was supposed to be pure HA<sub>n</sub>, because it contained aggregated surface projections and a single band of V3(E) was revealed by SDS-PAGE. Their results are compatible with our findings indicating that the V3(E) of JE virus is more easily aggregated to form larger structures than the envelope proteins of Semliki Forest virus (Helenius and Söderlund, 1973).

Helenius and Söderlund (1973) reported that TX-100 treatment released the nucleocapsid from virion forming a peak in the sucrose gradients. Because we did not use radiolabeled virion, we could not locate the position of JE virus core or its RNA in the sucrose gradients, nor the position of V2(C) was clarified by the staining. On the other hand, V1(M) protein was found at the bottom of the gradient, which might have formed large aggregates or complexed with virion RNA and V2(C). However, the optical density profile showed high peaks of OD<sub>254</sub> near the top of the gradient indicating the release of virion RNA on one hand and the aggregation of V1(M) on the other hand. These problems should be clarified by further studies.

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精製日本脳炎ウイルスから Triton X-100 処理によって得られた血球凝集活性を有する外被膜糖タンパク V3(E) 分画

Srivastava, A. K., 五十嵐 章 (長崎大学熱帯医学研究所ウイルス学部門)

精製された日本脳炎 (JE)ウイルス粒子を中性表面活性剤 Triton X-100 (TX-100) 処理した後に蔗糖密度勾配遠心法によりウイルス粒子の外被膜糖タンパク V3(E) を含む分画が得られた。ウイルスタンパク当り 1.3-75 mg の TX-100 で処理するとウイルス粒子は破壊され、

ウイルスの ELISA 抗原は TX-100 非処理の対照ウイルス粒子よりも蔗糖密度勾配遠心法により遅く沈降するピークを形成した。ウイルスタンパクに対する TX-100 の比率が大きいほど、破壊されたウイルス抗原の沈降速度は小さくなった。このウイルス抗原のピークには JE ウイルスの血球凝集活性が存在しており、ドデシル硫酸ソーダ存在下のポリアクリルアミドゲル電気活動およびウエスタンブロット法ではこの分画には JE ウイルス構造タンパクのうちの V3(E) のみが認められた。ネガティブ染色した電子顕微鏡試料では、高濃度の TX-100 処理によって得られた ELISA 抗原には 7-8 nm の単位構成要素から成る 30-40 nm の凝集塊が観察され、低濃度の TX-100 処理では 10-15 nm の単位構成要素から成る 240 nm 以上の凝集塊が観察された。これに対して TX-100 非処理の対照ウイルス試料には直径 40-45 nm の球型粒子が認められた。

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