Separation of Structural Protein of Japanese Encephalitis Virus by Column Chromatography

Ashok Kumar SRIVASTAVA, Yoshikazu AIRA and Akira IGARASHI

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

Abstract: Attempts were made to separate structural proteins of Japanese encephalitis (JE) virus from purified and disrupted virion or virus-infected *Aedes albopictus*, clone C6/36, cell homogenate. The specimens were applied on the columns containing various materials, such as glass wool, DEAE-Sephacel, Sepharose 4B and 6B, Sephadex-G 150, and Hydroxylapatite with various elution buffers. The eluted peak fractions of OD_{280} were further analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining. In several fractions, the presence of a protein band was identified by SDS-PAGE with estimated molecular weight (Mw) of 54K, which corresponds to the envelope glycoprotein V3 (E) of JE virus. These fractions were obtained from disrupted virion by Sepharose 6B or Sephadex G-150, and also from infected cell homogenate by Sepharose 6B or Hydroxylapatite columns. These fractions were associated with virus antigenicities measured by the ELISA. The best separation of V3 (E) was observed in Sepharose 6B column fractions obtained from the virion disrupted by SDS and 2-mercaptoethanol (2ME). While, disrupted but unfractionated virion showed 2 stained bands with Mw of 54K and 8K, the latter corresponded to virion membrane protein V1 (M).

Key words: Column chromatography, Japanese encephalitis, Structural proteins

INTRODUCTION

Japanese encephalitis (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 viral envelope contains glycoprotein V3 (E) with Mw of 54K, and membrane protein V1 (M) with Mw of 8K. While, viral nucleocapsid contains core protein V2 (C) with Mw of 14K (Stollar, 1969; Shapiro *et al.*, 1971). So far several procedures have been documented to isolate and separate envelope glycoproteins without loss of their biological activities or antigenicities for other flaviviruses or alphaviruses of Togaviridae (Trent, 1977; Hashimoto and Simizu, 1979; Yamamoto and Simizu, 1980; Poliquin and Shore, 1980; Omar and Koblet, 1985).

For JE virus, Takegami *et al.* (1982) separated V1, V2, and V3 by SDS-PAGE and renatured V3 antigenicities by removing SDS by ion-exchange resin. However, the

Received for Publication, October 13, 1987.
Contribution No. 1989 from the Institute of Tropical Medicine, Nagasaki University

recovery of the V3 appeared to be relatively low. In this paper we tried to isolate structural proteins of JE virus, especially, V3 from purified virion as well as virus infected cells by several column chromtography using suitable buffer system.

Materials and Methods

Cells: *Aedes alhopictus*, clone C6/36, cells (Igarashi, 1978) were grown at 28℃ 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 purfied from infected fluid by polyethelene glycol precipitation and ultracentrifugation through sucrose gradients in STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) 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_{254} were used as purified virion.

Cell homogenate: Infected C6/36 cells on microcarrier (Cytodex-1, Pharmacia, Sweden) were collected after 7 days of infection and homogenized in STE buffer containing 0.1% SDS or 1% Nonidet P-40, using a glass-homogenizer. The homogenate was centrifuged at 3,000 rpm for 15 minutes at 4℃, and the supernatant was used as the source of the virus proteins.

Column Chromatography: Glass wool column chromatography (1.5×12cm) of disrupted virion was performed according to Yamamoto and Shimuzu (1980). Hydroxylapatite column (1.5×12cm) was used to separate detergent-solubilized infected cell proteins by gradient or step-wise elution with phosphate buffer containing 0.1% SDS. DEAE Sephacel column (1.6×15cm) was used to separate proteins from disrupted virion or infected cell homogenate as anion exchanger by NaCl gradient in elution buffer containing 0.1% NP40. Sephadex-G 150, Sepharose 4B and Sepharose 6B columns (all 1.5×65cm) were utilized for gel filtration of proteins from disrupted virion or cell homogenate. Jacketed columns (Pharmacia, K16/20 or K16/70) were packed with gel suspension in appropriate buffer system. The specimen was equilibrated with the same buffer, while outer jacket was circulated with cooling water at 4℃. Specimen of approximately 1ml volume was applied and was eluted with either by the gradient system or the same buffer which was used for the equilibration at flow rate of 10−12 ml/hr. OD_{280} of the elute was continuously recorded by Toyo UNICON 540M and fractions of 5ml volume were collected by a fraction collector, Toyo SF-100P.

ELISA: The procedure as described by Voller *et al.* (1976) was used, with anti-flavivirus monoclonal antibody at 1:16,000 dilution as catching antibody and the same monoclonal antibody which was conjugated to horseradish peroxidase by Wilson and Nakane's method (1978) as detecting antibody at 1:1000 dilution, respectively.

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 sloubilzed under reducing condition in 0.125M Tris-HCl, pH 6.8,

containing 2% SDS and 1% 2ME by heating at 100℃ for 1 minute. After electrophoresis, the protein bands were visualized by staining with 0.1% CBB in 10% acetic acid and 30% methanol by diffusion, followed by destaining in 10% acetic acid and 30% methanol.

Chemicals: Acrylamide, bisacrylamide and 2ME were the products of Wako Pure Chemicals Industries Ltd. Japan. The peroxidase, type Ⅵ, was purchased from Sigma Chemicals Co. Ltd. Hydroxylapatite was the product of Bio Rad Co., and Sephadex G-150, Sepharose 4B and 6B were purchased from Pharmacia, Sweden.

RESULTS

Table 1 summarizes the experimental results using various column materials and buffer systems. Glass wool column did not adsorb almost any proteins from the disrupted virion, and the single peak eluted by NaCl gradient did not show virus antigenicities. Nor the protein bands corresponded to the structural proteins of JE virus were identified by the SDS-PAGE. Similar negative results were also obtained by DEAE Sephacel and Sepharose 4B chromatography of disrupted virion or cell homogenate, although 3 peaks of OD_{280} were recorded. Sepharose 6B gel filtration of the purified virion disrupted by SDS and 2ME eluted by STE containing 0.1% gave 4 OD peaks, with ELISA antigen and V3 band in the third peak (Fig. 1 & 2). Also, 3 OD peaks were separated from infected cell homogenate by Sepharose 6B column using borate buffer containing 0.1% NP40 and chased with 1M NaCl. In this case, ELISA antigen and V3 band were found in the 1st OD peak which was contaminated with other proteins as well. On the other hand, the same column could not give fractions with ELISA antigens or V3 band from disrupted viron or infected cell homogenate when elution was performed with phosphate buffer containing 0.1% NP40, although 2-3 OD peaks were observed. Sephadex G—150 column could also separate purified virion disrupted by NP40 into 5 OD peaks by elution with 0.1M NaCl, 0.01M Tris-HCl, 0.1% NP40, pH 7.4. In this case, ELISA antigen and V3 band were observed in the third peak, however, the V3 band was not clear enough. Also, Hydroxylapatite column could separate proteins from infected cell homogenate into 4 peaks by gradient elution with phosphate buffer. Although the ELISA antigen and V3 band were found in the third peak, they were contaminated with some other bands of possible cellular proteins. On the other band, step-wise elution of similar infected cell homogenate by the same column separated the proteins into 3 peaks, but none of them was associated with ELISA antigen or V3 band.

Comparative evaluation of the results in Table 1 shows that the best preparation of JE virus V3 was obtained by gel filtration of purified virion disrupted by SDS and 2ME and fractionated by Sepharose 6B column eluted by STE containing 0.1% NP40, pH 7.4. The peak fraction with ELISA antigen and V3 protein was analysed by the SDS-PAGE (lane 2 in Fig. 2) along with control starting material of the purified virion which was disrupted with SDS and 2ME (lane 1, Fig. 2) followed by the CBB staining. In Fig. 2, control virion in lane 1 showed two bands with Mw of 54K and 8K for V3 and V1 but V2

Table. 1 Separation of JE virus structural proteins by various columns and buffers

Column	Size (cm)	Sample	Buffer & pH	Separation of virion structural proteins
Glass wool	1.5×12	virion disrupted by 1% NP40	0 − 1 M NaCl gradient in 0.01 M Tris-HCl, 0.001M EDTA, 1 % NP40	single OD peak ELISA(−) SDS-PAGE(−)
DEAE Sephacel	1.6×15	Virion disrupted by 1 % NP40	0 − 1 M NaCl gradient in 0.01 M Tris-HCl, 0.001 M EDTA,0.1% NP40	3 OD peaks ELISA(−) SDS-PAGE(−)
DEAE Sephacel	1.6×15	infected cell homogenate in STE 1 % NP40	0 − 1 M NaCl gradient in 0.005 M potassium phosphate, 0.1% NP40, pH 8	3 OD peaks ELISA(−) SDS-PAGE(−)
DEAE Sephacel	1.6×15	virion disrupted by 1 % NP40	0 − 1 M NaCl gradient in 0.005 M potassium phosphate, 0.1% NP40, pH 8	3 OD peaks ELISA(−) SDS-PAGE(−)
Sepharose 4 B	1.5×65	virion disrupted by 1 % NP40	0 − 1 M NaCl in 0.01 M Tris-HCl 0.001 M EDTA, pH 7	3 OD peaks ELISA(−) SDS-PAGE(−)
Sepharose 6 B	1.5×65	virion disrupted by 1 % SDS, 1% 2 ME	0.1 M NaCl in 0.01 M Tris-HCl, 0.001 M EDTA, 0.1% NP40, pH 7.4	4 OD peaks one of them was ELISA(+)and V 3 in SDS-PAGE
Sepharose 6 B	1.5×65	infected cell homogenate in STE disrupted by 1 % NP40	0.005M sodium phosphate, 0.1% NP40, pH 7, chased by 1 M NaCl in above buffer	2 OD peaks ELISA(−) SDS-PAGE(−)
Sepharose 6 B	1.5×65	pooled virion disrupted by 1 % NP40 in STE	0.005 M potassium phosphate, 0.1% NP40 pH 8, chased by 1 M NaCl in the above buffer	3 OD peaks ELISA(−) SDS-PAGE(−)
Sepharose 6 B	1.5×65	infected cell homogenate in STE disrupted by 1 % NP40	0.01 M borate buffer, 0.1% NP40, pH 9, chased by 1 M NaCl in the above buffer	3 OD peaks one of them was ELISA(+)and V 3 in SDS-PAGE
Sepharose 6 B	1.5×65	infected cell homogenate disrupted by 1 % NP40	0.05 M phosphate buffer, 0.1%% NP40 pH9, chased by 1 M NaCl in the above buffer	3 OD peaks ELISA(−) SDS-PAGE(−)
Hyrdoxylapatite	1.5×15	infected cell homogenate in STE 1 % NP40	0.001−0.25 M sodium phosphate buffer 0.1% SDS, 0.1 M PMSF pH 7.4	4 OD peaks, one of them was ELISA(+)and V 3 in SDS-PAGE
Hydroxylapatite	1.5×15	infedted cell homogenate in STE, by 1 % SDS	eluted by 0.001 M, 0.005 M and 0.5 M sodium phosphate, 0.1% SDS, pH 7.4	3 OD peaks ELISA(−) SDS-PAGE(−)
Sephadex G-150	1.5×65	virion disrupted by 1 % NP40	0.1 M NaCl 0.01 M Tris-HCl, 0.1% NP40, pH 7.4	5 OD peaks, one of them was ELISA(+)and V 3 in SDS-PAGE

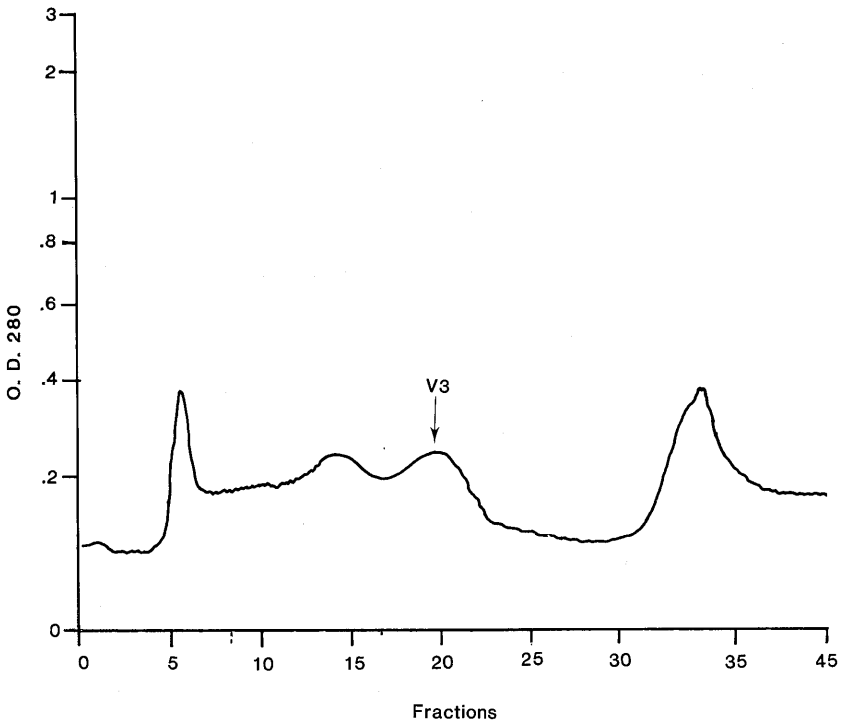

Fig. 1. Elution pattern of purified JE virion disrupted by SDS and 2ME from Sepharose 6B. One ml of the purified JE virion was solubilized by 1% SDS and 1% 2ME at room temperature for 5 minutes and then applied on the column (1.5×65cm) of Sepharose 6B equilibrated with STE with 0.1% NP40, pH 7.4 and eluted with the same buffer.

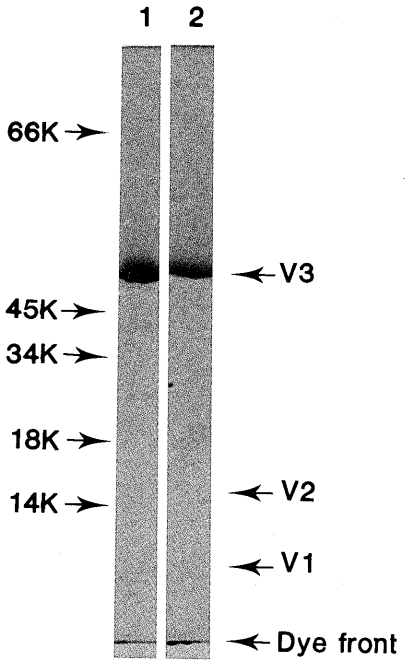

Fig. 2. SDS-PAGE of purified virion (lane 1) and fraction 19 from Fig. 1 (lane 2). Specimens were solubilized by 2% SDS and 1% 2 ME at 100℃ 1 minute in 0.125 M Tris-HCl buffer, pH 6.8, before electrophoresis.

could not be visualized by the staining of the gel. On the other hand, lane 2. fraction 19 from the gel filtration, showed only a single band of Mw 54K for V3.

Discussion

In our laboratory, structural proteins of JE virus have been separated by SDS-PAGE (Srivastava and Igarashi, 1985; Srivastava *et al.*, 1987), and tried to isloate V3 as Takegemi *et al.* (1982) reported. Also we have experienced that separation of solubilized JE virus was not efficient by using isoelectric focusing as reported by Trent (1977), and Hashimoto and Simuzu (1979). The former procedures were not efficient in the recovery of V3 protein, and the latter required quite a long time and the separation of each structural proteins was not clear.

We examined the combinations of several columns with several buffer systems in order to separate the V3 protein from purified virion or infected cell homogenate. As described in the Results, gel filtration by Sepharose 6B column gave best separation of V3 with antigenicities. This method is simple and efficient for large scale purification of the envelope glycoproteins without loss of its antigenicity. Therefore, this method might be applicable to other group of arboviruses with slight modification in elution buffer.

The reason why V2 was not stained well by SDS-PAGE in the control preparation before fractionation is not clear. However, purified virion under electron micrography showed that the preparation consisted of mostly complete particles (Srivastava and Igarashi, 1987). Our previous finding showed that the mobility and staining of V2 in SDS-PAGE as well as its reactivity with polyclonal anti-JE mouse serum was reduced for the virion disrupted under reducing condition compared with nonreducing condition (Srivastava *et al.*, 1987). Through our present studies, we used the SDS-PAGE for the specimens prepared under reducing condition. The negative finding of V2 in the SDS-PAGE result might have some relationship with this findings. Since Takegami *et al.* (1982), however, showed clear staining of V2 protein for Nakayama strain of JE virus in SDS-PAGE, our negative result might also be due to the difference in the virus strain used in the experiments. These questions should be answered by using radiolabeled virion or more sensitive detection method like Western blotting.

Acknowledgement

The first author was supported by the Monbusho-scholorship from the Ministry of Education, Science and Culture of Japan.

References

1) Friedman, R. M. (1968): Structural and nonstructural proteins of an arbovirus. J. Virol., 2., 1076−1080.

2) Hashimoto, K. & Simizu, B. (1979): Isolation of the structural proteins of Western equine encephalitis virus by isoelectric focusing. Arch. Virol. 60, 299−309.

3) Hori, H., Morita, K., Igarashi, A., Yoshida, I. & Takagi, M. (1986): Oligonucleotide fingerprint analysis of Japanese encephalitis virus strains isolated from Thailand with refernce to effect of passage histories on the fingerprints. Acta Virol., 30, 353−359.

4) Igarashi, A. (1978): Isolation of Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol., 40, 531−544.

5) Laemmli, U. K. (1970): Cleavage of structural protein during the assambly of the head of becteriophage T 4. Nature, 227, 680−685.

6) Omar, A. & Koblet, H. (1980): One-step separation of the components of Semliki Forest virus by cation exchange chromatography. J. Virol. Method, 12, 71−83.

7) Poliquin, L. & Shore, G. C. (1980): A method and selective recovery of membrane glycoproteins from dodecyl sulphate and urea. Analyt. Biochem., 109, 460−465.

8) Shapiro, D., Brandt, W. E., Cardiff, R. D. & Russell, P. K. (1971): The proteins of Japanese encephalitis virus. Virology, 44, 108−124.

9) Srivastava, A. K. & Igarashi, A. (1975): Comparison of Japanese encephalitis virus isolates from Japan and Thailand by peptide mapping. Trop. Med. 27, 269−275.

10) Srivastava, A. K., & Igarashi, A. (1987): Preparation of haemagglutinin of Japanese encephalitis virus by Triton X-100 treatment. Trop. Med., 29, 127−137.

11) Srivastava, A. K., Aira, Y., Mori, C., Kobayashi, Y. & Igarashi, A. (1987): Antigenicity of Japanese encephalitis virus envelope glycoprotein V3 (E) and its cyanogen bromide cleaved fragments examined by monoclonal antibodies and Western blotting. Arch. Virol. 96, 97−107.

12) Stoller, V. (1969): Studies on the nature of dengue viruses. Ⅳ. The structural proteins of type 2 dengue virus. Virology, 39, 426−438.

13) Studier, F. M. (1973): Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79, 237−248.

14) Takegami, T., Miyamoto, H., Nakamura, H. & Yasui, K. (1982): Biological activities of the structural proteins of Japanese encephalitis virus. Acta Virol., 26, 312−320.

15) Voller, A., Bidwell, O. & Bartlet, A. (1976): Microplate enzyme immunoassay for immunodiagnosis of viral infection. pp 506-512. *In* N. R. Rose & N. Friedman (ed.). Manual of Clinical Immunology, American Society of Microbiology. Washington, D. C.

16) Wilson, M. B. & Nakane, P. K. (1978): Recent development in periodate method of conjugating horseradish peroxidase (HRPO) to Immonofluorescence and related staining techniques. Elsevier/North-Holland Biochemical Press, Amsterdom.

17) Yamamoto, K. & Simizu, B. (1980): Purification of the envelope glycoproteins of Western equine encephalitis virus by glass wool chromatography. Applied Environ. Microbiol., 40, 240−243.

カラムクロマトグラフィーによる日本脳炎ウイルス構造蛋白質の分離

ASHOK KUMAR SRIVASTAVA, 姶良　義一, 五十嵐　章（熱帯医学研究所）

精製した日本脳炎（JE）ウイルス粒子を溶解した試料，或はウイルス感染ヒトスジシマカ培養細胞クローン C6/36 の乳剤から JE ウイルスの構造蛋白質を分離することを試みた．試料の分離にはガラスウール，DEAE-Sephacel, Sepharose 4B 又は 6B, Sephadex G-150, 及び Hydroxylapatite カラムを種々の緩衝液と組み合わせて使用した．カラムから溶出され280nmの吸光度でピークを示した分画を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泳動（SDS-PAGE）で分離し，Coomassie Brilliant Blue で染色した．いくつかの分画には JE ウイルス外被膜糖蛋白質 V3 (E) と同じ分子量（54K）を示すバンドが SDS-PAGE で検出された．これらの分画は，破壊した精製ウイルス粒子から Sepharose 6B 又は Sephadex G-150 カラムを用いて，或はウイルス感染細胞乳剤から Sepharose 6B 又は Hydroxylapatite カラムを用いて分離され，ELISA によってウイルス抗原性を有することが示された．ウイルス粒子を SDS と2メルカプトエタノール（2ME）で溶解後，Sepharose 6B カラムを用いた場合 V3 (E) は最もよく分離された．ウイルス粒子を溶解してカラムで分離する前の試料には SDS-PAGE で分子量 54K のバンドと V1 (M) 蛋白質に相当する分子量 8K の二つのバンドが染色された．

熱帯医学　第29巻　第4号　187－194頁，1987年12月