

Expression of the dengue virus structural proteins in *Pichia pastoris* leads to the generation of virus-like particles

Richard Joseph Sugrue,¹ Jianlin Fu,¹ Josephine Howe² and Yow-Cheong Chan²

Dengue Virus Group, Institute of Molecular and Cell Biology¹, and Department of Microbiology², National University of Singapore, 10 Kent Ridge Crescent, Republic of Singapore

We have expressed cDNA encoding the dengue virus structural proteins in *Pichia pastoris* by chromosomal integration of an expression cassette containing the dengue virus structural genes (CprME). The yeast recombinant E protein migrated during SDS-PAGE as a 65 kDa protein when analysed by Western blotting and radioimmunoprecipitation, which is the expected molecular mass for correctly processed and glycosylated E protein. Treatment with endoglycosidases showed that the recombinant E protein was modified by the addition of short mannose chains. The E protein migrated with a buoyant density of 1.13 g/cm³ when analysed using sucrose density gradient centrifugation. Spherical structures with an average diameter of 30 nm, whose morphology resembles dengue virions, were observed in the purified fractions using transmission electron microscopy. Furthermore, the virus-like particles were immunogenic in animals and were able to induce neutralizing antibodies. This is the first report that expression of the structural genes of a flavivirus in yeast is able to generate particulate structures that resemble virions.

The dengue virus genome encodes three structural and seven non-structural proteins and is initially translated as a single polyprotein from which each protein is subsequently released by cellular and virus proteolytic activity (Chambers *et al.*, 1990). The structural proteins consist of a capsid (C) and two membrane proteins, E and M (or its precursor prM), which are translated in the order CprME at the beginning of the polyprotein. Co-expression of the flaviviral envelope E and prM proteins in a variety of animal cell lines generates virus-like particles (VLPs) which induce neutralizing antibodies

against infectious virus (Konishi *et al.*, 1992; Pincus *et al.*, 1992). Yeast represents an alternative eukaryotic expression system for the generation of flaviviral glycoproteins and previous studies have demonstrated that Japanese encephalitis virus (JEV) E protein expressed in *Saccharomyces cerevisiae* can elicit neutralizing antibodies in experimental animals (Fujita *et al.*, 1987). Yeast as an expression host offers distinct advantages over mammalian expression systems both in terms of scalability and productivity as well as the proven safety of yeast-derived viral proteins for pharmaceutical applications, e.g. hepatitis B vaccine (Cregg *et al.*, 1987). We have selected the methylotropic yeast *Pichia pastoris* to express dengue virus structural proteins since several reports have shown that this yeast, unlike *S. cerevisiae*, is capable of expressing glycoproteins that are both non-hyperglycosylated and lack a terminal 1,3-linked mannose addition (Grinna & Tschopp, 1989; Trimble *et al.*, 1991). Both of these are undesirable modifications that severely limit the potential pharmaceutical applications of recombinant glycoproteins expressed in *S. cerevisiae*.

We have inserted cDNA encoding the structural genes of dengue virus type 1 (DEN-1) Singapore strain S275/90 (Fu *et al.*, 1992) into the *P. pastoris* expression vector pHIL-D2 (Invitrogen). Two primers, DIR81E (5' AGCAGAATTCTGATGAACAAC 3') and DIFYE (5' TTACGAATTCCTATTACGCTTGAACCA 3'), were used to generate a 2.3 kb PCR fragment containing flanking *EcoRI* restriction sites, using pFA/1 as the template. The template pFA/1 contains the cDNA sequence of the DEN-1 (S275/90) structural genes (CprME, nts 81–2402). Sequencing of the 2.3 kb fragment, by the dideoxy sequencing method, confirmed the sequence of the entire CprME coding region, which is predicted to encode a polypeptide of approximately 100 kDa. This 2.3 kb fragment was ligated to the corresponding *EcoRI* site in pHIL-D2 to generate pHIL-D2/CprME (Fig. 1a). The vectors pHIL-D2 and pHIL-D2/CprME were linearized by *NotI* digestion and used to transform spheroplasts of the *P. pastoris* strain GS115. The stable transformants GS115/D2 and GS115/CprME were obtained using pHIL-D2 and pHIL-D2/CprME, respectively, by methods previously described (Cregg *et al.*, 1985).

The transformants GS115/D2 and GS115/CprME were incubated at 30 °C for 48 h in minimal glycerol medium

Author for correspondence: Richard Sugrue.

Fax +65 779 1117. e-mail mcbsrj@leonis.nus.sg

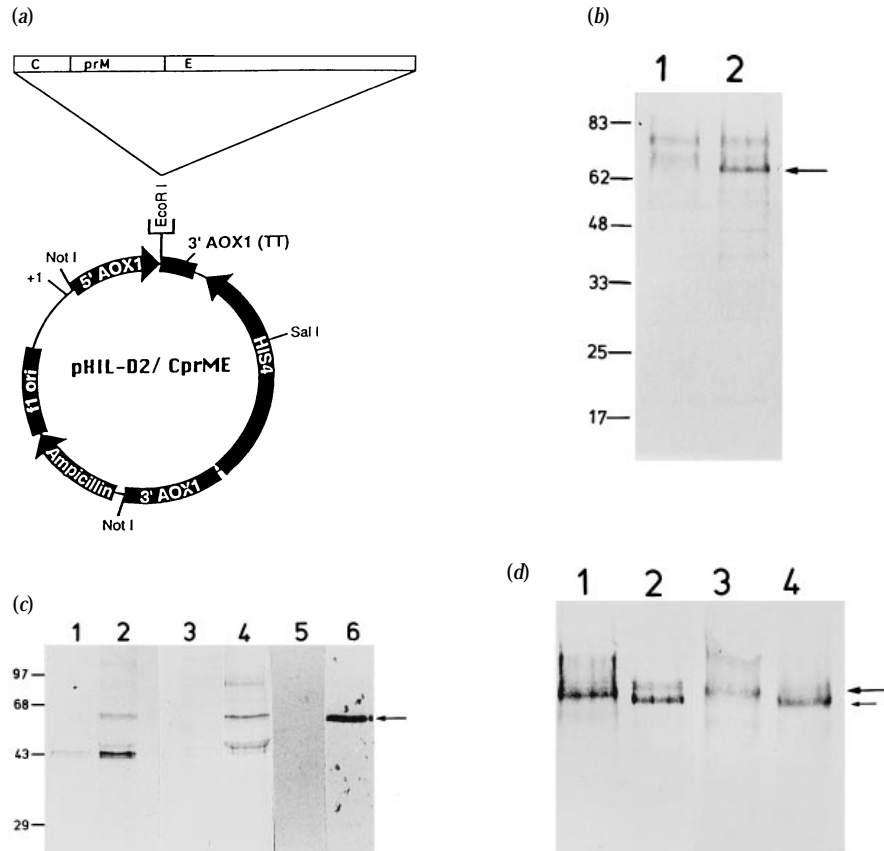


Fig. 1. Biochemical and antigenic analysis of recombinant E protein. (a) Construction of pHIL-D2/CprME. 5' AOX1, methanol oxidase promoter; HIS4, histidinol dehydrogenase. (b) Transformants GS115/D2 (lane 1) and GS115/CprME (lane 2) were harvested and the cells disrupted by vortexing with glass beads (Cregg *et al.*, 1987). The lysate was clarified by centrifugation at 11 000 *g* for 20 min and analysed by Western blotting using the polyclonal antibody RT951. Molecular masses are in kDa. (c) RIPA of [³⁵S]methionine-labelled E protein using RT951 (lanes 1–4) and the monoclonal antibody HAW#1 (lanes 5 and 6). Lane 1, mock-infected C6/36 mosquito cells; lane 2, DEN-1-infected C6/36 mosquito cells; lane 3, GS115/D2; lane 4, GS115/CprME; lane 5, GS115/D2; and lane 6, GS115/CprME. (d) Western blot analysis of glycosidase-treated recombinant E protein using RT951. The clarified supernatant from GS115/CprME was denatured at 100 °C for 10 min in 0.5% SDS, 1% β -mercaptoethanol. Portions of the lysate were made up in either 50 mM sodium phosphate, 1% NP40, pH 7.5 (lanes 1 and 2, respectively) and 50 mM sodium citrate, pH 5.5 (lanes 3 and 4, respectively) and incubated at 37 °C for 14 h with or without PNGase F (lanes 1 and 2, respectively) and Endo H (lanes 3 and 4, respectively). Lane 1, mock-treated; lane 2, 500 U PNGase F (New England Biolabs); lane 3, mock-treated; lane 4, 2000 U Endo H (New England Biolabs). Longer arrows, E protein; shorter arrow, deglycosylated E protein.

(MGY) (1.3% w/v, yeast nitrogen base; 1% v/v, glycerol; 0.4 μ g/ml biotin). Expression from the methanol oxidase promoter was induced by replacing MGY with minimal methanol medium (MM) (1.3% w/v, yeast nitrogen base; 0.5% v/v, methanol; 0.4 μ g/ml biotin) and continuing the incubation at 30 °C for a further 96 h. Expression of recombinant E protein was detected by Western blot analysis using a rabbit polyclonal antibody (RT951) which recognizes the E protein in DEN-1 (S275/90)-infected cells. Following methanol induction, the immunoblot revealed the presence of a specific 65 kDa antigenic band in GS115/CprME cultures, corresponding to the expected molecular mass for a correctly processed glycosylated E protein (Fig. 1*b*). In contrast, no recombinant DEN-1 E protein was detected in the culture medium despite repeated screening. Additionally, we compared

the recombinant E protein expressed in *P. pastoris* with the E protein in DEN-1-infected mosquito cells by radioimmuno-precipitation (RIPA) using RT951 and a mouse monoclonal antibody raised against the E protein of DEN-1 (Haw#1). Analysis of the immunoprecipitates by SDS-PAGE confirmed the identity of the specific radiolabelled 65 kDa species in GS115/CprME (Fig. 1*c*), which migrated identically to the E protein expressed in DEN-1-infected cells. Previous reports using the vaccinia virus expression system suggest that co-expression of the C protein together with prM and E has an inhibitory effect on E protein secretion (Konishi *et al.*, 1991; Pincus *et al.*, 1992; Fonsaca *et al.*, 1994), possibly as a result of incomplete processing of the capsid protein. We therefore constructed an additional recombinant vector, pHIL-S1/prME, which uses the yeast pho5 signal sequence rather than the prM

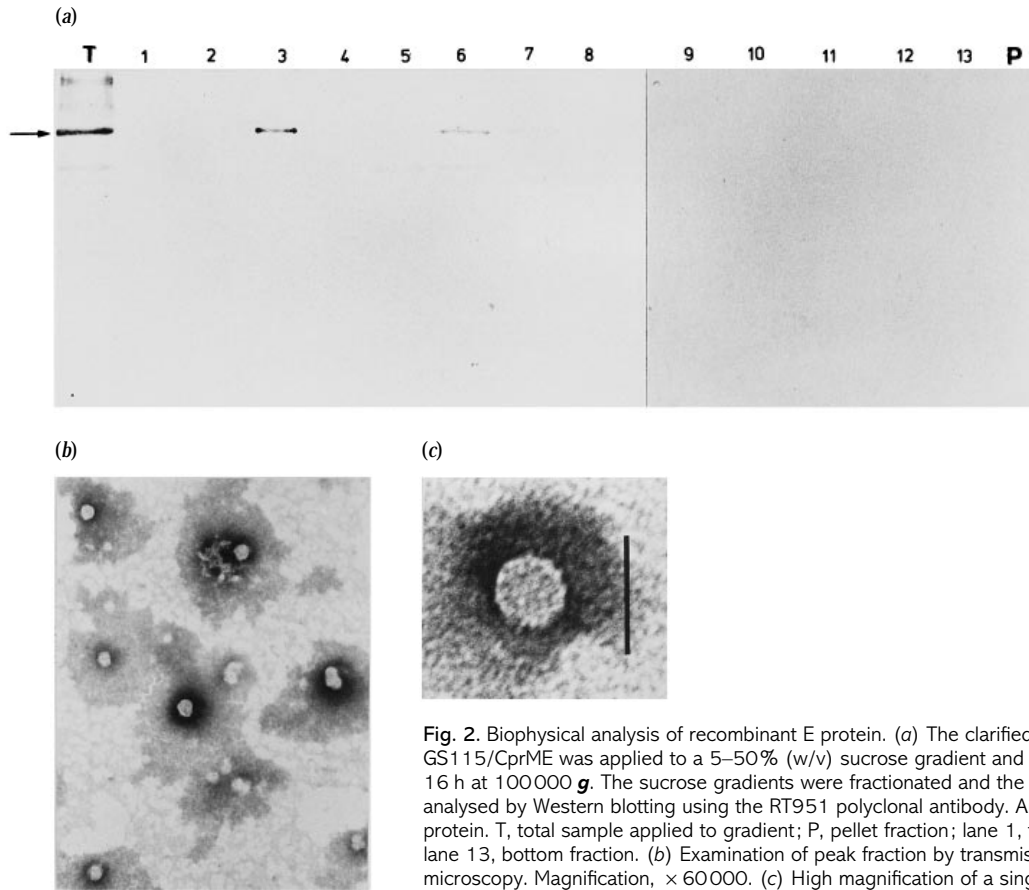


Fig. 2. Biophysical analysis of recombinant E protein. (a) The clarified lysate from GS115/CprME was applied to a 5–50% (w/v) sucrose gradient and centrifuged for 16 h at 100000 g. The sucrose gradients were fractionated and the individual fractions analysed by Western blotting using the RT951 polyclonal antibody. Arrow indicates E protein. T, total sample applied to gradient; P, pellet fraction; lane 1, top fraction; and lane 13, bottom fraction. (b) Examination of peak fraction by transmission electron microscopy. Magnification, $\times 60000$. (c) High magnification of a single VLP. Bar, 50 nm.

virus signal sequence that is present in pHIL-D2/CprME (Chambers *et al.*, 1990). The E protein expressed by cultures transformed with either pHIL-D2/CprME or pHIL-S1/prME was indistinguishable when analysed by Western blotting and endoglycosidase treatment (R. J. Sugrue, unpublished results), suggesting that co-expression of the capsid protein with the viral envelope proteins has no significant adverse effect on E protein processing in *P. pastoris*. Analysis of the culture medium by Western blotting indicated that this second transformant did not secrete recombinant E protein into the culture medium.

Although flaviviruses exhibit considerable heterogeneity in the pattern of E protein glycosylation, this produces no significant effect on antigenicity (Winkler *et al.*, 1987; Guirakhoo *et al.*, 1989). In contrast, several reports have suggested that some recombinant viral glycoproteins expressed in *P. pastoris*, e.g. HIV-1 gp120 (Scorer *et al.*, 1993), undergo hyperglycosylation, which has the potential to significantly alter both protein antigenicity and immunogenicity. The glycosylation state of the recombinant E protein was therefore analysed using two glycosidases, PNGase F and Endoglycosidase H (Endo H). PNGase F is able to remove the entire carbohydrate moiety from proteins modified by *N*-linked glycosylation, whereas Endo H specifically removes

high mannose chains. The recombinant E protein was sensitive to treatment with both glycosidases (Fig. 1*d*), indicating that the protein had been modified by the addition of small mannose chains via *N*-linked glycosylation without the addition of complex sugars. The size difference between the glycosylated and deglycosylated recombinant E protein was approximately 6 kDa, which is consistent with the modification of both potential glycosylation sites in the E protein, further demonstrating the absence of hyperglycosylation. Previous studies have demonstrated that the E protein in mature DEN-1 exhibits a mixed pattern of glycosylation (Fonsaca *et al.*, 1994), where only one of the *N*-linked glycans is modified by the addition of complex sugars. Our data suggest that *P. pastoris* is unable to modify this second glycosylation site, which is presumably a result of the different glyco-transferases present in yeast and animal cells.

We have previously observed that DEN-1 E protein expressed in *P. pastoris* undergoes extensive proteolytic degradation by an undefined yeast protease activity (R. J. Sugrue, J. L. Fu & Y. C. Chan, unpublished results). This is similar to earlier reports which showed that expression of the JEV E protein in *S. cerevisiae* underwent proteolytic degradation (Fujita *et al.*, 1987). However, we clearly show that co-expression of the E protein with the other DEN-1 structural

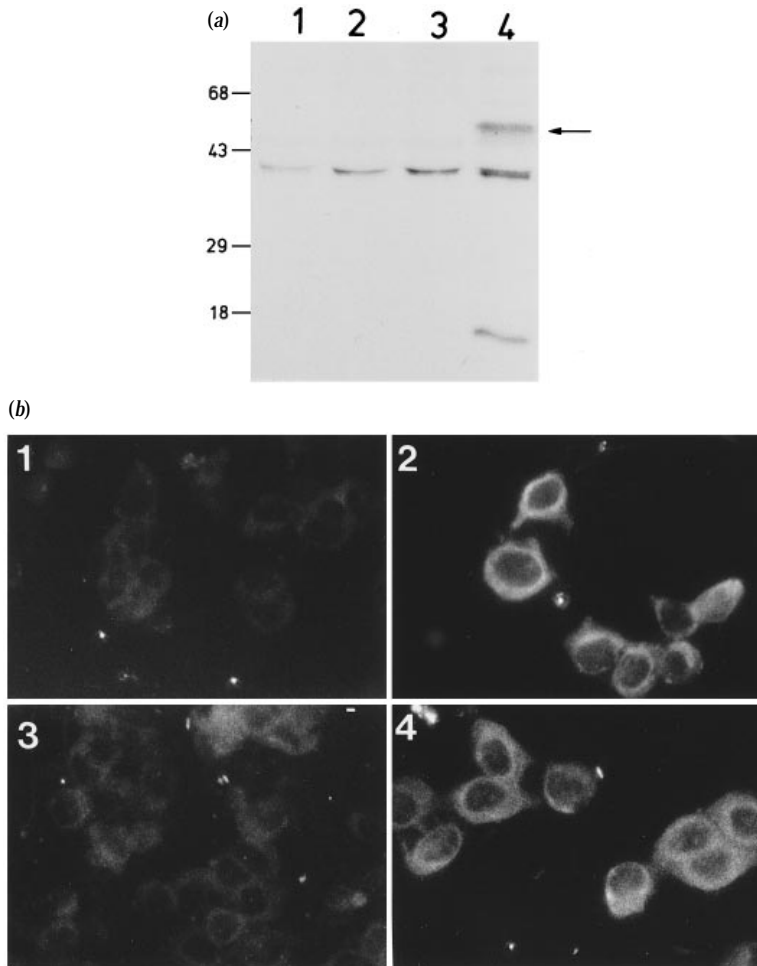


Fig. 3. Immunogenic analysis of VLPs. (a) RIPA analysis of [³⁵S]methionine-labelled DEN-1-infected Vero cells using antiserum RT1583. Detergent extracts prepared from mock-infected (lanes 1 and 3) and DEN-1-infected (lanes 2 and 4) vero cells were immunoprecipitated using preimmune serum RT1583 (lanes 1 and 2) and RT1583 (lanes 3 and 4). Arrow indicates E protein. Molecular masses are in kDa. (b) Immunofluorescence analysis of antiserum RT1583. DEN-1-infected Vero cell monolayers were fixed in acetone and incubated with preimmune serum RT951 (lane 1), RT951 (lane 2), preimmune serum RT1583 (lane 3) and RT1583 (lane 4) for 1 h at 37 °C, after which they were washed extensively with PBS. A fluorescein-conjugated donkey anti-rabbit IgG was added and incubation continued for a further 1 h. The monolayers were washed with PBS and cells examined using fluorescence microscopy.

proteins in *P. pastoris* is able to protect the E protein from this degradation process. Previous studies have shown that VLPs are generated when the flaviviral structural proteins are co-expressed in animal cell lines (Mason *et al.*, 1991; Konishi *et al.*, 1992; Pincus *et al.*, 1992). We wished to determine if similar VLPs were formed in *P. pastoris*, which could account for the resistance of the E protein to degradation. The yeast-expressed recombinant E protein was therefore analysed using a combination of 5–50% (w/v) sucrose density gradient centrifugation and transmission electron microscopy. The sucrose gradients were fractionated and the individual fractions assayed for the presence of E protein by Western blot analysis using antibody RT951 (Fig. 2*a*). The immunoblot showed that the peak fraction containing recombinant E protein migrated to a position in the gradient corresponding to a buoyant density of approximately 1.13 g/cm³ (Fig. 2*a*, fraction 3), which is similar to values obtained for flaviviral VLPs expressed in animal cell culture (Schalich *et al.*, 1996). This peak fraction accounted for > 90% of the antigenic recombinant E protein in the gradient. A faster migrating E protein fraction was also observed (Fig. 2*a*, fraction 6), which appeared to co-migrate with a 50 kDa antigenic protein. This suggests that an

association between these two antigenic species may be responsible for the increase in buoyant density. A sample of each fraction was fixed in glutaraldehyde and stained with uranyl acetate prior to analysis by transmission electron microscopy. We observed the presence of spherical particles with uniform morphology (Fig. 2*b*) and size distribution (approximately 30 nm) in the peak fraction (Fig. 2*c*). These particulate structures were observed only in gradient fractions showing positive staining for E protein by Western blot analysis and were not detected in cultures transformed with the parent vector, pHIL-D2. Additional studies using monoclonal antibody HAW#1 in immune-electron microscopy confirmed the presence of E protein in the VLPs (R. J. Sugrue, J. L. Fu, J. Howe & Y. C. Chan, unpublished results). Although the yeast-expressed VLPs were morphologically similar to infectious virus they were significantly smaller than infectious DEN-1 (average diameter 50 nm) (Murphy, 1980), which may reflect differences in the structural organization between the VLPs and infectious virus.

The failure to detect the DEN-1 E protein in the culture medium suggests that the VLPs expressed in *P. pastoris* were not secreted. Previous studies have shown that expression of

hepatitis B virus envelope proteins in *S. cerevisiae* produces 20 nm VLPs which accumulate in the cell (Kitano *et al.*, 1987; Kobayashi *et al.*, 1988; Kuroda *et al.*, 1992). This inability to secrete the VLPs expressed in yeast is presumably due to the complex nature of the yeast cell wall. It is therefore likely that the size of the DEN-1 VLPs (30 nm) is sufficient to prevent the secretion of VLPs by *P. pastoris*.

The purified VLPs obtained from the peak fraction were used to immunize two rabbits with six doses of approximately 25 µg per dose. The antiserum from each animal (RT1583 and RT1585) was assayed for the presence of DEN-1 antibodies by using both RIPA and immunofluorescence (IFA) assays. The results obtained with RT1583 are presented in Fig. 3(a, b). RT1583 was able to specifically immunoprecipitate a 65 kDa and an 18 kDa protein in detergent extracts prepared from DEN-1-infected cells (Fig. 3a). These are the expected sizes for the dengue virus E and prM proteins, respectively. We did not detect the C protein in these cell lysates, suggesting that the immune response is primarily directed against the E and prM proteins. Additional studies using IFA showed that RT1583 was able to recognize dengue virus structural proteins in whole cells infected with DEN-1, producing a strong cytoplasmic staining pattern (Fig. 3b). On the basis of our IFA data, we estimated that RT1583 and RT1585 had an antibody titre of 1:2560 and 1:625, respectively. We employed the plaque-reduction assay, using the DEN-1/S275/90 stain (25 p.f.u. per well), to estimate the level of neutralizing antibodies present in RT1583. We estimated that RT1583 had a neutralization titre of 1:10, which compares with a neutralization titre of < 1:5 for RT1585 and 1:40 for the control serum, RT951. These data collectively demonstrate the immunogenicity of the VLPs and show that they are able to induce virus-neutralizing activity.

In conclusion, we report here that expression of the DEN-1 structural genes (CprME) in *P. pastoris* leads to the generation of particulate structures that contain E protein and resemble dengue virions. The recombinant E protein is processed in an identical manner to that in virus-infected cells and is further modified by the addition of short mannose chains via N-linked glycosylation. These VLPs show immunogenicity and are able to elicit neutralizing antibodies. This expression system therefore offers an alternative method for generating particulate dengue virus E protein. We are currently using this system to express other constructs containing DEN-1 structural proteins.

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