Characterization and Isolation of Structural Polypeptides in Haemagglutinating Encephalomyelitis Virus

By P. E. CALLEBAUT AND M. B. PENSAERT

Laboratory of Virology, Faculty of Veterinary Medicine, University of Gent, Casinoplein 24, B-9000 Gent, Belgium

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SUMMARY

Haemagglutinating encephalomyelitis virus (HEV), a member of the coronavirus family, was purified and analysed by SDS–polyacrylamide gel electrophoresis. It was shown to contain eight polypeptides, seven of which were glycosylated. They had apparent mol. wt. of 180000 (GP 180), 130000 (GP 130), 120000 (GP 120) 76000 (GP 76), 64000 (VP 64), 54000 (GP 54), 32000 (GP 32) and 31000 (GP 31). Electrophoresis of virus samples dissociated under varying conditions showed that GP 54 and GP 120 could be interpreted as larger products of GP 31 and GP 32 and of GP 76, respectively. GP 76 also appeared as a dimer with a mol. wt. of 140000 (GP 140) in the absence of β-mercaptoethanol. Subviral particles, obtained by treatment with bromelain, banded at a slightly lower density than the intact virus and lacked surface projections. Analysis of these particles indicated that GP 180, GP 130 and GP 76 are associated with the virus projections. A small part of GP 31 and GP 32 also appeared to protrude from the lipid envelope, since 20% of each molecule was sensitive to digestion. Two glycoproteins, GP 130 and GP 76, were solubilized with the detergent Triton X-100 and separated by rate zonal centrifugation. According to its activity in indirect haemagglutination tests, GP 76 was considered to be a monovalent haemagglutinin subunit.

INTRODUCTION

Haemagglutinating encephalomyelitis virus (HEV) is a porcine coronavirus that causes vomiting and wasting disease and/or motor disturbances in suckling piglets (Andries et al. 1978). Members of the coronavirus family are grouped mainly on the basis of their similar electron microscopic appearance (Tyrrell et al. 1975). However, little information is available on the chemical composition of most of these viruses.

Reports on the structural proteins of coronaviruses agree on the presence of an inner core protein with a mol. wt. of approx. 50000, a polypeptide of approx. mol. wt. 30000 associated with the inner region of the envelope and a glycopeptide of approx. mol. wt. 100000 associated with the virus projections. However, all coronavirus species appear to contain a highly variable number of additional polypeptides, the total number ranging from four in mouse hepatitis virus (MHV; Sturman, 1977) to 16 in avian infectious bronchitis virus (IBV; Bingham, 1975). This makes it very difficult to compare the different coronavirus species with regard to their structural proteins. Many of these apparent variations in chemical composition may be due to different methods used for virus cultivation and purification or different procedures used for protein-analysis. Artificial by-products appeared in IBV and MHV polypeptide preparations even under the current conditions of analysis (MacNaughton & Madge, 1977; Sturman, 1977).
It was the purpose of the present study to examine the polypeptide composition of the porcine coronavirus, HEV, by means of a high resolution polyacrylamide gel electrophoresis method. The effect of varying preparative conditions on the electrophoretic migration of the polypeptides was examined. This paper also reports the isolation of individual glycoproteins associated with the surface projections, using a non-ionic detergent treatment.

**METHODS**

**Virus cultivation.** The Belgian HEV isolate, designated VW 572 and described earlier (Pensaert & Callebaut, 1974) was used as the 14th and 15th passages in primary pig kidney (PPK) cells. These were the second and third passages following cloning of the virus through three successive plaque passages.

The virus was cultivated in PPK cells as described previously (Pensaert & Callebaut, 1974). Cells were grown in Bellco 835 cm² roller bottles, rolled at 12 rotations/h. Confluent monolayers were pre-treated with Eagle’s minimum essential medium (Earle’s salts; MEM) containing 50 µg/ml DEAE-Dextran (Pharmacia, Sweden) for 1 h at 37 °C and inoculated at a m.o.i. of approx. 0.1 TCID₅₀/cell. After 1 h at 37 °C, 50 ml of maintenance medium was added, consisting of MEM, 2% foetal bovine serum, antibiotics and 0.2 M-HEPES, pH 7.5. After 48 h at 37 °C, the medium was harvested and clarified at 2000 g.

**Purification of HEV.** Polyethylene glycol 6000 was added to a concentration of 5% (w/v) and the suspension was stirred for 2 h at 0 °C. The precipitated virus was collected by centrifugation at 3000 g for 20 min and suspended in 0.005 M-phosphate buffer, pH 7.0, to give a 100-fold concentration. This material was centrifuged through linear 10 to 40% Urografin (Schering) density gradients at 76,400 g in a Beckman SW 41 Ti rotor for 70 min (Gschwender et al. 1975). The virus was further purified by batch chromatography on hydroxylapatite (Bio Rad Lab., Richmond, Calif., U.S.A.; Bernardi, 1971). Virus from the gradients was mixed with the packed gel in the proportions of about 30000 haemagglutinating units of virus per ml of gel and adsorbed for 45 min at 0 °C. Contaminating material was removed by washing the gel three times with 0.005 M-phosphate. The virus was eluted by incubation with 0.15 M-phosphate buffer, pH 7.0, for 30 min at 0 °C. Virus was concentrated by sedimentation at 30000 g in a Beckman Type 42.1 rotor for 2 h.

**Electron microscopy.** A drop of virus was placed on a Formvar coated grid, excess liquid was removed by blotting and a drop of 2% potassium phosphotungstate, pH 6.1, applied for negative staining. Specimens were examined in a Zeiss EM 95-2 microscope at an instrumental magnification of 28000 and an acceleration voltage of 60 kV.

**Bromelain treatment of HEV.** Purified virus was treated with 1.3 mg/ml of bromelain (Sigma Chemical Co., St Louis, Mo., U.S.A.) for 1 h at 37 °C as described by Compans et al. (1970). Control preparations consisted of virus incubated at 37 °C without enzyme. The virus suspensions were re-purified on linear 25 to 50% (w/v) sucrose gradients at 76,400 g for 12 h. Gradient fractions were measured for protein by absorbance at 280 nm using a Vitatron MPS photometer. Solution density was calculated from the refractive index. The peak fractions were pelleted at 30900 g for 2 h.

**Dissociation of virus protein.** Standard dissociation conditions were essentially as described by Maizel (1971). Protein samples were solubilized in 0.060 M-tris-phosphate, pH 6.7, containing 1% SDS, 0.1% β-mercaptoethanol (β-ME) and 0.005% bromophenol blue. The mixtures were heated at 100 °C for 1 to 2 min and after cooling were applied directly to gels. When indicated in the text, SDS-protein-mixtures were left unreduced by omission of β-ME. Reductive alkylation of protein samples with iodoacetic acid was performed by the method of Maizel (1971). Non-reduced proteins were alkylated according to Shapiro (1967).

**Polyacrylamide gel electrophoresis (PAGE).** The high pH discontinuous SDS-poly-
acrylamide system described by Maizel (1971) was used. The resolving gels, approx. 9 cm long and 6 mm in diam., contained 10% polyacrylamide, pH 8.8. The 3% spacer gels, pH 6.8, were 1.5 cm long. Electrophoresis was carried out at 75 V until the tracking dye had migrated to 1 cm from the bottom of the gel (about 4 h). For protein staining, gels were fixed and stained with 0.2% Coomassie brilliant blue R250 and 7% glacial acetic acid in methanol:water (50:50, v/v). Destaining was accomplished by incubating the gels in 7% acetic acid-25% methanol for 3 h, and further in 7% acetic acid-5% methanol. For lipid staining, gels were immersed in freshly prepared saturated Sudan Black B and 0.05% (w/v) sodium hydroxide in 60% ethanol for 2 h, destained with 50% ethanol for 2 h and rehydrated in distilled water (Uriel et al. 1964). Carbohydrate staining was by the method described by Zacharius et al. (1969). Gels were scanned at 1 cm/min at 530 nm. The relative amount of stain bound by the different polypeptides was estimated by integration of the peaks in the staining profile. The apparent mol. wt. of polypeptides was determined according to Weber & Osborne (1969). Bovine haemoglobin, porcine pancreatic trypsin, porcine gastric pepsin and bovine serum albumin, monomer and dimer (Sigma Chemical Co.), were used as mol. wt. markers.

Triton X-100 treatment of HEV. Purified virus was treated with Triton X-100 following in general the method described by Scheid et al. (1972). Triton X-100 was added to a final concentration of 2% (v/v) in 0.02 M-tris-HCl buffer, pH 7.2, plus 0.001 M-EDTA (TE buffer). The mixture was gently sonicated twice for 10 s (MSE 150 Watt Ultrasonic disintegrator) and allowed to stand for 30 min at room temperature. The turbid solution was centrifuged at 110000 g for 1 h to remove any material which was not dissolved. The clear supernatant fluid was collected and layered on to a continuous gradient of 5 to 25% sucrose, which contained 2% Triton X-100 and TE buffer. Following centrifugation at 205000 g for 14 h, samples of each gradient fraction were assayed by the protein determination method of Lowry et al. (1951) with 1% SDS in the reaction mixture (Helenius & Simons, 1972). Gradient fractions containing protein were dialysed overnight against TE-buffer. The proteins were precipitated with cold n-butanol, then 0.060 M-tris-phosphate, pH 6.7, or phosphate buffered saline (PBS) was added and the precipitate was dissolved by sonic treatment twice for 10 s at 0 °C.

Haemagglutination tests. Haemagglutination (HA) titrations were performed in round-bottomed microtitre trays. Fifty μl of serial twofold dilutions of virus in PBS diluent were mixed with an equal vol. of 0.5% chicken erythrocytes. Tests were read after 1 h at room temperature.

Indirect HA (IHA) tests were also done in the microtitre system. Twenty-five μl volumes of test material were mixed with 25 μl 1% chicken erythrocytes and incubated for 2 h at room temperature. One HA unit of purified virus in 50 μl was added and, following re-incubation for 1 h at room temperature, tests were observed for inhibition of HA.

RESULTS

Analysis of HEV polypeptides

The polypeptides of purified virus, separated on SDS–polyacrylamide gels, were stained for protein, carbohydrate or lipid. The electropherograms are shown in Fig. 1 and revealed a minimum of eight polypeptides. Seven of them proved to be glycopolypeptides (GP) but none contained lipid. The smallest polypeptides, with apparent mol. wt. of 31000 and 32000 (GP 31 and GP 32), accounted for approx. 27% of the total amount of protein-bound stain. GP 31 was consistently found as a shoulder on the leading edge of GP 32. Efforts to improve the resolution by increasing the gel length or changing the gel concentration (7.5% and 13%, polyacrylamide) were not successful. A glycopolypeptide with an apparent mol. wt.
of 54,000 (GP 54) comprised about 11% of the total protein. A non-glycosylated polypeptide with a mol. wt. of 64,000 (VP 64) was found in the largest amount, i.e. 37%. The largest polypeptides had apparent mol. wt. of 76,000 (GP 76), 120,000 (GP 120), 130,000 (GP 130) and 180,000 (GP 180) and comprised 4, 4, 13 and 4% of the total protein, respectively. These data, together with the estimations of the relative number of polypeptide copies per virion, are listed in Table 1.

Together with the bromophenol blue marker, a faint peak of proteinaceous material was found. However, in gels with increased polyacrylamide concentration this band disappeared and no additional peaks were resolved, indicating that this material was very heterogeneous. A zone migrating in front of the dye marker behaved like glycolipid, as it was stained both by periodic acid-Schiff reagent and Sudan Black B.

**Susceptibility to protease treatment**

Treatment of intact virus with bromelain was used as an indirect method to identify the proteins located on the surface of the virus envelope. Purified enzyme-treated virus banded in the 1.18 g/ml region of a sucrose density gradient. Electron microscopy revealed that this band contained spikeless but fully enveloped particles which were aggregated in large clumps (Fig. 2). They also lacked haemagglutinating activity. The non-treated control virus had a buoyant density of 1.19 g/ml in sucrose and retained its intact morphology as well as its haemagglutinating activity.
Analysis of HEV structural polypeptides

Fig. 2. Electron micrographs of purified HEV, prepared by negative staining. (a) Untreated control; (b) treated with bromelain, 1.3 mg/ml for 1 h at 37 °C.

Table 1. The molecular weights of the polypeptides and their relative contribution to the total protein content of intact and bromelain-digested HEV, strain VW 572

<table>
<thead>
<tr>
<th>Mol. wt x 10^-3</th>
<th>Percentage of total stain bound*</th>
<th>Relative number of polypeptide copies per virion†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact virus</td>
<td>Bromelain treated virus</td>
</tr>
<tr>
<td>180</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>130</td>
<td>—</td>
<td>13</td>
</tr>
<tr>
<td>120</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>76</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>64</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td>54</td>
<td>—</td>
<td>11</td>
</tr>
<tr>
<td>31/32</td>
<td>—</td>
<td>27</td>
</tr>
<tr>
<td>25</td>
<td>—</td>
<td>35</td>
</tr>
</tbody>
</table>

* The amount of stain bound per polypeptide was determined by integration of the peaks in the staining profile on 10% polyacrylamide gels.
† The relative number of polypeptide copies per virion was computed from the ratio between the percentage of stain bound by the polypeptide and its mol. wt. The values were normalized to 1 for GP 180 in intact virus and to 31 for VP 64 in bromelain digested virus.

The results of the SDS–PAGE analysis are presented in Fig. 3 and Table 1 and showed that only VP 64 was unaffected by bromelain; it bound about 52% of the total stain. All original glycoproteins were lost and two new polypeptide species were found with mol. wt. of 25000 (p' 25) and 46000 (p' 46). They comprised 35% and 13% of the total stain, respectively. The amount of VP 64 was considered to be unchanged upon bromelain treatment and was used as an internal standard to calculate the relative numbers of polypeptide molecules per particle as presented in Table 1. None of the polypeptides was stained by periodic acid-Schiff reagent.
Fig. 3. SDS-PAGE of polypeptides of purified HEV digested with bromelain as described. The sample was dissociated as indicated in the legend to Fig. 1. Migration is from left to right and the arrow indicates the position of the bromophenol blue marker.

Table 2. Effects of varying dissociation conditions on the percentage* of stain bound per virion polypeptide of HEV, strain VW 572, analysed by SDS-PAGE

<table>
<thead>
<tr>
<th>Dissociation treatment</th>
<th>Polypeptide species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GP 180</td>
</tr>
<tr>
<td>2 min at 100 °C, 0.1% β-ME</td>
<td>4</td>
</tr>
<tr>
<td>15 min at 100 °C, 0.1% β-ME</td>
<td>3</td>
</tr>
<tr>
<td>30 min at 22 °C, 0.1% β-ME</td>
<td>3</td>
</tr>
<tr>
<td>Non-reductive alkylation</td>
<td>2</td>
</tr>
<tr>
<td>Reductive alkylation</td>
<td>2</td>
</tr>
</tbody>
</table>

* The percentage of stain bound per polypeptide was determined by integration of the peaks in the staining profile on 10% polyacrylamide gels.

Effects of dissociation conditions on HEV polypeptides

As aggregates or degradation products of polypeptides may be formed during sample treatment before electrophoresis, the effect of varying dissociation on the PAGE profile and on the percentage composition of HEV preparations was examined. The results are shown in Fig. 4 and Table 2 respectively. In virus samples alkylated without reducing agent, only six polypeptide species were found: GP 31, GP 32, VP 64, GP 130, GP 180 and GP 140 (a new glycopolypeptide species with an apparent mol. wt. of 140000). Similar
Fig. 4. SDS–PAGE of polypeptides of purified HEV treated under different conditions following solubilization in 1% SDS: alkylated with iodoacetamide in (a) the absence and (b) the presence of β-mercaptoethanol; (c) incubated at 22 °C for 30 min in the presence of 0.1% β-mercaptoethanol; (d) boiled for 15 min with β-mercaptoethanol. Migration is from left to right; the arrows show the position of the bromophenol blue marker.
patterns were obtained when samples were boiled in the absence of β-ME. Reductive alkylation of virus proteins resulted in the disappearance of GP 140, which appeared to be replaced by GP 120 and GP 76. Solubilized virus, kept at 22 °C for 30 min in the presence of 0.1 % β-ME, had also lost GP 140 but contained an enlarged GP 130 peak; apparently GP 130 co-migrated with a slightly faster migrating form of GP 140. When SDS–protein samples were boiled for 2 min with 0.1 % β-ME, eight polypeptide species were resolved (Fig. 1) due to the further appearance of GP 54, accompanied by a concomitant decrease in the amount of GP 31/32. This decreasing effect was directly related to the duration for which the sample was boiled, as shown by boiling for 15 min; this severe treatment also produced a diffuse increase of staining, retained in the upper third of the gel.

**Isolation of two surface glycoproteins**

In an initial attempt to find out which biological activities are associated with the different virus proteins, the virus was fractionated and virus proteins were isolated in their active form. This was accomplished by disrupting the virus with the non-ionic detergent Triton X-100, as described in Methods. Rate zonal centrifugation of the solubilized material in sucrose gradients containing Triton X-100 produced two peaks of proteinaceous material (Fig. 5). A broad zone was also found at the top of the gradient which was recorded as protein. However, since no peaks appeared with the latter when subjected to PAGE analysis, this may have been caused by the presence of substances interfering with the protein determination method used.

By standard HA tests no HA-activity was detectable across the gradient. However, to detect monovalent haemagglutinin subunits, IHA tests were carried out with samples of each gradient fraction. By this procedure the slower sedimenting peak I component was shown to have IHA-activity. After removal of Triton X-100 by butanol treatment the HA-activity was lost, but the addition of Triton X-100 restored it. No IHA-activity could be demonstrated in peak II.

PAGE analyses of the polypeptide composition of peak I and peak II material from the gradient are shown in Fig. 6. When a sample of peak I protein was applied in the non-
Analysis of HEV structural polypeptides

Fig. 6. PAGE of polypeptides obtained by velocity sedimentation of the HEV proteins solubilized by Triton X-100. Fractions comprising peaks I and II from the gradient shown in Fig. 5 were pooled, dialysed and precipitated as described. Peak I protein was solubilized in 1% SDS and boiled for 1 min in (a) the absence and (b) the presence of β-mercaptoethanol. (c) Peak II protein was boiled for 1 min in the presence of 1% SDS without β-mercaptoethanol. Migration was from left to right; the location of the bromophenol blue marker is shown by the arrows.

reduced form, whether alkylated with iodoacetic acid or not, it migrated as a single major polypeptide corresponding to GP 140. If the protein was reduced before analysis and either alkylated or not, two major components were found which were considered to be identical with GP 120 and GP 76 found in complete virus preparations under the same conditions. Each of them comprised about 50% of the total amount of stain bound. Samples of peak II protein, analysed under identical conditions, were always found to migrate as a single major polypeptide corresponding to GP 130. A minor component with a mol. wt. of approx. 180,000 was present in peak I and peak II preparations under all test conditions. The amount of this was greatly decreased in reduced samples, suggesting that it was an artefact, presumably an aggregate.
DISCUSSION

PAGE analysis of HEV strain VW 572 reveals eight polypeptide species under standard conditions. The results show that some of these species are different forms of the same molecules, so that purified particles of HEV appear to contain a minimum of six polypeptide species: GP 31, GP 32, VP 64, GP 76, GP 130 and GP 180.

The non-glycosylated polypeptide, VP 64, is completely unaffected by bromelain digestion of the virus. This indicates that VP 64 is located entirely within the virion and probably represents a core protein.

The glycopeptides GP 31 and GP 32 were found to behave remarkably similarly under the various treatments described and are therefore treated as a doublet, designated GP 31/32. It was observed that the amount of GP 31/32 is decreased upon boiling in the presence of β-ME and that GP 54 together with some large heterogeneous material appears. This suggests that GP 31/32 forms larger products, possibly aggregates, depending on the conditions of sample treatment.

A similar observation has been made with the smallest glycoprotein E1 of MHV strain A59 on boiling in SDS (Sturman, 1977). It may be that coronavirus polypeptides give rise to artificial products by hydrophobic interactions under certain conditions of sample preparation. This is also suggested by the finding that severe dissociation treatments of IBV preparations resulted in an increase of the number of peaks in the electropherogram from 7 to 12 (MacNaughton & Madge, 1977) and perhaps even to 14 (Collins et al. 1976) or 16 (Bingham, 1975).

The relative number of copies of polypeptide p' 25, which appeared after bromelain treatment of HEV, is very close to the number of GP 31/32 copies in the intact virus preparations, analysed under identical conditions. This indicates that p' 25 is derived from GP 31/32 as a result of the digestion of about 20% of the original molecule by bromelain. Based on similar observations, the p' 46 polypeptide in bromelain-treated virus preparations appears to be derived from GP 54, which was shown to be a larger form of GP 31/32; p' 46 therefore appears to be an aggregate of p' 25. The present results suggest that 20% of the GP 31/32 molecule represents an external part and the remainder (p' 25) is a large internal part. The latter appears to be protected against protease activity by the virus envelope. Since the bromelain-resistant part is a homogeneous non-glycosylated polypeptide, the appearance of GP 31/32 as a doublet is likely to be related to the presence of the external, carbohydrate containing region of the molecule. The fact that the inner part of the molecule still gives rise to a larger product indicates that it is of a hydrophobic nature.

The finding that bromelain treatment causes a loss of a 20% portion of the GP 31/32 molecule is very similar to that reported on the E1 polypeptide of MHV strain A59 (Sturman, 1977). On the other hand, it is known that the membrane-associated glycopolypeptides of TGEV and the human coronaviruses OC 43 and 229 E are not measurably digested by bromelain (Hierholzer et al. 1972; Garwes & Pocock, 1975; Hierholzer, 1976). This suggests that the arrangement of glycoprotein in the envelope of HEV is similar to that of MHV, but different from that in the other species mentioned. Although the members of the coronavirus family have a rather uniform morphology, this characteristic may prove to have implications with relation to the outer structure of different coronavirus species.

The HEV glycoproteins GP 76, GP 130 and GP 180 are completely removed on bromelain treatment and therefore appear to be associated with the projections on the surface of the virus envelope.

In complete virus preparations, GP 140 molecules are partly converted into GP 76 by boiling with β-ME, indicating that GP 76 molecules are covalently linked by disulphide bonds, giving rise to a GP 140 dimer in the absence of reducing agent. The remaining part
of the GP 140 molecules is converted into GP 120 on reduction; GP 120 may represent an aggregation product of the GP 76 monomer molecules, formed either by hydrophobicity or by other interactions. In agreement with this interpretation is the finding that the isolated GP 76, the peak I protein obtained by the Triton X-100 procedure, forms a distinct larger product both in the absence and presence of reducing agent. However, in order to establish a more definite relationship between these polypeptides, peptide mapping should be performed. The results of our IHA tests with GP 76 strongly suggest that this polypeptide specifically adsorbs on to virus receptors on red cells and is responsible for the haemagglutinating activity of complete virions. As it loses its HA-activity upon removal of detergent, this protein probably has a great tendency to aggregate.

The second protein isolated by Triton X-100 treatment and present in peak II, GP 130, is stable under all conditions tested, indicating that it is not a product of GP 76. It is therefore considered as a separate spike-component, although its function remains obscure.

The present results on the structural polypeptides of the VW 572 strain of HEV generally confirm the observations made by Pocock & Garwes (1977) on the serologically closely related, if not identical, FS 255 strain (Pensaert & Callebaut, 1974). The mol. wt. estimations were slightly lower than those described in the present report, but the differences do not appear to be significant. However, some findings are different. A homogeneous glycopeptide GP 25 was detected in the FS 255 strain. This polypeptide probably corresponds to the membrane-associated glycopeptides GP 31 and GP 32 of the VW 572 strain. Since GP 31 and GP 32 may vary only in carbohydrate content, this difference is not necessarily a reflection of a variation in genomic complexity between these strains. However, it may represent a difference in the membrane structure of both strains. The organization of the VW 572 membrane may resemble more closely that of TGEV and MHV strain JHM, since their membranes were shown to contain two polypeptides (Garwes & Pocock, 1975; Wege et al. 1979).

A further difference is related to the virus haemagglutinin. Based on virus treatment with dithiothreitol, Pocock (1978) concluded that the haemagglutinating activity of the FS 255 strain was associated with a glycopolypeptide having a mol. wt. of 125000 (GP 125). GP 125 is therefore probably analogous to GP 140 of the VW 572 strain. However, unlike the labile GP 140 dimer, GP 125 contains no interpeptide disulphide bonds since it was found to be stable even under highly reducing conditions and since a polypeptide comparable to the GP 76 monomer of the VW 572 strain was not detected.

From the present results it cannot be concluded whether these differences are due to strain variations or to methods for virus culturing and analysis.

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