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Coronavirus IBV glycopolypeptides: locational studies using proteases and saponin, a membrane permeabilizer

David Cavanagh¹, Philip J. Davis¹ and Darryl J.C. Pappin²

¹ Houghton Poultry Research Station, Houghton, Huntingdon, Cambs, PE17 2DA, and

² Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

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Summary

[³⁵S]methionine-labelled avian infectious bronchitis virus (IBV) (strain 41) and its purified protein components and virions of IBV-Beaudette were incubated with 10 proteases. Several proteases hydrolysed all or some of the membrane glycopolypeptide (M; M_r 30K) and removed about 1.3K of peptide from the amino-(N)-terminus plus both glycans, as determined by SDS-polyacrylamide gel electrophoresis. N-terminal analysis of [³H]isoleucine-labelled M after hydrolysis by bromelain revealed that the first nine residues had been removed. After the virions had been permeabilised with saponin, a further 2.5K decrease in molecular weight was produced and this was shown to be from the carboxy-(C)-terminus. When considered with the hydrophobicity plot analysis of the amino acid sequence of M (Boursnell, M.E.G. et al., 1984, *Virus Res.* 1, 303–313) these results suggest that as few as 9–20 N-terminal amino acid residues may protrude at the outer membrane surface and that there is a highly protease sensitive sequence of an estimated 20–25 residues at the C-terminus of M exposed in the lumen of the virion. S2 but not S1 was cleaved to a major glycopolypeptide of approximately 71K by several proteases, and to 76K by trypsin. N-terminal sequencing of the 71K glycopolypeptide revealed that it had the same N-terminus as intact S2. After hydrolysis in the presence and absence of saponin it was concluded that S2 is very sensitive to hydrolysis near its carboxy terminus at residues close to the outer membrane surface.

coronavirus IBV, glycopolypeptides, proteases, saponin

Introduction

Avian infectious bronchitis coronavirus (IBV) has three protein structural elements; an internal nucleocapsid (N) protein and two envelope-associated proteins, the membrane (M) and spike (S) glycoproteins. Preliminary studies using the protease bromelain indicated that M protruded only a little at the outer virion surface while most of S was exposed (Cavanagh, 1981). S comprises two glycopolypeptides, S1 and S2 (Cavanagh, 1983b), derived from a precursor glycopolypeptide (Stern and Sefton, 1982a). One function of S2 is to anchor S in the membrane (Cavanagh, 1983c).

Studies with another coronavirus, murine hepatitis virus (MHV), have indicated that part of M protrudes at the inner membrane surface where it probably interacts with the ribonucleoprotein (Sturman et al., 1980; Rottier et al., 1984).

The purpose of the present study was to determine if proteases could be used to elucidate further the location of the M and S glycopolypeptides of IBV. To enable proteases to enter the virions the virus membrane was permeabilized using the nonionic detergent saponin (Weigel et al., 1983).

Methods

Virus preparation and analysis

Virus was radiolabelled with [^3H]isoleucine or with [^{35}S]methionine with or without D-[6- ^3H]glucosamine hydrochloride (Amersham International) in de-embryonated eggs (IBV strains M41 and Beaudette; Cavanagh 1981, 1983a) or in chick kidney cells (IBV-Beaudette only; Stern et al., 1982) and purified in sucrose gradients containing 100 $\mu\text{g}/\text{ml}$ of bovine serum albumin (BSA). [^{35}S]methionine-labelled S, N and M proteins of IBV-M41 were prepared using nonionic detergent and sucrose gradient centrifugation (Cavanagh, 1983b). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide slab gels (Laemmli, 1970) and fluorographs were prepared with sodium salicylate (Chamberlain, 1979) using Kodak XAR or Fujimex RX X-ray film, or in tube gels which were processed for scintillation counting (Cavanagh, 1981).

Proteolysis of IBV proteins

Proteases and inhibitors were obtained from Sigma unless stated otherwise. Each enzyme was used at several concentrations and for several 37°C incubation periods; the highest concentrations used are shown in parentheses: papain (type IV; 0.2 mg/ml); pronase E (type XIV, from *Streptomyces griseus*; 1 mg/ml); subtilisin (type VIII, from *Bacillus subtilis carlsbergensis*; 1 mg/ml); proteinase K (type XI, from *Tritirachium album*; 1 mg/ml); trypsin (type XI, treated with diphenyl carbamyl chloride; from bovine pancreas; 20 mg/ml); *Staphylococcus aureus* V8 protease (Miles; used with 5 mM calcium acetate and 5 mM magnesium acetate; 0.9 mg/ml); chymotrypsin (type VII, treated with tosyl-lysine chloromethyl ketone (TLCK) from bovine pancreas; 1 mg/ml); thermolysin (type X, from *Bacillus thermoproteolyticus*

rokko; 2 mg/ml); elastase (type I, from porcine pancreas; 2 mg/ml); bromelain (Calbiochem; 1 mg/ml). Protease inhibitors used were: leupeptin (synthetic); TLCK; α_2 -macroglobulin (Boehringer); and phenylmethylsulfonyl fluoride (PMSF), dissolved in propan-2-ol.

Virus or viral proteins direct from sucrose gradients in NET buffer (100 mM NaCl/1 mM EDTA/10 mM Tris-HCl, pH 7.4) containing 100 μ g/ml of BSA were incubated at 37°C with or without 0.1% saponin (Sigma) with proteases at the concentrations and for the times reported in the Results section. Protease activity was stopped by the following procedure. PMSF was added to 2.5 mM followed by leupeptin and α_2 -macroglobulin, each at 0.75 mg/ml. TLCK at 1 mg/ml was used to inhibit trypsin. After 10 min at room temperature the samples (volume about 50 μ l) were held in a boiling waterbath for 2 min. SDS and 2-mercaptoethanol were added to 2% each and the samples heated at 100°C for a further 2 min. Samples were stored at -20°C prior to analysis by SDS-PAGE.

Amino-terminal sequencing

[³H]Isoleucine-labelled IBV-M41 was incubated with 50 μ g/ml of bromelain for 30 min at 37°C with 0.1% saponin. After separation of the polypeptides by SDS-PAGE the products of the hydrolysis of S2 and M were electroeluted (Binns et al., 1985) and the location of [³H]isoleucine residues determined by automated solid-phase Edman degradation (Cavanagh et al., 1986).

Results

Effect of proteases on the M protein

With the exception of elastase and thermolysin all proteases were used with purified S, N and M proteins, with and without saponin, in addition to intact and saponin-treated virions. This was partly to help distinguish between cleavage products of N and M after hydrolysis of virions and is illustrated only for bromelain (Fig. 1, a-i).

The polypeptide moiety of M of IBV has an apparent M_r in SDS-PAGE of 23K and is heterogeneously glycosylated, the major glycosylated species being approx. 30K (Stern and Sefton, 1982b; Cavanagh, 1983b). Chymotrypsin, elastase, thermolysin, trypsin and V8 protease had no observed effect on M when added to virions of IBV-M41. IBV-Beaudette was also exposed to trypsin, chymotrypsin and protease V8 but without any effect on M.

Bromelain cleaved M to a polypeptide of slightly lower M_r than the small amount of unglycosylated M (23K) which is present in virions (Fig. 1, a,b). In most experiments almost all of M was hydrolyzed to this product. A polypeptide of indistinguishable M_r was generated by subtilisin (Fig. 1,k), pronase E and proteinase K (not shown). However, only a small amount of M was hydrolyzed by these enzymes even after several hours of incubation. The mean molecular weight, with standard deviation, of this polypeptide from 18 experiments was 21.7K \pm 0.3K.

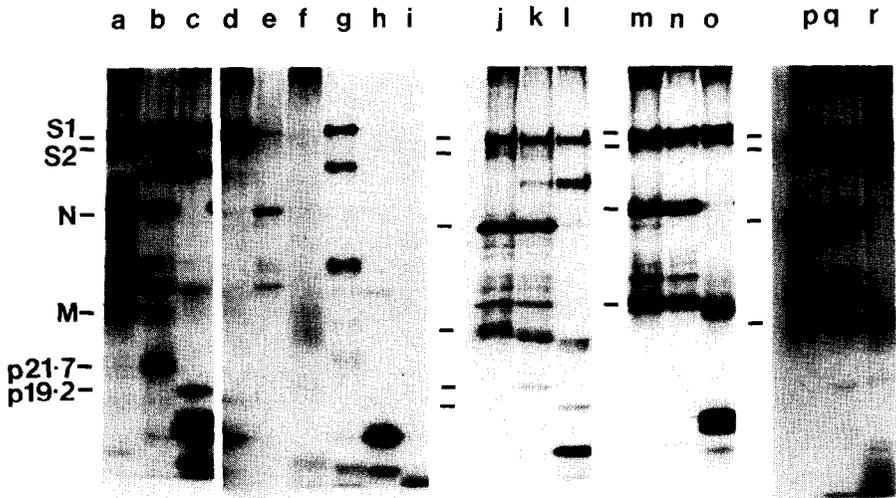


Fig. 1. SDS-PAGE of [³⁵S]methionine-labelled IBV-M41 and purified proteins after incubation with proteases at 37°C for 30 min in the presence and absence of 0.1% saponin: (a-c) virus plus (a) no enzyme, (b) 1 mg/ml bromelain and (c) bromelain plus saponin; (d,e,f) S, N and M alone, respectively, and (g,h,i) with 0.2 mg/ml bromelain, respectively; (j) virus alone and with (k) 1 mg/ml subtilisin and (l) subtilisin plus saponin; (m) virus alone and with (n) 0.2 mg/ml papain and (o) papain plus saponin; (p) virus alone and with (q) 1 mg/ml trypsin and (r) with trypsin plus saponin.

After addition of 0.1% saponin to virus suspensions the virus remained intact but with increased permeability to stain (Fig. 2). SDS-PAGE of radiolabelled, pelleted, saponin-treated virus showed that the spikes had been retained. When proteases were added to virus in the presence of 0.1% saponin the N protein was hydrolyzed, showing that the proteases had entered the virion. Some virus preparations contain polypeptides of molecular weight less than that of N which have been shown by peptide mapping to be related to N (Stern and Sefton, 1982a; Cavanagh and Davis, unpublished observation). Polypeptides of the same molecular weight were present in preparations of purified N (Fig. 1, e).

p21.7 was no longer present after virions with saponin had been treated with bromelain (Fig. 1, c), subtilisin (Fig. 1, l), proteinase K and pronase E (not shown); instead there was a polypeptide of 19.2K (p19.2). In some experiments there was also observed a minor polypeptide of M_r 17.3K. In the absence of saponin most of M had remained uncleaved by subtilisin (Fig. 1, k), proteinase K and pronase E (not shown) while none of M was hydrolyzed by papain (Fig. 1, n) and protease V8 (not shown). With saponin present the M_r of M was decreased from 30K to 27–28K (Fig. 1, l,o) with an additional polypeptide of about 25–26K generated by proteinase K in some experiments (not shown). In the case of V8 protease with both IBV-M41 and IBV-Beaudette only about 50% of M was cleaved to p27–28 under the conditions used (not shown).

The location of the cleavage of the N-terminus of M by bromelain was determined by N-terminal sequencing of [³H]isoleucine-labelled p19.2, obtained after

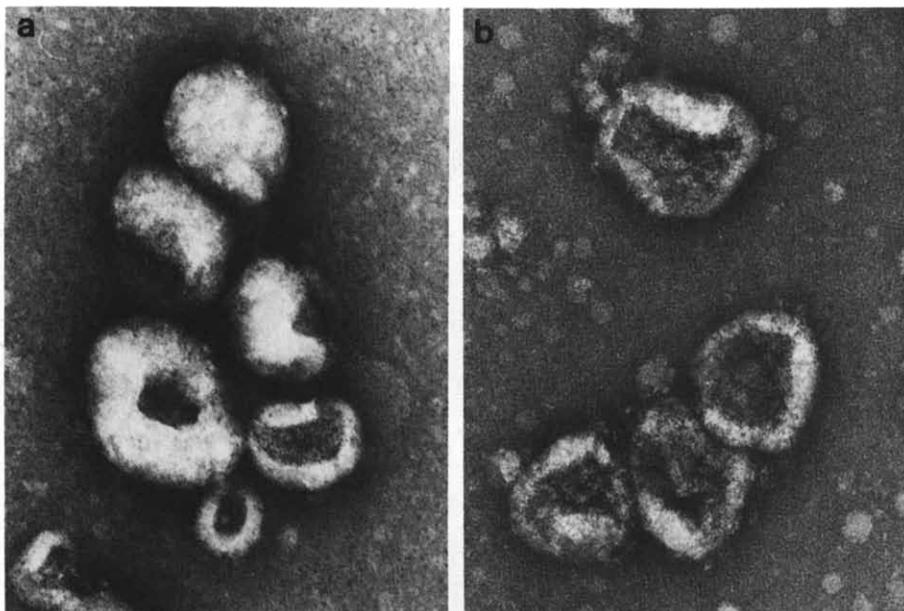


Fig. 2. Comparison by electron microscopy of IBV-M41 (a) without and (b) with 0.1% saponin. Spikes were not visible in most fields of control and saponin-treated virus. The virus was stained with uranyl acetate.

hydrolysis of virus by bromelain in the presence of saponin. (Saponin was included so as to be able to obtain hydrolysis of all S2 molecules, as described below.) Edman degradation revealed isoleucine residues at positions 15, 24 and 25 from the N-terminus of p19.2 (Fig. 4A). As explained in the Discussion this indicates that p19.2 lacked the first 9 N-terminal residues present on intact M.

As described below the hydrolysis of S2 was enhanced in the presence of saponin and the cleavage sites appeared to be adjacent to the outer membrane surface. Consequently it was necessary to demonstrate that the additional hydrolysis of M in the presence of saponin was a consequence of the removal of residues from the C-terminus of M. To achieve this IBV-M41 was labelled with [^{35}S]methionine and [^3H]glucosamine in order to label the glycans of M, the potential glycosylation sites of which are known for IBV-Beaudette to be located at residues 2 and 5 from the N-terminus (Bournnell et al., 1984). After exposure of the virus to several proteases in the presence and absence of saponin the $^{35}\text{S}/^3\text{H}$ dpm ratios were calculated. The p27-28 generated by papain, subtilisin and V8 protease had virtually the same $^{35}\text{S}/^3\text{H}$ ratio as the unhydrolyzed M (Table 1), indicating that the glycans had not been removed. p21.7 generated by bromelain was not associated with glycans; the small amount of ^3H in p21.7 was probably associated with amino acid residues, since a similar $^{35}\text{S}/^3\text{H}$ ratio was obtained for the non-glycosylated N polypeptide (Table 1). These results confirm that in the presence of saponin the proteases removed peptides from the C-terminus of M and indicate that this might be located in the lumen of the virion.

TABLE 1

$^{35}\text{S}/^3\text{H}$ dpm RATIO OF M AND N POLYPEPTIDES BEFORE AND AFTER PROTEOLYSIS OF [^{35}S]METHIONINE- AND [^3H]GLUCOSAMINE-LABELLED IBV-M41 WITH AND WITHOUT SAPONIN

Gel	Protease	Saponin	Polypeptide	M_r ($\times 10^{-3}$)	$^{35}\text{S}/^3\text{H}$ ratio, mean (S.D.) ^a
1	none	-	M	30	1.6 (0.1)
2	bromelain	-	M	30 ^b	1.8 (0.2)
2	bromelain	-	M	21.7	5.9 (0.5)
3	papain	-	M	30	1.9 (0.2)
4	papain	+	M	27/28	1.1 (0.2)
5	subtilisin	-	M	30	1.6 (0.2)
6	subtilisin	+	M	27/28	1.6 (0.4)
1	none	-	N	54	4.8 (0.1)
2	bromelain	-	N	54	5.6 (0.1)
3	papain	-	N	54	6.5 (0.3)
5	subtilisin	-	N	54	5.1 (0.4)
7	none	-	M	30	1.4 (0.3)
8	V8	-	M	30	1.4 (0.1)
9	V8	+	M	30	1.2 (0.1)
9	V8	+	M	27/28	1.5 (0.2)

^a The mean ratio (and S.D.) of four gel slices is presented.

^b In this experiment some of M was not hydrolyzed by bromelain.

Effect of proteases on the S protein

Variability in the S1 and S2 content of IBV-Beaudette preparations precluded the use of this strain for study of the effect of proteases on S1 and S2. Thermolysin and elastase had no effect on S1 or S2 when added to virions of IBV-M41. Papain, which in the presence of saponin did hydrolyze M, had no effect on S1 or S2 in the presence or absence of saponin (Fig. 1, m-o). Chymotrypsin hydrolyzed some S1 and S2 to give 4 products which ranged from approximately 61-74K; the extent of hydrolysis was not increased in the presence of saponin (not shown). Bromelain (Fig. 1, b), subtilisin (Fig. 1, k), proteinase K and pronase E (not shown) in the absence of saponin cleaved some of S2 but none of S1. Electrophoresis of samples in one slab gel showed that the molecular weight of the polypeptides generated was very similar for all four enzymes; the mean M_r from 17 determinations was 71.0 ± 1.2 (p71). Only with bromelain was the majority of S2 sometimes cleaved while very little of S2 was cleaved by pronase E. In contrast, bromelain (Fig. 1, c), subtilisin (Fig. 1, l), and proteinase K (not shown) hydrolyzed most or all of S2 to p71 in the presence of saponin. The amount of S2 cleaved by pronase E was greater when saponin was present (not shown). After 30 min at 37°C trypsin had hydrolyzed little or no S2 in the absence of saponin (Fig. 1, q) although a small proportion of S2 had been hydrolyzed to a 76.1K glycopolypeptide (p76.1) after 4 h (not shown). No further hydrolysis of S2 was obtained when 20 mg/ml of enzyme was used at 37°C for 4 h. Addition of saponin resulted in the hydrolysis of a greater proportion of S2 (Fig. 1, r). In some instances the p71 band was more intense than the S2 band from which it was derived. This was because S2 forms a broader band than p71 (and S1).

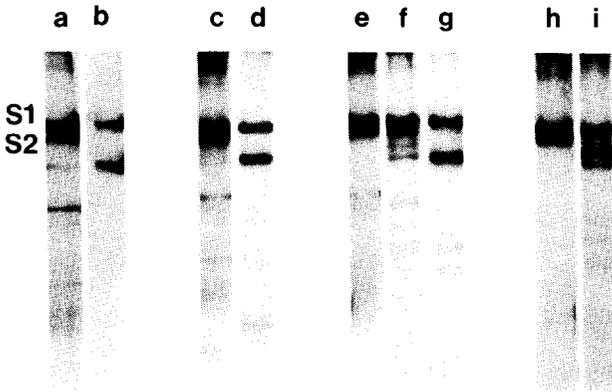


Fig. 3. SDS-PAGE of [35 S]methionine-labelled purified spike after incubation without (a,c,e,h) and with proteases for 30 min at 37°C; (b) 1 mg/ml subtilisin; (d) 1 mg/ml pronase E; (f) 0.02 mg/ml papain; (g) 0.2 mg/ml papain; (i) 1 mg/ml trypsin. Some of the tracks containing unhydrolyzed S1 and S2 (a,c,e) contain minor bands; these had arisen from samples in one or both adjacent tracks. The minor bands in the other tracks were from hydrolyzed S2.

Purified S was also treated with proteases with and without saponin. Bromelain (Fig. 1, c), subtilisin (Fig. 3, b), pronase E (Fig. 3, d) and proteinase K (not shown) hydrolyzed all of S2 to p71 plus lesser amounts of smaller glycopolypeptides. Some S2 was hydrolyzed at least twice by 0.02 mg/ml of papain to glycopolypeptides of approximately 75K and 71K (Fig. 3, f) whereas with 0.2 mg/ml of papain only the 71K product was observed (Fig. 3, g). Trypsin hydrolyzed most of S2 to p76.1 while some was cleaved further to p71 (Fig. 3, i). The presence of saponin with the purified S did not increase the extent of hydrolysis of S2 by papain (0.02 mg/ml) or trypsin (not shown).

N-terminal analysis of [3 H]isoleucine-labelled p76.1, generated by hydrolysis of IBV-M41 virion-associated S2 by bromelain, showed that p76.1 had isoleucine residues at positions 2, 19, 25 and 28 from the N-terminus (Fig. 4B). Intact S2 of IBV-Beaudette also has isoleucine residues at positions 2, 19, 25 and 28 (Binns et al., 1985). This distribution of isoleucine residues, which occurs only once, also exists in S2 of IBV-M41, as deduced from nucleotide sequence analysis (M.M. Binns, pers. comm.). This unequivocally shows that the bromelain-cleaved S2 has an intact N-terminus.

When the virions which had been exposed to proteases were dissociated by SDS in the absence of mercaptoethanol p71 was still obtained, showing that it was not linked by disulphide bonds to the remainder of S2.

In some experiments with bromelain, papain, subtilisin, proteinase K and pronase E the limited proteolysis of IBV glycopolypeptides described above was much more extensive, for S1 in addition to S2. In these experiments N was also hydrolyzed in the absence of saponin. Observation of the results of many experiments indicated that virus was best used as soon as possible after preparation and with incubation times of less than 1 h.

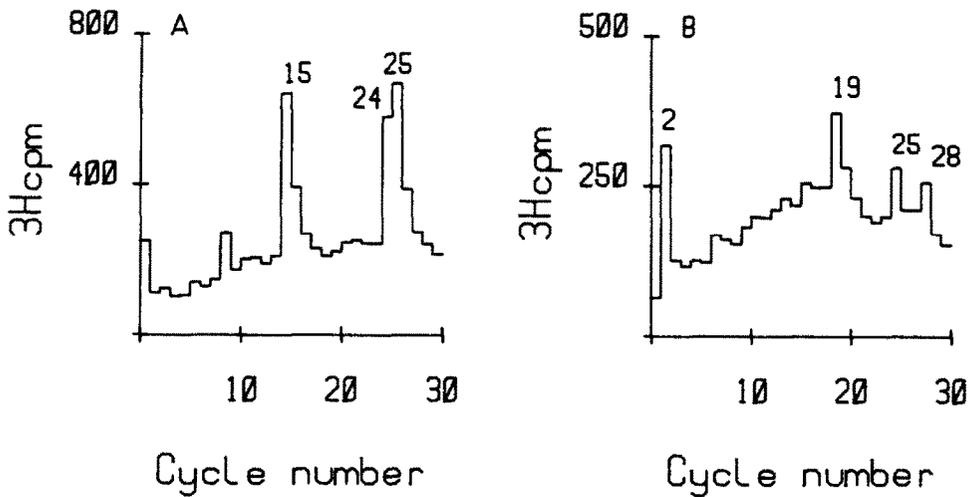


Fig. 4. Partial N-terminal amino acid sequencing of IBV polypeptides recovered after treatment of [^3H]isoleucine-labelled IBV-M41 virions with bromelain in the presence of saponin. The radiolabelled polypeptides were covalently attached to 170 Å DITC-glass and subjected to solid-phase Edman degradation. (A) p19.2 derived from M; (B) p71 derived from S2.

Discussion

The glycans of IBV M protein are of the N-linked, predominantly high mannose type (Stern and Sefton, 1982; Cavanagh, 1983a) and are attached to the N-terminus of M (Stern et al., 1982), indicating that the N-terminus is located beyond the outer surface of the virus membrane. This is confirmed by the finding that p21.7 generated by proteases lacks glycans. Nucleic acid sequencing of the IBV-Beaudette M gene has shown that there are only two potential glycosylation sites of the type Asn-X-Thr/Ser in M, at positions 2 and 5 from the N-terminal (Bournell et al., 1984); partial amino acid sequencing has shown that the N-terminal methionyl residue is not present in the virion-associated M protein (Cavanagh et al., 1986). The difference in M_r of 7K between the 23K non-glycosylated M precursor and the major glycosylated form of 30K indicates that both glycosylation sites are linked to glycans. In this communication several proteases are shown to be able to remove a glycopeptide from the N-terminus of M, such that the large fragment of M had an M_r of about 1.3K less than that of the 23K unglycosylated M polypeptide. N-terminal sequencing of p19.2 generated from the M of IBV-M41 by bromelain showed that isoleucine residues (I, in single-letter amino acid code) were present at positions 15, 24 and 25 from the N-terminus of p19.2 (Fig. 4A). Inspection of the deduced amino acid sequence of M of IBV-Beaudette (Bournell et al., 1984), part of which is shown in Fig. 5, shows that the first three isoleucine residues are at positions 24, 33 and 34 from the N-terminus. Comparison of this data shows that bromelain had cleaved M up to residue 10. The M_r of the first 9 residues of the IBV-Beaudette M polypeptide is 1140. This is in good agreement with the 1.3K estimate for the decrease in M_r of the polypeptide moiety of M caused by several proteases.

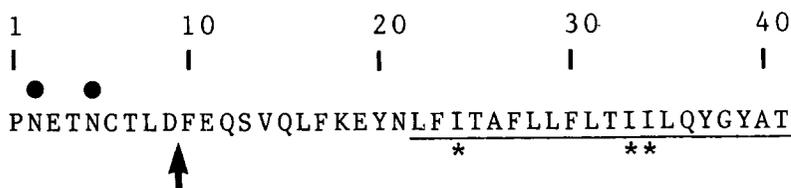


Fig. 5. Sequence of the first 41 N-terminal amino acid residues of the M glycopolyprotein of IBV-Beaudette (Boursnell et al., 1984). The asterisks (*) indicate the isoleucine (I) residues detected by partial amino acid sequencing of p19.2, generated by bromelain hydrolysis of [³H]isoleucine-labelled IBV-M41. The arrow indicates the position cleaved by bromelain. The closed circles indicate the two glycosylation sites of M.

The resistance of M to chymotrypsin, for which there is a potential target phenylalanyl (code F) residue at position 10 from the N-terminus (Boursnell et al., 1984; Fig. 5) and resistance to protease V8 which has target glutamyl (code E) residues at positions 3 and 11 in the IBV-Beaudette M polypeptide suggests that residues 10 and 11 might not be exposed at the virus surface. Potential target residues for hydrolysis by chymotrypsin (phenylalanyl and tyrosyl (code Y) residues) are situated at residues 17, 20, 23, 27 and 30, and by trypsin (lysyl residue, code K) at position 18 (Fig. 5). Since hydrolysis did not occur at these sites it is possible that no more than the first 20 or so N-terminal residues, and possibly as few as 9 residues, may protrude at the outer membrane surface. This conclusion is consistent with the identification, by analysis of sequence data, of three extensive hydrophobic sequences in the N-terminal half of M and the prediction that these three stretches span the membrane (Boursnell et al., 1984). The hydrophobic sequence nearest the N-terminus starts at residue 20. It is possible that some of the resistance of M to chymotrypsin, trypsin and V8 protease was because the tertiary structure of the polypeptide concealed some target residues. Whether M is an oligomeric protein is not known. If it is then quaternary structure might also have hidden some target residues.

The residues between the first and second hydrophobic sequences contain three residues which are potential targets for hydrolysis by trypsin. Our finding that trypsin does not cleave virion-associated M even in the presence of saponin indicates that these residues are not exposed at either membrane surface. IBV-Beaudette M has a glutamyl residue at position three from the N-terminus (Boursnell et al., 1984). However, V8 protease, which hydrolyzes at the carboxyl side of glutamate residues (Houmard and Drapeau, 1972) did not cleave M at that position; such hydrolysis would have been detected because it would have resulted in the loss of a glycan. This suggests that the glutamyl residue may be protected by the adjacent glycans.

In an elegant piece of work Rottier et al. (1984) translated the MHV M gene in the presence of microsomes such that the M protein became located in the microsomal membrane. Using proteinase K and saponin they observed that approximately 2.5K and 1.5K of polypeptide were removed from the N- and C-termini, respectively. The similarity of the results obtained after treatment of IBV and MHV with proteases correlates well with the prediction, derived from nucleotide sequence analysis, that the tertiary structure of the IBV and MHV M polypeptide is very

similar (Armstrong et al., 1984; Bournsnel et al., 1984). The hydrophobic sequence nearest the N-terminus of MHV M protein starts at residue 25 in contrast to residue 20 for IBV. The apparent difference in molecular weight of the peptide moiety removed from the N-terminus of the IBV and MHV polypeptide may thus reflect a real difference in the amount of polypeptide which protrudes at the outer membrane surface of these two viruses. In addition to the similar hydrophobic character of the N-terminal half of the IBV and MHV M polypeptide, nucleotide sequence analysis indicates that the C-terminal half is neither strongly hydrophobic nor hydrophilic in both viruses. The C-terminal half of M may, therefore, be adjacent to, but not completely buried in, the inner surface of the virus membrane (Armstrong et al., 1984). The finding that the C-terminal end of M was hydrolyzed only in the presence of saponin suggests that the last 20–25 or so residues are exposed in the lumen of the virion. It cannot be ruled out completely, however, that these residues might be in the membrane and be exposed when saponin was present.

Virion-associated S2 was hydrolyzed to a 71K glycopolypeptide by several proteases and a 76K glycopolypeptide by trypsin. Since these glycopolypeptides had the same N-terminus as intact S2 the cleavage of S2 had occurred near the C-terminal. This cleavage occurred at the outside of the virions since there was no evidence for hydrolysis of the nucleocapsid protein or of the C-terminus of M. With the occasional exception of bromelain, only a proportion of S2 was cleaved. Hydrolysis by bromelain can result in the removal of S from virions (Cavanagh, 1981). The quantitative hydrolysis of S2 by some proteases in the presence of saponin, or when purified S was used, suggests that the cleavage locations were adjacent to the outer membrane surface and were completely exposed when the membrane was disorganised by saponin. This may have resulted directly by the movement of lipids adjacent to S or indirectly by configurational change in S when the lipids were rearranged.

The finding that urea removed S1 but not S2 from IBV led to the proposal that S2 anchored S in the viral membrane (Cavanagh, 1983c). This conclusion has been strengthened by nucleotide sequence analysis of the spike precursor gene of IBV-Beaudette (Binns et al., 1985) which is of the same serotype as IBV-M41. The 71 C-terminal residues (M_r 7.7K) comprise a hydrophilic tail (27 residues) followed by a 44 residue stretch with a very hydrophobic character. This arrangement is typical of the membrane-anchoring sequences of the spike proteins of several other viruses. The M_r of S2 minus the C-terminal 71 residues is approximately 76.3K. Trypsin hydrolyzed S2 of IBV-M41 to a glycopolypeptide of 76.1K, especially in the presence of saponin. Inspection of the IBV-Beaudette sequence shows that the first target residues for trypsin (arginyl and lysyl) after the 44 residue highly hydrophobic sequence are at positions 72, 76 and 81 from the C-terminus (Binns et al., 1985). Cleavage at one or more of these residues would result in the generation of a peptide of 71–80 residues of M_r about 8–9K and a large glycopolypeptide of about 75–76K. Since trypsin did generate such a glycopolypeptide, especially with saponin present, and since these basic residues are conserved in IBV-M41 (M.M. Binns, pers. comm.), this suggests that S2 was cleaved in the region of 71–80 residues from the C-terminus and that this region is close to but possibly not completely exposed at the outer

membrane surface. The presence of a protease sensitive site near the point of membrane insertion is a feature of the HA₂ glycopolyptide of the influenza virus haemagglutinin (Brand and Skehel, 1972). The HA₂ of A/Hong Kong/1968 influenza virus is cleaved by bromelain at a position which is 10 residues away from the uncharged anchoring domain (Wilson et al., 1981). The sites at which hydrolysis generates the 71K glycopolyptides may be only a few residues upstream from the proposed trypsin cleavage site. S2 has a potential glycosylation site 88 residues from the C-terminus. If hydrolysis occurred at the N-terminal side of this site, effectively removing a glycosylated peptide from S2, then the residual S2 would have an M_r of about 71K. Thus, it can be speculated that the residues immediately after the 71 residue C-terminal anchor sequence are very close to the outer virion surface.

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