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PROTEASE SUSCEPTIBILITY OF HUMAN A INFLUENZA VIRUS POLYPEPTIDES

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Summary. – The proteolytic susceptibility of polypeptides of four antigenically distinct subtypes of influenza A virus strains of human origin was studied. The extent of degradation of polypeptide molecules of strains A/PR/8/34 (H0N1) (PR), A/FM/1/47 (H1N1), A/Singapore/1/57 (H2N2) and A/Hong Kong/8/68 (H3N2), assessed by densitometry of gels after sodium dodecylsulfate polyacrylamide gel electrophoresis was variable by treatment with trypsin. Also, sequential treatment of PR strain initially with phospholipase D followed by proteases of different specificities suggested differences in susceptibility of surface and internal polypeptide molecules. The significance of these results is discussed.

Key words: influenza A viruses; proteases; polyacrylamide gel electrophoresis

Introduction

Previous studies with influenza viruses have demonstrated that proteases produce changes in surface morphology and biological activities of the particles (Kendal *et al.*, 1969; Rott, 1977). The question arises whether influenza-polypeptides differ in susceptibility to proteases. The WHO (Assaad *et al.*, 1979) has suggested that influenza viruses should be systematically examined to assess the usefulness of protease markers in establishing subtype relationships. We therefore determined the susceptibility of polypeptides of representative antigenic subtypes of influenza A viruses to proteases by polyacrylamide gel electrophoresis (PAGE).

Materials and Methods

Four strains of influenza virus, namely A/PR/8/34 (H0N1) (PR), A/FM/1/47 (H1N1) (FM) A/Singapore/1/57 (H2N2) (Sing) and A/Hong Kong/8/68 (H3N2) (Hong Kong), were grown in the allantoic cavities of 10-day old chick embryos, concentrated by centrifugation and purified by rate zonal centrifugation (1.5 hr at 90.000 × g) in discontinuous sucrose gradients. The opalescent band harvested from the 32.5–37.5 % zone was recentrifuged through a 2-step (10–20 %) gradient. Virus pellets were resuspended in 0.05 M Tris-HCl buffer (pH 7.2) and assayed for

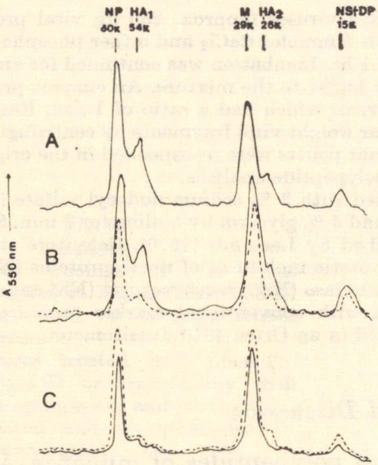

Fig. 1.

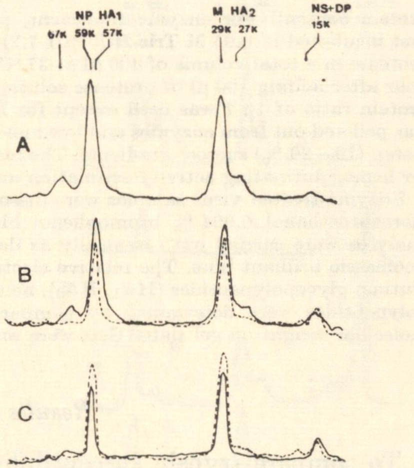

Fig. 2.

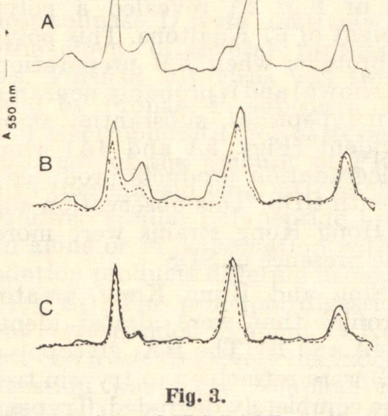

Fig. 3.

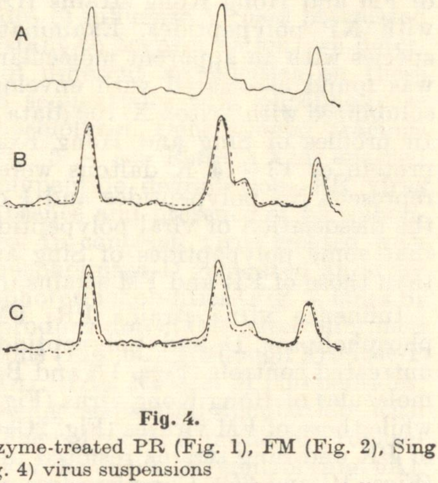

Fig. 4.

Densitometer tracings of SDS-PAGE profiles of enzyme-treated PR (Fig. 1), FM (Fig. 2), Sing (Fig. 3) and Hong Kong (Fig. 4) virus suspensions

A — Untreated viruses; B — viruses treated with phospholipase D (——) and sequentially with phospholipase D and trypsin (— — —); C — viruses treated with trypsin for 1 hr (— — —) and 2 hr (———).

Migration from left to right.

NP — nucleoprotein; HA_1 and HA_2 — haemagglutinin glycopolypeptides; M — matrix polypeptides; NS + DP — nonstructural polypeptides and degradation products. The figures below these designations refer to molecular weights in kilodaltons.

protein content. For enzyme treatment, purified viruses (approx. 250 μg viral protein) were first incubated in 0.05 M Tris-HCl (pH 7.2) with 2 mmoles $CaCl_2$ and either phospholipase D or protease in a total volume of 400 μl at 37 °C for 1 hr. Incubation was continued for an additional hour after adding 100 μl of protease solution or buffer to the mixture. An enzyme protein: viral protein ratio of 1 : 7 was used except for PR virus which had a ratio of 1 : 12. Residual virus was pelleted out from enzymes and low molecular weight viral fragments by centrifuging through 2-step (10—20 %) sucrose gradients. The resultant pellets were resuspended in the original buffer for hemagglutinating activity estimation and polypeptide analysis.

Enzyme-treated virus samples were dissociated with 2 % sodium dodecyl sulfate (SDS), 2 % mercaptoethanol, 0.004 % bromophenol blue and 4 % glycerol by boiling for 2 min. SDS-PAGE analyses were carried out essentially as described by Laemmli (1970). Gels were stained with Coomassie brilliant blue. The relative electrophoretic mobilities of nucleoproteins (NP), hemagglutinin glycopolypeptides (HA_1, HA_2), neuraminidase (NA), nonstructural (NS) and matrix (M) polypeptides were determined by comparison with conventional marker proteins of known molecular weight on gel slabs. Gels were scanned in an Ortec 4810 densitometer.

Results and Discussion

To compare trypsin susceptibility of polypeptides of influenza A viruses, densitometer tracings of SDS-PAGE profiles of enzyme-treated viruses were made for evaluating the relative amount of each viral polypeptide. Visual comparison of the polypeptide profiles of untreated PR and Sing strains suggested an overall similarity, except that the electrophoretic migration rates of HA_2 and M polypeptides were reversed (Figs. 1A and 3A). The gel profile of untreated FM polypeptides qualitatively corresponded to the profile obtained for the Hong Kong strain (Figs. 2A and 4A). In gels of FM and Hong Kong strains HA_1 glycopolypeptides tended to comigrate with NP polypeptides. Examination of Fig. 2A revealed a polypeptide species with an apparent molecular weight of 67 Kdaltons. This polypeptide was found associated with envelope proteins when FM preparations were solubilized with Triton X-100 (data not shown) and is probably neuraminidase. In profiles of Sing and Hong Kong polypeptides, substantial amounts of protein of 13—14 K daltons were evident (Figs. 3A and 4A) which may represent NS polypeptides and I or degradation products produced during the dissociation of viral polypeptides with SDS. This observation suggested that some polypeptides of Sing and Hong Kong strains were more labile than those of PR and FM strains in the presence of SDS.

Influenza virus strains PR, FM, Sing and Hong Kong, treated with phospholipase D had polypeptide profiles that were almost identical to untreated controls (Figs. 1A and B — 4A and B). The HA_1 glycopolypeptide molecules of Hong Kong virus (Fig. 4C) were refractory to trypsin treatment while those of FM viruses (Fig. 2C) were completely degraded. Trypsinization of PR and Sing strains resulted in extensive degradation of HA_1 molecules (Figs. 1C and 3C). Complete loss of HA_2 molecules was noted in the profile of Sing polypeptides (Fig. 3C) following one hour of incubation with trypsin, whereas the extent of HA_2 degradation by this enzyme was variable in the other three strains (Figs. 1C, 2C and 4C). Further, to investigate the trypsin sensitivity of internal viral polypeptides, the different influenza subtypes were sequentially treated with phospolipase D and trypsin. The results

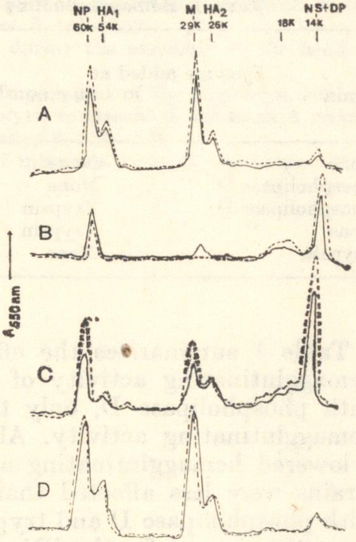

Fig. 6.

Densitometer tracings of PR virus amples analyzed in Fig. 5 (Plate XXII)

——— Untreated or protease-treated viruses

- - - - Viruses treated with phospholipase D or sequentially with phospolipase D and protease

A — Untreated and phospholipase D-treated viruses

B — Pronase-P treatments

C — Subtilisin treatments

D — Thermolysin treatments

indicate that trypsin had little effect on internal NP and M polypeptides although surface HA_1 and HA_2 molecules were affected to varying degrees (Figs. 1B—4 B).

Since the internal NP and M polypeptides of influenza viruses pretreated with phospholipase D were relatively resistant to trypsin action, we determined the effect of several proteases of different specificities on these molecules. Purified PR virus was treated sequentially with phospholipase D followed by Pronase-P, subtilisin or thermolysin. Densitometer tracings (Fig. 6) of electrophoretic gel patterns (Fig. 5 — see Plate XXII) enabled the assessment of the amount of PR polypeptide degradation. PR virus exposed to thermolysin (Fig. 6D) in the absence and presence of phospholipase D showed similar polypeptide profiles. In conttast, virus treated with subtilisin alone or in combination with phospholipase D generated an array of degradation products differing in electrophoretic mobilities (Fig. 5, lanes S_1 and $PD_2 + S_1$). The principal digestion product detectable after subtilisin treatment corresponded to about 14 Kdaltons (Fig. 6C). Although Pronase-P treatment of virus extensively degraded HA_1, HA_2, NP and M polypeptides, their digestion was more complete following sequential incubation with phospholipase D and Pronase-P (Fig. 6B). The polypeptide profile of Pronase-P treated virus differed markedly from that of the untreated control (Fig. 6A). in relative amounts of detectable polypeptides and degradation products Presumably, phospholipase D — Pronase-P treatment of PR virus produced polypeptide fragments with molecular weights less than 10 K which cannot be resolved in our gel system. However, two major degradation products with apparent molecular weights of 18 K and 14 K (Fig. 6B) were generated.

Table 1. Hemagglutinating activity of enzyme-treated influenza A viruses

Enzyme added at 0 min	Enzyme added at 60 min	Activity (HA units/0.05 ml) A/PR/8/34 (H0N1)	A/FM/1/47 (H1N1)	A/Singapore/ /1/57 (H2N2)	A/Hong Kong/8/68 (H3N2)
None	None	800,000	16,000	32,000	16,000
Phospholipase D	None	3,200,000	16,000	32,000	16,000
Phospholipase D	Trypsin	1,600,000	<20	16,000	8,000
None	Trypsin	160,000	<20	12,800	8,000
Trypsin	None	40,000	<20	6,400	8,000

Table 1 summarizes the effect of phospholipase D and trypsin on the hemagglutinating activity of influenza A strains. Of the viruses treated with phospholipase D, only the PR strain showed a fourfold increase in hemagglutinating activity. All viruses following exposure to trypsin had a lowered hemagglutinating activity, although Singapore and Hong Kong strains were less affected than strains PR and FM. Sequential treatment with phospholipase D and trypsin resulted in minimal inhibition of hemagglutination except for the FM strain.

The electrophoretic data reported on tryptic digests of influenza subtypes indicate that the extent of degradation of hemagglutinins was variable. Differences in susceptibility of HA_1 and HA_2 glycopolypeptides to trypsin action among influenza A strains might be expected. Analyses of several other protease digests of PR virus prepared with enzymes of different specificity revealed differences in their capacity to degrade hemagglutinins (Fig. 5). This finding suggests that the particular tertiary structure of PR-hemagglutinins may determine which peptide bonds are hydrolyzed by specific proteases. Reginster *et al.* (1975/76) reported that when glycoprotein spikes of PR virus were partially removed by caseinase C, the enzyme digested about 50 % of the M-polypeptide content of the particles. We observed that sequential treatment of PR strain initially with phospholipase D to hydrolyze supposedly protective lipids followed by the addition of Pronase-P resulted in complete fragmentation of M and HA_2 polypeptides.

Although there are differences in the cleavability of influenza polypeptide molecules of different subtypes by proteases, this finding is of considerable interest in the context of enzyme markers for establishing subtype relationships. However, further studies on several influenza strains of the major subtypes will be necessary to establish whether proteases with different specificities will be useful for the characterization of subtype relationships.

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