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INHIBITION OF SUBTILISIN BY INFLUENZA VIRUS INFECTED ALLANTOIC FLUIDS

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Summary. — Allantoic fluids harvested from embryonated chicken eggs infected with reference strains of influenza A viruses were analysed for subtilisin inhibitor activity. While all acid heat-treated and nontreated virus-infected fluids could reduce subtilisin activity, fluids of FM and Bangkok strains had the greatest inhibitory ability. The degree of subtilisin inhibition closely paralleled the appearance of infectious Bangkok and FM virus in allantoic fluid. Maximum levels were achieved at 48 hr post-infection (p.i.) Ultracentrifugation analyses indicated that the bulk of thermostable inhibitor(s) of 48 hr Bangkok and FM infectious fluids remained in the supernatant rather than sedimenting with the viral pellet.

Key words: subtilisin inhibitor; influenza viruses; allantoic fluid

Introduction

In the preceding paper (Ewasyshyn and Sabina, 1986), it was shown that during replication of different strains of influenza viruses a marked reduction in allantoic fluid protease activity resulted when near maximum yields of infectious virus were attained. This observation led us to speculate that thermostable protease inhibitor(s) in 48 hr virus fluids were present. Evidence for this was provided by inhibitor studies using acid heat treated 48 hr allantoic fluids of six influenza strains to block the activity of several serine proteases. A clear specificity for inhibition of subtilisin only was demonstrated by all virus fluids. In this communication, we present more information of the biological activities of subtilisin inhibitor(s) associated with 48 hr influenza virus fluids.

Materials and Methods

Strains of influenza A viruses employed in this study included A/PR/8/34 (H1N1) (PR), A/Bangkok/1/80 (H3N2) (Bangkok), A/FM/1/47 (H1N1) (FM), A/Turkey/Ontario/7732/66 (H5N9) (Turkey) and A/Brazil/8/78 (H1N1) (Brazil). The methods used for this work are described in previous papers (Ewasyshyn and Sabina, 1983, 1986).

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Table 1. Per cent inhibition of different concentrations of subtilisin by 48 hour influenza infected allantoic fluids

Treatment	Conc. of subtilisin (mg/ml)	Virus[a]					
		PR	Brazil	FM	Bangkok	Turkey[b]	Control
		% inhibition of subtilisin[d]					
Nontreated	10^{-2}	0	0	15	9	0	0
Acid-heated[c]	10^{-2}	0	0	20	15	0	0
Nontreated	10^{-3}	20	88	100	100	35	0
Acid-heated	10^{-3}	22	85	100	100	35	0
Nontreated	5×10^{-4}	75	89	100	100	93	0
Acid-heated	5×10^{-4}	74	85	100	100	95	0

[a] Eggs were allantoically inoculated with approximately 25 $TCID_{50}$ of Turkey virus and 40 $TCID_{50}$ of the other influenza viruses.
[b] Turkey virus infected allantoic fluid was harvested at 24 hr p.i.
[c] Allantoic fluid was heated at 85 °C at pH 1 for 10 minutes prior to readjustment to pH 7.5.
[d] Fluid was preincubated with subtilisin for 1 hr at 35 °C prior to the addition of substrate.

Results and Discussion

To further analyze influenza virus infected allantoic fluids which contain subtilisin inhibitory activity, we subjected nontreated and heated-acidic 24 or 48 hr fluids to different concentrations of subtilisin. Table 1 shows that preincubation of 24 and 38 hr fluids of Turkey and PR viruses, respectively, could not appreciably depress subtilisin activity at a concentration of 10^{-3} mg/ml. However, enzyme activity was completely blocked by FM and Bangkok viral fluids. Control noninfected allantoic fluid was unable to inhibit subtilisin activity. Heat treatment of acidic 48 hr infectious fluids did not significantly increase the level of subtilisin inhibition. Whereas the inhibitory ability of 48 hr fluids of influenza H1N1 strains on the initial peak of protease activity produced during virus replication was about 2-fold greater than that of 24 hr fluids (Ewasyshyn and Sabina, 1986). These results suggest that 48 hr allantoic fluid of various influenza viruses contain different thermostable inhibitory capabilities.

We compared the extent of subtilisin inhibition in the presence of different amounts of nontreated and acid heated 48 hr virus allantoic fluid protein. The results indicated that the inhibitory effect on subtilisin was dependent on the protein concentration of the viral fluids (Fig. 1). The subtilisin concentrations used in this study were based on the ability of 48 hr allantoic fluid to produce maximum inhibition of enzyme activity. In all cases, a linear relationship existed between the percent inhibition and quantity of protein present. To ensure that any exogenous protein, if present, would not interfere with subtilisin activity, various amounts of bovine serum albumin were added to control fluids. The presence of bovine serum albumin had no effect on subtilisin activity. Also, the possibility that hemolyzed red blood cells

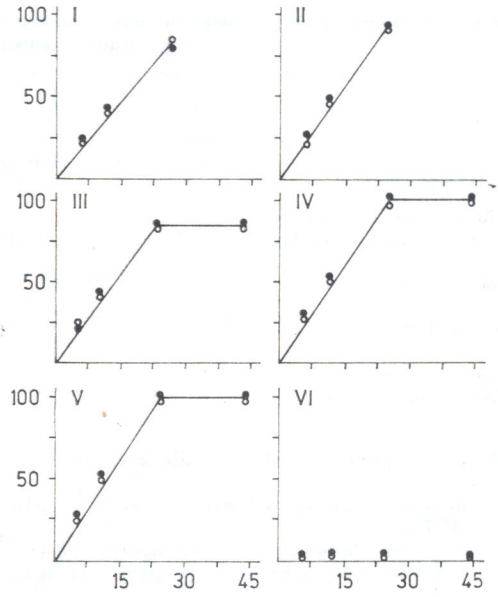

Fig. 1.

Analysis of subtilisin inhibition by 48 hr allantoic fluid protein of various influenza A viruses

The per cent inhibition of subtilisin by different amounts of nontreated (○) and acid-heated (●) 48 hr allantoic fluid protein, except for 24 hr allantoic fluid in the case of Turkey virus, was determined. Conditions of acid heat treatment and preincubation of allantoic fluids with subtilisin as in Table 1. Samples were incubated with herring protamine as substrate and thereafter fluorometrically assayed for residual subtilisin activity. The inhibitory ability of allantoic fluids of PR virus (I), Turkey virus (II) and noninfected fluid containing increasing amounts of bovine serum albumin (VI) was tested with 5×10^{-4} mg/ml subtilisin. A 2-fold higher subtilisin concentration was used to test infectious fluids of Brazil (III), Bangkok (IV) and FM (V) viruses. Abscissa: μg protein; ordinate: % subtilisin inhibition.

in highly infectious allantoic fluids could account for the observed subtilisin inactivation was investigated. When increasing concentrations of lysed chick embryo red blood cells were added to control allantoic fluid, no effect on the activity of 5×10^{-4} mg/ml subtilisin was evident. Other unidentified factor(s) must therefore be involved in the inhibition of subtilisin.

Because 48 hr Bangkok and FM strains had the greatest subtilisin inhibitory ability, the level of inhibition was monitored at various time points throughout their replicative cycles. As seen in Figs. 2-I and II, there was a progressive increase in the percent inhibition of 10^{-3} mg/ml subtilisin

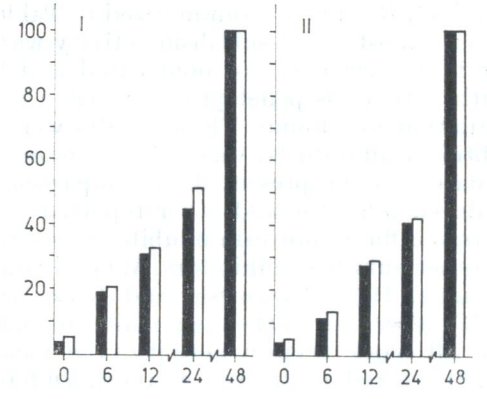

Fig. 2.

Subtilisin inhibition during the course of infection with Bangkok and FM strains Allantoic fluid was harvested at various time points after infection with about 40 $TCID_{50}$ of Bangkok (I) and FM (II) strains. Experimental design as in Fig. 1. Open bars represent the percent inhibition of subtilisin (10^{-3} mg/ml) and solid bars represent nontreated fluids.

Abscissa: hr p.i.; ordinate: % subtilisin inhibition

Table 2. Comparison of subtilisin inhibitory ability in 48 hour Bangkok and FM supernatants and virus pellets

Sample	Per cent inhibition of subtilisin activity[a]			
	Bankgkok virus		FM virus	
	Nontreated	Acid-heated[b]	Nontreated	Acid-heated
48 hour virus-infected allantoic fluid uncentrifuged	100	100	100	100
Supernatant fraction	88	79	76	84
Virus pellet[c]	16	15	5	4
48 hour control non-infected allantoic fluid	0	0	0	0

[a] Fluid was preincubated with subtilisin (10^{-3} mg/ml) for 1 hr at 35 °C prior to the addition of substrate.

[b] Fluid was subjected to heating at 85 °C at pH 1 for 10 min and subsequently readjusted to pH 7.5.

[c] Virus pellet obtained from cenrifugation of 48 hr infected allantoic fluid at 65,000 × g for 45 min was resuspended to its original titer using 48 hr control noninfected fluid.

during the course of Bangkok and FM virus infection. At all time points tested, the inhibitor(s) was unaffected by heating under acidic conditions. Maximum inhibitory levels were reached at 48 hr p.i. Moreover, the degree of subtilisin inhibition closely paralleled the appearance of infectious virus in the allantoic fluid.

Ultracentrifugation of 48 hr Bangkok and FM infected allantoic fluids was carried out to determine if the inhibitor(s) was associated with the viral pellets or remained in the supernatants. Subtilisin inhibitor ability of non-treated and heated-acidic 48 hr virus fluids of both supernatants and viral pellets were tested. In evaluating viral pellets, they were resuspended in control noninfected allantoic fluid to achieve a titer similar to that of 48 hr p.i. fluid. Results are summarized in Table 2. With supernatant fluid of both viruses, most of the subtilisin activity was blocked. Also, differences between the inhibitory effect of nontreated and heated-acidic fluids were minimal. With both virus pellet preparations, a relatively small amount of subtilisin inhibition was found. These results were interpreted as indicating that the subtilisin inhibitor(s) was not associated with the virus particle.

Based on the present data comparisons can be made between the virus--induced inhibitor and other reported inhibitors of subtilisin activity. The allantoic fluid subtilisin inhibitor is restricted in specificity when compared to other subtilisin inhibitors. While ovoinhibitor from hen egg white (Matsushima, 1958; Laskowski and Kato, 1980) and chicken plasma (Barett, 1974) as well as chicken egg white ovomacroglobulin (Kitamoto *et al.*, 1982) can all block trypsin activity, the virus-induced subtilisin inhibitor was unable to reduce trypsin activity. Differences also exist in the stability of

the subtilisin inhibitor. Unlike ovomacroglobulin (Donovan *et al.*, 1969) allantoic fluid subtilisin inhibitor is unaffected by heating under acidic conditions. This virus-induced inhibitor appears to be extremely thermostable. Additional properties of the allantoic fluid subtilisin inhibitor will become apparent following its isolation and purification.

It will be important to determine the role of thermostable subtilisin inhibitor(s) released into the allantoic fluid during influenza virus infection. If the inhibitor has a major function in maintaining infectivity of the virus by protecting against proteolytic degradation, interference with inhibitor synthesis may be of significant therapeutic value.

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