Isolation and Partial Characterization of an Antiviral Proteolytic Fraction from the Venom of *Echis Carinatus Sochureki*

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Abstract: The venom of the viper *Echis carinatus sochureki* suppresses the hemolytic activity of Sendai virus on human erythrocytes, when pre-incubated with the virions prior to their binding to cells. A fraction (C1), with an IC_{50} of 1.25 μg/ml, was isolated from the venom. Fraction C1 possesses strong azocollase, azocaseinase and gelatinase activity. The proteolytic and anti-hemolytic potency of C1 depends on the period and temperature of incubation. Its antiviral activity is inhibited by Sodium-EDTA but not by PMSF. SDS PAGE of Sendai virus incubated with fraction C1 shows disappearance of several of the virion high molecular weight bands. We suggest that inhibition of the hemolytic activity of the virions is probably a result of the cleavage of viral surface proteins, such as the hemagglutinin-neuraminidase glycoprotein found on the virion envelope that mediates the absorption of the virus to cells.

INTRODUCTION

Snake venoms are complex mixtures of toxins and enzymes with different activities on many biological systems. Among other components they include cytotoxins [1-3], anti-bacterial [4] and anti-viral factors [5-7]. Among several viperid snake venoms screened for antiviral effects, the snake venoms of *Echis carinatus suchureki* and *Echis coloratus* were found to abolish the hemolysis of human erythrocytes caused by Sendai virus [5]. The active antiviral factor found in the venom of *Echis coloratus* was isolated and characterized [6]. This factor, termed Echinibin-1, is a 25-kDa metalloproteinase glycoprotein, which interferes with the virus adsorption to the cells resulting in the suppression of the hemolytic, hemagglutinating and lethal activities of Sendai virus.

Sendai virus is an enveloped virus, which belongs to the Parainfluenza family. This group of viruses includes pathogens that can cause serious respiratory tract diseases [8]. Parainfluenza is one of three genera of the Paramyxoviridae family, which includes additional important human pathogens, such as Measles, Mumps, and Respiratory Syncytial Virus (RSV). The first two stages, which allow the penetration of these enveloped viruses into animal cells, are the attachment and the fusion of the virions with the target cell membrane. These steps are mediated by two glyco-proteins found on the virion envelope - the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins [9]. Inhibition of any of these two steps results in the loss of virus infectivity.

The present work deals with the isolation and partial characterization of fraction found in the venom of the snake *Echis carinatus sochureki* that suppresses the hemolytic activity of Sendai virus.

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MATERIALS AND METHODS

Venom

The venom of *Echis carinatus sochureki* was purchased from Latoxan Laboratories, 05150 Rosans, France.

Virus

Sendai virus (Z strain) was injected into the allantoic sac of 10-11 day-old chicken embryo, harvested after 48 h from the allantoic fluid and stored at -70°C until used.

Blood

Fresh, human blood was obtained from a blood bank and stored for up to one month at 4°C. Prior to use, erythrocytes were washed five times with phosphate buffer saline, pH 7.2 (PBS), and were diluted to 2% (v/v) with the same buffer (10^8 cells per ml).

Estimation and Concentration of Proteins

Protein content was estimated by measuring the absorbance at 280 nm in a Gilford U.V. spectrophotometer, using a value of $A\ 1\%_{280} = 14$.

Hemolysis Assay

One milliliter of washed 2% erythrocytes (10^8 cells) were incubated with Sendai virion aliquots for 15 min at 4°C and then washed twice with cold PBS. The pellets were resuspended in 1 ml PBS and incubated for another two hr at 37°C in a shaking bath (90 strokes/min), followed by centrifugation at 1000 x g. The hemolysis was determined by measuring the absorbance of the supernatant at 540 nm.

Determination of Antiviral Activity

The effect of the venom and the various fractions on the viral activity was tested in three series of experiments. All

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experiments were done in duplicates, and at least in three separate sets, as follows:

Pre-Treatment of the Virus with the Venom

Samples of Sendai virions, each containing 70 Hemagglutinating Units (HAU), were incubated with various concentrations of the venom or its fractions in 50 µl PBS for 1 h at 37°C. Then one ml containing 10^8 washed human erythrocytes was added to each tube and the hemolysis assay was carried out as described above. As controls, erythrocytes were incubated with the venom or venom fractions only without virus, or with sample of Sendai virions in PBS without venom.

Pre-Absorption of the Erythrocytes with Sendai Virus before the Addition of Venom

Duplicate samples of 10^8 washed human erythrocytes were incubated with the Sendai virions for 15 min at 4°C. Excess virus was removed by washing twice with cold PBS and the pellets were resuspended in 1ml of cold PBS. Various concentrations of the venom were then added and the mixtures were incubated for another 2 hrs at 37°C in a shaking bath and centrifuged. The hemolysis was determined by measuring the absorbance of the supernatant at 540 nm.

Pre-Treatment of the Erythrocytes with Venom before the Addition of the Virus

Duplicate samples of 10^8 washed human erythrocytes were incubated with various concentrations of the venom in one ml PBS for one hr at 37°C. The cells were then washed twice with PBS before the addition of Sendai virions to each erythrocyte sample and the hemolysis was tested as described above. As controls, untreated erythrocytes were incubated with PBS or with the virus only.

Proteolytic Activity

Proteolytic activity was tested on three different substrates: azocoll, azocasein and gelatin. Aliquots containing different concentrations of the venom fractions were incubated separately with 10 mg of azocoll (insoluble dye - protein complex), in one ml of 0.1M phosphate buffer, pH 7.2, for 2 hr at 37°C in a shaking bath (120 strokes per min). The mixtures were centrifuged and the azocollase activity was estimated by measuring the absorbance of the clear supernatant at 520 nm. Caseinase activity was determined by incubating various concentrations of the venom fractions with 2% azocasein in one ml of 0.4M *Tris* buffer, pH 8.5, for 2 hr at 37°C in a shaking bath. The reaction was stopped by precipitating the undigested azocasein with 5% final concentration of trichloroacetic acid (TCA). The mixtures were centrifuged and the proteolytic activity was estimated by measuring the absorbance of the clear supernatant at 400 nm. Gelatinase activity was examined as follows: 50 µl PBS containing 80 µg of the isolated active fractions were applied to an undeveloped black and white Kodak film and incubated for 2 hr at 37°C in a moist chamber. The film was then washed thoroughly with tap water. A transparent spot on an opaque background indicated gelatin digestion. Trypsin alone (125 µg/ml) was used as control.

Purification of the Antiviral Factors from the Crude Venom

(i) Gel filtration on a Sephadex G-50

100 mg of lyophilized *Echis sochureki venom* were dissolved in 1 ml of phosphate buffer, 0.01M, pH 7.2, centrifuged at 5000 x *g* and applied on a Sephadex G-50 column (1 x 90 cm). The elution was performed with the same buffer at a flow rate of 2 ml/cm^2/h and the effluent was collected in 2-ml fractions. Each fraction was examined for antiviral activity as described above.

(ii) Chromatography on CM-Sepharose

The active fractions of the previous step were pooled and subjected to column chromatography on CM-Sepharose (5 x 0.6 cm) equilibrated with 0.01M phosphate buffer (pH 7.2). The column was washed by the same phosphate buffer before elution with a gradual increase of salt concentration (up to 0.3M NaCl). Each fraction was examined for antiviral activity as described above.

(iii) Chromatography on DEAE-Sepharose

The active fractions of the previous step were pooled and subjected to column chromatography on DEAE-Sepharose (10 x 1.0 cm) equilibrated with 0.01M phosphate buffer (pH 7.2). The column was washed by the same phosphate buffer before elution with a gradual increase of salt concentration (up to 0.3M NaCl). Each fraction was examined for antiviral activity as described above.

SDS-PAGE

The antiviral fraction alone (10 µg), virus alone (corresponding to 70 HAU), or virus (70 HAU) incubated for various periods of time with the antiviral fraction (10 µg), were run separately on 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) dissolved in 0.05M *Tris*-glycine buffer, pH 8.3. under denaturating conditions according to the SDS polyacrylamide gel electrophoresis (PAGE) method [10]. The gel was stained with 0.025% Coomassie brilliant blue in 25% isopropanol and 10% acetic acid.

RESULTS

Antiviral Activity of *Echis Carinatus Sochureki* Crude Venom

The effect of the crude venom on the hemolytic activity of Sendai virus was examined on human erythrocytes in three different experimental settings as described in Materials and Methods.

The hemolytic activity of the virions was inhibited in a dose response and temperature dependant manner when the virions were pre-treated with the venom of *Echis carinatus sochureki* (Fig. **1**). The inhibitory concentration that reduced the virus hemolytic activity by 50% (IC_{50}) was 30 µg/ml when the virions were pre-incubated with the venom for 2 hr at 37°C. However, prolonged incubation for up to 24 hr at 37°C or at 30°C resulted in an IC_{50} of 1 µg/ml and 5 µg/ml, respectively. Comparable results, showing less that 5% deviation in the IC50 values, were obtained in two additional

similar experiments. The effect of the temperature and the time of incubation on the IC_{50} indicated that the antiviral activity of the venom was probably a result of enzymatic digestion of a viral component(s).

Addition of the venom to erythrocytes, which were pre-absorbed with Sendai virus, did not affect the hemolysis caused by the pre-absorbed virions. Similarly, pre-treatment of the erythrocytes by up to 100 µg/ml of the venom before the addition of the virus did not affect the hemolysis caused by the Sendai virions. These results indicated that the venom inhibited the hemolytic activity of the virus by interacting with a free (non-attached) viral particle rather than with a cell component.

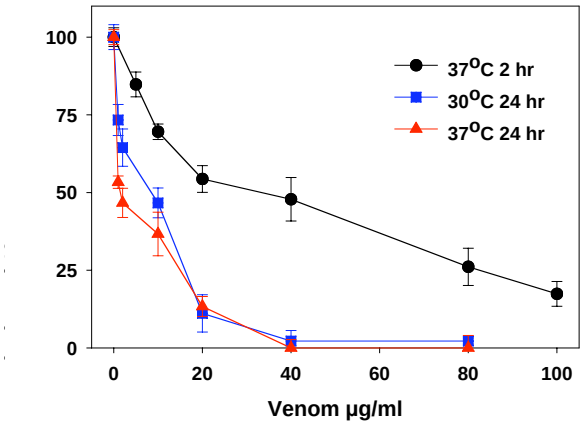

Fig (1). Effect of temperature and the incubation period on the antiviral activity. 100 µl samples containing 70 HAU of Sendai virus with various venom concentrations in 0.01M phosphate buffer, pH 7, were incubated at 30°C or 37°C for 2 hr or 24 hr. An hemolysis assay was carried out with the mixture, as described in the Methods section.

Isolation of the Active Factor from the Venom

We assumed that the venom active factor is a protein. This assumption was based on the above results, which indicated that the active fraction is an enzyme. In addition, the fact that Echinibin-1, the antiviral factor that we have previously isolated from the venom of a closely related snake, *Echis coloratus*, is a protein also contributed to this assumption [6]. Therefore, the various components of the *Echis sochureki* venom were fractionated by protein size chromatography, followed by ion and cation exchange chromatography of the antiviral active fractions (as detailed in the methods section).

Following chromatography on G-50 Sephadex, the antiviral activity was found in fractions 38-45 (Fig. **2A**) with a 12 fold increase in the specific activity of the pool (Table **1**). After subjecting the pooled fractions 38-45 to a CM-Sepharose column, the antiviral activity was found in the first peak C1 that was not absorbed to the column (Fig. **2B**). This peak showed a further 2-fold increase in the specific activity (Table **1**). Further fractionation of Fraction C1 on a DEAE-Sepharose column resulted in 3 peaks. None of the three peaks showed antiviral activity, unless they were pooled together, obtaining a similar specific activity as fraction C1.

Table 1. Isolation of the Antiviral Factor

IC_{50} Step (µg/ml)	Total	Protein (mg)	Specific Activity	Total Units	Yield % of Recovery
Crude Venom	100	30	1	3333	100
G-60 (S2)	6.25	2.5	12	2500	75
CM-Sepharose (C1)	1.5	1.2	24	1200	36

The IC_{50} was determined by incubating 70 HAU of fraction at each step of purification for 2 hr at 37°C described in the Methods. The standard deviation was Sendai virus with various concentrations of the antiviral and the mixture was examined in a hemolysis assay, as less than 5%.

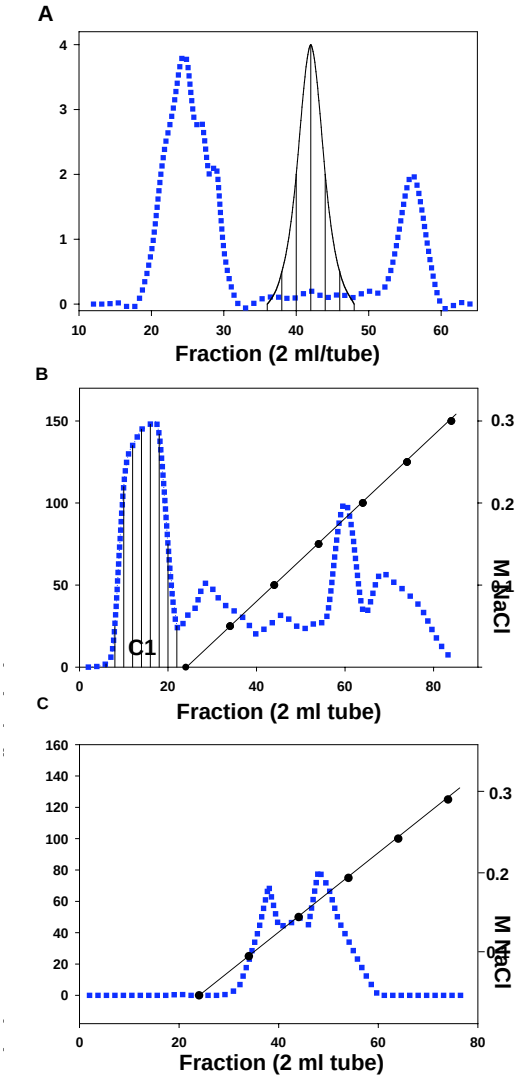

Fig (2). Isolation of the antiviral fractions from the venom of *Echis carinatus sochureki.* A) Whole venom was applied to a Sephadex G-50 column. The antiviral activity was found in fractions 38-45 (shadowed peak). B) The pooled fractions 38-45 (S2) of the previous step were subjected to CM-Sepharose column. The antiviral activity was found in the first peak C1 (shadowed peak) that was not absorbed to the column. C) Fraction C1 of the previous step was subjected to a DEAE-Sepharose column. None of the eluted fractions showed antiviral activity.

Characterization of the Active Fraction

SDS-PAGE electrophoresis of fraction C1 revealed one main band of 23.5 kDa and two other weaker bands with molecular weights of 27.5 and 18 kDa. The antihemolytic activity was thermo-labile: while no effect was observed on the antihemolytic activity of fraction C1 heated up to 50°C for 30 min, about 50% of the antihemolytic activity was lost after heating at 60°C; and was completely destroyed after heating at 80°C (Fig. **3A**). Similarly, exposure of fraction C1 to pH 5 or 8.8 for 2 hr resulted in about 50% reduction of the antihemolytic activity, and exposure to extreme pH such as 2.8 or 12.5 resulted in a complete loss of the antihemolytic activity (Fig. **3B**).

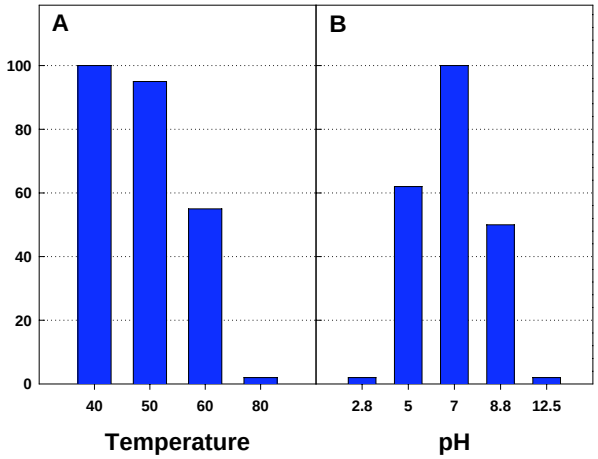

Fig. (3). A) Thermal stability of the antiviral activity. Samples containing 10 µg of fraction C1 were incubated for 30 min at various temperatures and cooled before they were incubated with Sendai virions for 24 hr at 30°C. The hemolytic activity of the incubated mixture containing the Sendai virions was examined by an hemolysis assay as described. B) Stability of the antiviral activity at various pH values. Samples containing 10 µg of fraction C1 were incubated with various buffer solutions having different pH values for 2 hr at room temperature. Each mixture was titrated to pH 7 by 0.5M phosphate buffer before incubated with Sendai virus and an hemolysis assay was carried out as described. Each experiment was performed twice and similar results were obtained.

As shown in Fig. (**4A**) and Fig. (**4B**), fraction C1 possesses azocollase, azocaseinase and gelatinase activities.

The finding that Echinibin-1, the antiviral factor isolated from *Echis coloratus*, is a metalloproteinase, indicated that specific proteolytic activity is needed for the inhibition of Sendai virus. Indeed, the antihemolytic activity of fraction C1 could be inhibited with 125 µg/ml EDTA, but not with 1 mM PMSF (Fig. **5**), which indicate that the antiviral fraction is not a serine protease but rather a metalloproteinase. The specificity of C1 towards the viral components is the 30-fold lower concentrations needed to digest the viral components (see below) than those needed (30 µg/ml) for complete digestion of azocoll or azocaseine (data not shown). In addition, while 1.2 µg/ml of fraction C1 inhibited 50% of the hemolytic activity of the virus (Table **1**), a hundred-fold concentration of trypsin (125 µg/ml) did not affect the hemolytic activity of the virions.

SDS PAGE of Sendai virions under reductive conditions resulted in multiple bands with molecular weights ranging between 30 to 200 kDa (Fig. **6c**). Incubation of the virus alone at 30°C overnight did not change this profile (Fig. **6e**).

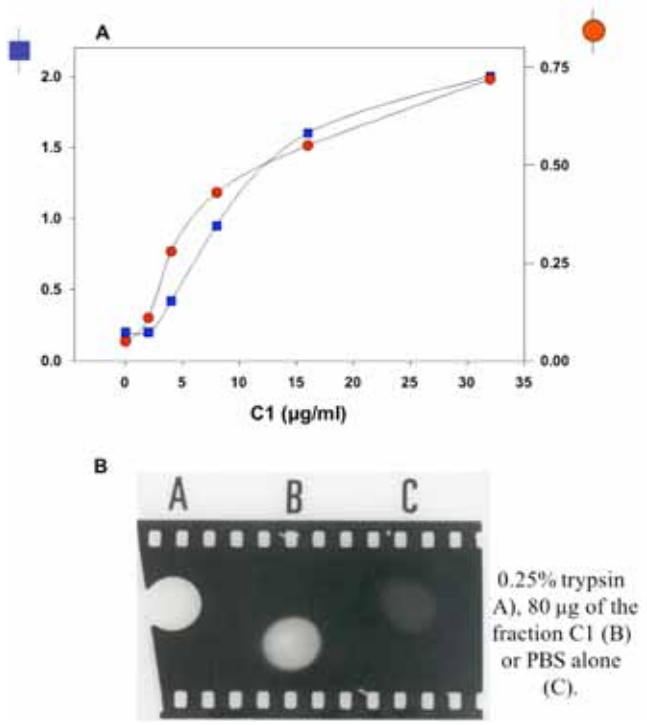

Fig. (4). A) Azocollase, azocaseinase and B) gelatinase activities of fraction C1 were examined as described in the methods section.

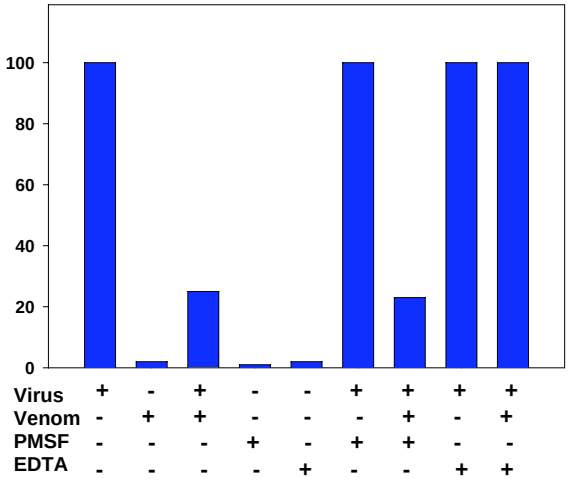

Fig. (5). Antiviral activity of fraction C1 after incubation with EDTA or PMSF. 10 µg aliquots of the venom fraction C1 were incubated with 1mM PMSF or with 125 µg/ml EDTA for 1 hr at 37°C. Then, each mixture was incubated with the Sendai virions for 1 hr before a hemolysis assay was carried out as described. PBS, EDTA or PMSF alone were added to the virions, as controls. Each experiment was performed twice and similar results were obtained.

However, incubation of the Sendai virions with fraction C1 resulted in the disappearance of several molecular weight bands already after 3 hr of incubation (e.g. see arrows in Fig. **6**). The longer the incubation period was, the stronger digestion of the viral bands occurred (Fig. **6g,i**), indicating

that the antiviral activity is related to the digestion of viral components.

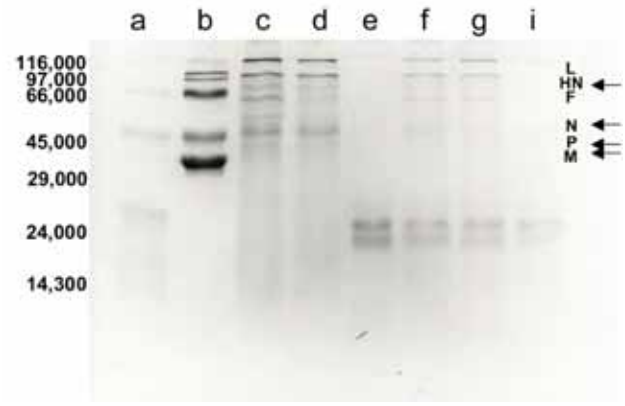

Fig. (6). SDS-PAGE of the Sendai virions incubated with fraction C1. Lanes a,b) markers with different Mol. wt; c) Sendai virus without pre-incubation; d) Sendai virus incubated overnight with PBS; e) Fraction C1 alone, without virions; f-i) Sendai virus incubated with fraction C1 for 20 min, 3 hr and overnight, respectively. L- Large protein; HN- Hemagglutinin-neurominidase protein; F- Fusion protein; N- Nucleocapsid protein; P- Phosphoprotein; and M- Matrix protein.

DISCUSSION

Among eleven viperid snake venoms that we screened for antiviral activity using a model of hemolysis of human erythrocytes by Sendai virus, the most powerful venoms were those of the snakes *Echis coloratus* and *Echis carinatus sochureki* [5]. A 25-kDa metalloproteinase antiviral factor, termed Echinibin-1, was isolated from the venom of *Echis coloratus* [6], which neutralizes Sendai virus by proteolytic digestion of viral components, including the HN glycoprotein responsible for the attachment of the virions to the target cells. The present work deals with the isolation of an antiviral fraction from the venom of *Echis carinatus sochureki*, another powerful antiviral venom [5]. The hemolytic activity of the Sendai virus is inhibited by more than 24 fold by the isolated active fraction C1 than by the crude venom. Further fractionation of this fraction with DEAE Sepharose resulted in a loss of the anti-hemolytic activity, indicating that all three fractions are needed for antiviral activity. SDS electrophoresis under denaturating conditions of C1 revealed that this fraction is composed of three bands with molecular weights of 18, 23.5 and 27.5 kDa. It is of interest that although *Echis carinatus sochureki* and *Echis coloratus* are closely related viper snakes, in the venom of the former snake a complex of 3 proteins is needed to neutralize the hemolytic activity of Sendai virus, while in the venom of the later, one protein, which exerts a similar mechanism of antiviral activity, is sufficient. The reason why these three factors do not show antiviral activity independently but only when they are combined is not clear, and further studies to elucidate the exact mechanism by which they exert their antihemolytic action are needed. Finding out if one on the three fractions obtained from the venom of *Echis carinatus sochureki* is similar to Echinibin-1, the antiviral protein isolated from the venom of *Echis coloratus*, should be investigated, for example by determining the sequence of the proteins, as well as by generating monoclonal antibodies against Echinibin-1 and determining its cross-reactivity with any of the three fractions now obtained from the venom of *Echis carinatus sochureki*.

As in the case of Echinibin-1, the antihemolytic activity is expressed only when the virions were pre-incubated with the active fraction. Once the virions were attached to the target cells, the antiviral fraction did not affect their following hemolytic activity.

Incubation of C1 with the virions, even for a short incubation of 20 min, resulted in the degradation of several viral protein bands. The hemagglutinin-neuraminidase (HN), matrix (M), phosphoprotein (P) and nucleocapsid (N) were completely digested already after 20 minutes of incubation. Longer incubation (overnight at 30°C) resulted in degradation of almost all viral protein bands. This included the large-polymerase (L) and fusion (F) glycoproteins, as the high molecular weight bands, corresponding to these glycoproteins disappeared (Fig. **6i**).

Interestingly, all bands, including those that represent the C1 fraction, fade over time, indicating a possible auto- and general proteolytic activity of the isolated proteins. Furthermore, no small molecular weight bands appear in the Coomassie blue stained gel (Fig. **6**) as well as in additional such similar experiments (data not shown), suggesting non-specific degradation of small proteins by the isolated C1 fraction. However, previously we did not find any correlation between the general proteolytic activity of several whole viperid venoms and their capacity to inhibit Sendai virus hemolytic activity [5]. Incubation of Sendai virus with 100 fold higher concentration of trypsin (125 μg/ml, compared to the IC_{50} of 1.2 μg/ml the active fraction), did not inhibit the hemolytic activity of Sendai virus. This result points to the increased specificity of the antiviral activity of fraction C1, rather than general strong proteolytic activity, as found in other venoms [5] or trypsin. The proteolytic and antihemolytic activities of the isolated fraction C1 were inhibited by EDTA but not by PMSF, indicating that the active fraction is a metalloproteinase.

Future studies should further characterize the proteolytic activities of fraction C1, which seems to possess sequence specificity. These studies should include purifying and sequencing the polypeptide fragments resulting from proteolysis. In addition, the capacity of fraction C1 to inhibit the hemolytic activities of other Sendai virus strains or of other viruses in other viral genus (e.g. Influenza A, New Castle Disease virus, etc.), should be studied.

CONCLUSIONS

This manuscript describes the isolation and partial characterization of a protein proteolytic fraction isolated from the venom of *Echis carinatus sochureki,* using Sendai virus as a substrate. The isolated fraction, C1, inhibits the hemolytic activity of Sendai virus through a mechanism of proteolysis in similar to that of Echinibin-1, a protein isolated from the venom of a closely related viper snake – *Echis coloratus*. It is therefore not surprising that the antiviral factors found in two closely related species have similar mechanisms of activity.

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