

A new approach to identification of African swine fever virus proteins

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Abstract. African swine fever virus (ASFV) is the sole member of Asfviridae family and it is responsible for African swine fever, which is highly contagious and fatal disease of pigs. Since the 1960s, inactivated vaccines, attenuated live vaccines, and subunit vaccines had been developed, but still there is no effective vaccine against it. In this study we used different methods of protein precipitation coupled with an RP-HPLC method to analyze the ASFV proteins. The aim of this work was to develop a sensitive reversed-phase HPLC method, applicable for the identification of specific proteins in the African swine fever vaccine. Two different stationary phases were used for protein separation and identification, *i.e.*, C4 and C18. These columns present different hydrophobic properties, with an impact on protein separation. In the aim of protein separation by HPLC, some methods of protein precipitation were used, as follows: saline and acid precipitation, trypsin action, and disulfide bonds reduction by dithiothreitol. The saline and acid protein precipitation were used at a medium value of saturation percent of ammonium sulfate and trichloroacetic acid, respectively, in a reaction medium (30–60% ammonium sulfate saturation and 40% trichloroacetic acid saturation, respectively). For trypsin treatment of proteins, a 1% aqueous solution of trypsin was used, so that viral proteins would undenatured. In the case of dithiothreitol, a 100 mM aqueous solution was used, so that secondary structure of proteins is maintained. Some major polypeptides with molecular weights of 10,000 – 70,000 Da were found by using the protein precipitation methods coupled with the proposed HPLC method. The cell culture medium used for viral particle growing was treated in the same manner as viral suspension. The protein precipitation coupled to the HPLC method has shown that only viral suspension contains polypeptides with molecular weights of 10,000 – 70,000 Da. Protein precipitation coupled to the HPLC proposed method applied to cell culture medium has not shown specific viral polypeptides and proteins, and the chromatographic profile is different from that of viral suspension.

Keywords: high performance liquid chromatography; African swine fever virus; structural proteins; vaccine; viral infection.

1. Introduction

African swine fever virus (ASFV) is an enveloped, large double stranded DNA virus, with icosahedral symmetry. It is the only member of the family Asfarviridae. The double stranded DNA genome consists of 170,000 – 190,000 bp (base pairs) [1, 2]. The ASFV genome encodes the proteins for viral assembly and replication, inhibition of cellular apoptosis and viral proliferation [3]. The ASFV genome encodes over 60 structural proteins and 100 non-structural proteins [4, 5]. The structure of ASFV includes an external envelope membrane, a capsid, an inner membrane, a core shell, and an inner core [6]. The protein composition is specific for every part of virion, including a range of 34–54 polypeptides, with a molecular weight in the range of 10,000–150,000 Da [1, 7]. The proteins of external envelope membrane are involved in viral attachment to the cell and in endocytosis [3, 8, 9]. The p72 protein is capsid specific, having an important role in antibody response [10]. It was observed that the blood of ASFV–infected pigs contain mostly p72 antigens [6]. The specific proteins of inner membrane are p12, p17, and p54. Protein p12 together with core shell specific proteins p14, p34, p150, p35, and p15 have an important role for the assembly of the viral capsid [7, 10, 11].

Previous immunological investigations have shown that the inner membrane protein p54 is secreted in the first stage of viral infection [1, 2, 7]. The SDS-PAGE studies of virion particle have shown the inner core specific p10 protein, which binding DNA [7].

Structural and functional analysis of these proteins is essential to the future development of antiviral vaccines. At present, there is no commercial vaccines, even the disease spreads rapidly and the mortality is 100% in swine [12].

The earlier studies about ASFV proteins purification were described by Polatnick et al. [13]. The importance of this method is the use of sucrose and cesium chloride gradients for purification of viral particles. Tabares et al. [14] have modified previous method by using Tween 80 and sucrose-NaCl gradient. After 2-mercaptoethanol treatment of viral suspension, the SDS – PAGE studies have shown that some polypeptides are eliminated (polypeptides VIII, XII, XXI, XXIV) [15]. Carrascosa et al. have reported a Percoll density gradient purification of ASFV [15]. The SDS-PAGE and immunoprecipitation test of ASFV proteins after purification have shown about 34 structural proteins of ASFV. Five major ASFV polypeptides, with molecular weights of 125,000 Da (VP1), 76,000 Da (VP20),

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50,000 Da VP3), 44,000 Da (VP4) and 39,000 Da (VP5) were found by treatment with Tween 80 and the use of 1 M NaCl in sucrose gradient [16].

In this study, for separation and identification ASFV proteins, differently methods for protein purification, another that previously described in literature, coupled with a RP-HPLC method were used. The protein precipitation methods were conducted so that only polypeptides with molecular weights of 10,000-70,000 Da sediments in the reaction medium. In this way, the specific viral proteins would separate from polypeptides of cell culture medium, and from another viral proteins. Two HPLC columns, C4 (with butyl group as functional group) and C18 (with octadecyl silyl group as functional group), having differently hydrophobic properties, were used in this work. The C4 column is less hydrophobic than C18, so that the proteins migrate easier through stationary phase particles. The adsorption at surface of less hydrophobic column is smaller than for a more hydrophobic one, leading to a better separation of proteins. The mobile phase played an important role in HPLC analysis. Two different mobile phases were used, as follows: a phosphate buffer saline, having a neutral pH (pH 7.2-7.4), and a mix of acetonitrile and 1% aqueous H_3PO_4 solution, having an acidic pH (pH 1.5-2.0). The chromatographic runs of molecular weight standard proteins have shown a better separation at low levels of mobile phase pH, with a resolution factor over 1.0 and a peak asymmetry value around 2.0. The temperature seems not to play a significant role for a better protein identification by HPLC; a good resolution was obtained at 30 °C as at 25 °C. The integrity of tertiary and secondary structure of protein depends on temperature, so that we performed the HPLC analysis at a medium value of temperature. The presence of VP 72 protein in viral suspension was confirmed by ELISA and SDS-PAGE. Starting from these results, we performed our studies for protein precipitation so that viral proteins with molecular weights around 70,000 Da or less were identified.

2. Experimental

2.1. Materials and reagents

The viral suspension of ASFV was obtained from Institute for Diagnosis and Animal Health (Bucharest, Romania). The research was performed by comparative tests, using a cells culture medium (Gibco MEM – Fischer Scientific). The molecular weight standards were used, as follows: Pierce Intact Protein Standard Mix (Thermo Fisher Scientific Baltics) and *p*-aminobenzoic acid (Merck). Acetonitrile, CH_3CN , (HPLC grade) was purchased from Fischer Scientific (Loughborough). Phosphoric acid, H_3PO_4 , was purchased from Merck. Milli Q water was obtained using a Milli Q Ultrapure Lab Water System (Millipore Merck). Dithiothreitol ($\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$), ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), trichloroacetic acid (CCl_3COOH), trypsin powder, sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), potassium chloride (KCl), Tris (tris hydroxymethyl aminomethane, $(\text{CH}_2\text{OH})_3\text{CNH}_2$), and hydrochloric acid (HCl) were purchased from Merck.

All solutions (40% CCl_3COOH , phosphate buffer saline PBS pH 7.2-7.4, 1% trypsin, 1% H_3PO_4 , 100 mM $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$, 200 mM Tris-HCl pH 8) were prepared in house using Milli Q water.

2.2. HPLC analysis

All analytical development was performed on a DIONEX ULTIMATE 300 HPLC system (Thermo Fisher Scientific, USA) with an RS pump, RS autosampler, RS Column Compartment, and RS Diode Array Detector.

As stationary phases, a Kromasil C4 (150 x 4.6 mm, 3.5 μm - Akzo Nobel) and Ascentis Express Peptide ES C18 (100 x 2.1 mm, 2.7 μm - Merck) analytical columns were used. For detection of viral proteins using a C4 reverse phase column, a gradient elution of CH_3CN /1% aqueous H_3PO_4 solution (v/v) (10% $\rightarrow$ 50% CH_3CN) was used, at the flow rate of 0.7 ml/min and a UV detection at 220 nm. The chromatography runs were performed at 30 °C and the injection volume was set to 20 μl . Viral proteins analysed by Express Peptide C18 column were run in an isocratic system consisting 100% phosphate buffer saline (PBS) solution, pH 7.2-7.4. The flow rate was maintained at 0.3 m/min and UV detection was set at 220 nm. The chromatography runs were performed at 25 °C and the injection volume was set to 5 μl . For filtering test probes, disposable syringe filters (Titan 3, 30 mm, PVDF, 0.45 μm - Thermo Fisher Scientific) were used. For the integration of analysis data, the Chromeleon 7.2.10 was used. The working standard Pierce Intact Protein Standard Mix was prepared with a concentration of 76 μg /500 μl mobile phase (CH_3CN /1% aqueous H_3PO_4 solution, 100/ 900 v/v, 100% PBS solution respectively). The working standard of *p*-aminobenzoic acid was prepared with a concentration of 0.1 mg/ml in the mobile phase (CH_3CN /1% aqueous H_3PO_4 solution, 100/900 v/v, 100% PBS solution respectively).

The viral proteins identification was performed by analysis of retention times of protein standards, by using the Chromeleon integrator. There is a linear dependence between retention time of protein and its molecular weight. Each component of protein standards, used in our study, has a specific retention time on the stationary phase. By using the relation between molecular weight of standard protein and its specific retention time, the molecular weights of viral proteins were calculated.

2.3. Purification of virus particles by saline precipitation

Saline precipitation of viral proteins was performed according to method described by Iordachescu et al. [17]. The virus fluids were diluted ten times in phosphate buffer saline (PBS) and then treated with $(\text{NH}_4)_2\text{SO}_4$, in two steps (from 30% to 60% saturation of $(\text{NH}_4)_2\text{SO}_4$). For each 5 ml of diluted virus suspension, 0.8 g of $(\text{NH}_4)_2\text{SO}_4$ were added. Saline precipitation was conducted at 4 °C, for 30 min, on an orbital shaker. The mixture was sonicated in water bath sonicator for 30 min, followed by centrifugation at 4000 g, at 4 °C. The pellets were resuspended in minimum volumes of PBS.

2.4. Purification of virus particles by acid precipitation

Acid precipitation of viral proteins was performed according to method described by Iordachescu et al. [18]. The virus fluids and cells culture medium were diluted ten times in phosphate buffer saline (PBS). For each ml of diluted virus fluids and each ml of diluted cells culture medium, 110 μ l of 40% CCl_3COOH aqueous solution were added. The mixtures were incubated at room temperature for 2 h and then centrifugated at 4000 g, at 4 °C. The pellets were resuspended in minimum volumes of PBS.

2.5. Purification of virus particles by trypsin action

Protein trypsin digestion was performed according to modified procedure adapted from the method described by Hustoft et al. [19]. A reaction mixture containing 1 ml virus fluids (previously diluted ten times in PBS) and 1 ml of 0.5 mg/ml of trypsin in 0.2 M Tris-HCl buffer pH 8 was prepared and incubated at 50 °C for 2 h. A reaction mixture containing 1 ml cells culture medium (previously diluted ten times in PBS) and 1 ml of 0.5 mg/ml of trypsin in Tris-HCl 0.2 M pH 8 buffer was prepared and incubated at 50 °C for 2 h. Each mixture was then centrifugated at 4000 g, at 4 °C and the pellets were resuspended in minimum volumes of PBS.

2.6. Disulfide bonds reduction by dithiothreitol

Reducing disulfide bonds of polypeptide chain was performed according to modified procedure adapted from the method described by McQueen et al. [20]. A reaction mixture containing 100 μ l PBS, 15 μ l of 100 mM $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$ (aqueous solution), 100 μ l virus fluids (previously diluted ten times in PBS), and 10 μ l distilled water was prepared and incubated at 95 °C for 5 min. After cooling, the mixture was diluted five times in $\text{CH}_3\text{CN}/1\%$ aqueous H_3PO_4 solution (100/900, v/v). A reaction mixture containing 100 μ l PBS, 15 μ l of 100 mM $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$ (aqueous solution), 100 μ l cells culture medium (previously diluted ten times in PBS), and 10 μ l distilled water was prepared and then incubated at 95 °C for 5 min. After cooling, the mixture was diluted five times in $\text{CH}_3\text{CN}/1\%$ aqueous H_3PO_4 solution (100/ 900, v/v).

2.7. Polyacrylamide gel electrophoresis

The proteins of viral suspension were dissociated by heating the sample in a 50 mM Tris buffer (pH 6.8) with 1% Zwittergent 3-14, 1% sodium dodecyl sulfate, $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$, 5% 2-mercapto ethanol, $\text{HOCH}_2\text{CH}_2\text{SH}$, for 5 min, in block heater. Electrophoresis was carried out in 4-20% polyacrylamide gels by the method of Laemmli [21], in the presence of marker proteins of known molecular weight (11,000 – 245,000 Da).

2.8. ELISA assay

The VP72 protein content of viral suspension was determined by double antibody sandwich ELISA technique, using monoclonal antibodies specific to VP72 of ASFV. The ELISA assay was performed by using Ingezim PPA DAS kit from GOLD STANDARD DIAGNOSTICS, Hungary. The plate is coated with a specific monoclonal antibody (Mab) to VP72 of the ASFV. In this way, when a sample is added into the

wells, if they contain antigen, the coated Mabs will catch it. After washing, a second Mab to VP 72 (specific for a different epitope) conjugated with peroxidase, is added. If there is an antigen captured into the wall, the conjugate will bind it and the peroxidase action will be detected after adding an adequate substrate. The samples with optical density higher than 0.30 must be considered positive to ASFV. The samples with optical density higher than 0.15 must be considered negative to ASFV.

3. Results and discussion

The aim of this study was to develop a RP-HPLC method for separation and identification ASFV proteins. For protein separation and purification, RP-HPLC is more sensitive and rapid method than other chromatographic methods. The RP-HPLC has a nonpolar stationary phase and an aqueous, moderately polar mobile phase. The protein separation depends on the hydrophobicity of mobile phase and the binding of dissolved protein to hydrophobic ligands of stationary phase [22]. Preliminary studies have involved trying C4 and C18 reversed-phased columns from different suppliers and testing several chromatographic conditions (composition of mobile phase, temperature, flow), with good chromatographic parameters (good symmetry, minimized peak tailing, good resolution, number of plates). The optimization of chromatographic separation is a critical factor for maximizing the separation of studied proteins. Optimal separation of proteins depends on the relationship between stationary and mobile phase, the ionic strength of mobile phase playing a key role. For a better choice of chromatographic system, the chromatographic profiles of protein standards used in our study were determined. By using a C4 column and an acidic pH (pH 1.5-2.0), all components of standards mix were resolved. A shorter alkyl chain (a butyl functional group) bonded to silica is less hydrophobic than a C18 column (18-carbon chain), leading to reduced carryover of nonpolar compounds. The resolution values were 2.0–3.0 and the peaks asymmetry of 2.0. By using a C18 column we have not obtained resolution between the components of protein standard, and some of them have crossed together through the column. The same results were obtained if we used a neutral pH in the presence of phosphate saline buffer (PBS) as mobile phase. Even the all components of protein standards were not resolved on a C18 column, the results at an acidic pH were better than at neutral one, with a resolution value at 1.0 and an asymmetry of 1.5–3.5. So that, we considered to choose a mobile phase with acidic pH (pH less than 2.0). Another important parameter for choosing the better chromatographic system was theoretical plates. By using the C4 column and a mobile phase with pH less 2.0, the values of theoretical plates were over 30000. In the case of C18 column and an acidic mobile phase, the theoretical plates values were in the same range, but by using a neutral mobile phase, these values are around 150. The same results (peaks asymmetry, resolution, theoretical plates) were obtained by running viral suspension and cell culture medium on C4 and C18 column, with acidic and neutral mobile phase. The value of UV detection

was set at 220 nm according to literature [22- 24]. Since the UV detection was the same, the experimental results indicated that the temperature not play a key role here. Based on our observations, we performed the purification and identification viral protein by RP-

HPLC on a C4 column and an acidic mobile phase ($\text{CH}_3\text{CN}/1\%$ aqueous H_3PO_4 solution, 100/900, v/v). At a lower pH of mobile phase, the adsorption-desorption steps on stationary phase are optimum than at a neutral pH (pH 7.2-7.4).

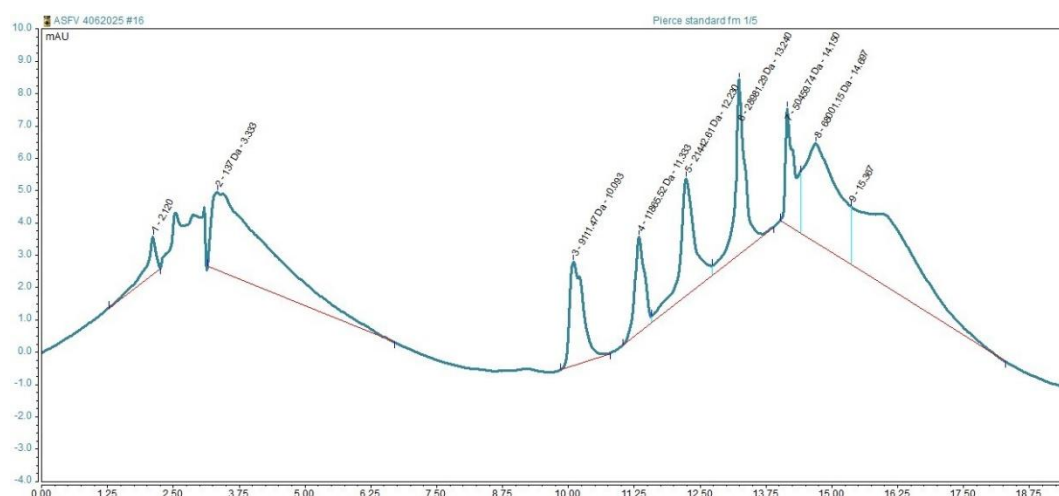


Figure 1. Chromatograms of *p*-aminobenzoic acid PABA (molecular weight 137 Da – retention time RT – 3.33 min) and Pierce Intact Protein Standard Mix (molecular weights of 9111.47 Da – Human IGF – retention time RT = 10.09 min; 11865.52 Da – Human Thioredoxin – retention time RT = 11.33 min; 21442.61 Da – Protein G – retention time RT = 12.23 min; 28981.29 Da – Bovine Carbonic Anhydrase II – retention time RT = 13.24 min; 50459.74 Da – Protein AG – retention time RT = 14.15 min; 68001.15 Da – E. coli Exo Klenow – retention time RT = 14.697 min). The HPLC runs were performed using a reverse phase C4. Graphical representation of peak area (mAU) vs retention time (min); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.

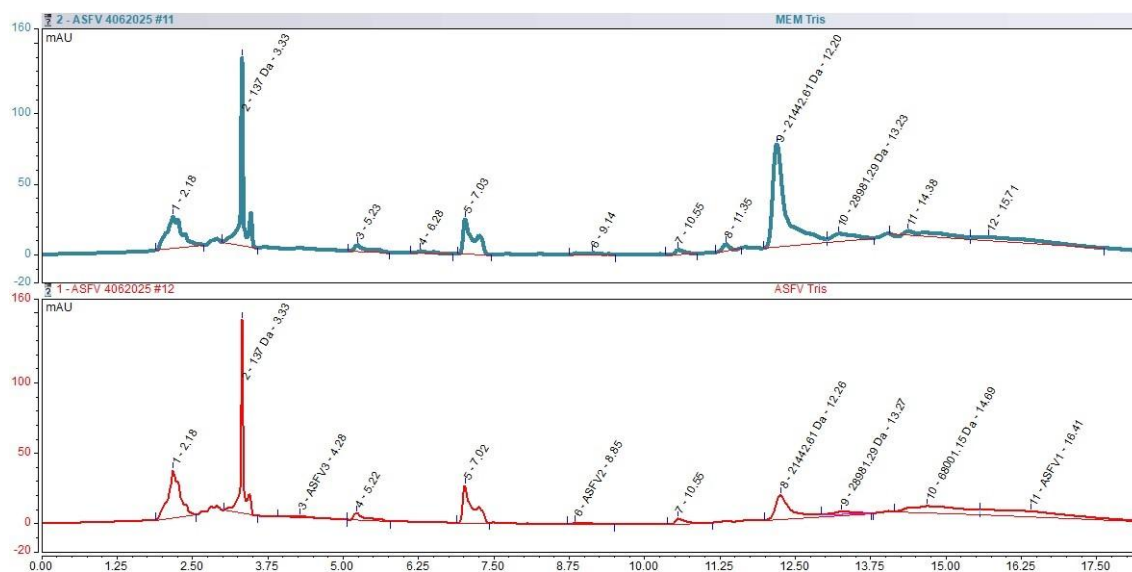


Figure 2. A comparison between chromatographic profile of ASFV suspension and cellular culture medium. The HPLC runs were performed using a reverse phase C4 (red – ASFV suspension, blue – cells culture medium; the p72 protein with molecular weight of 68,000–72,000 Da at a retention time RT = 14.69 min, other viral proteins at a retention time RT = 4.28 min, RT = 8.85 min, and RT = 16.41 min). Graphical representation of peak area (mAU) vs retention time (min); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.

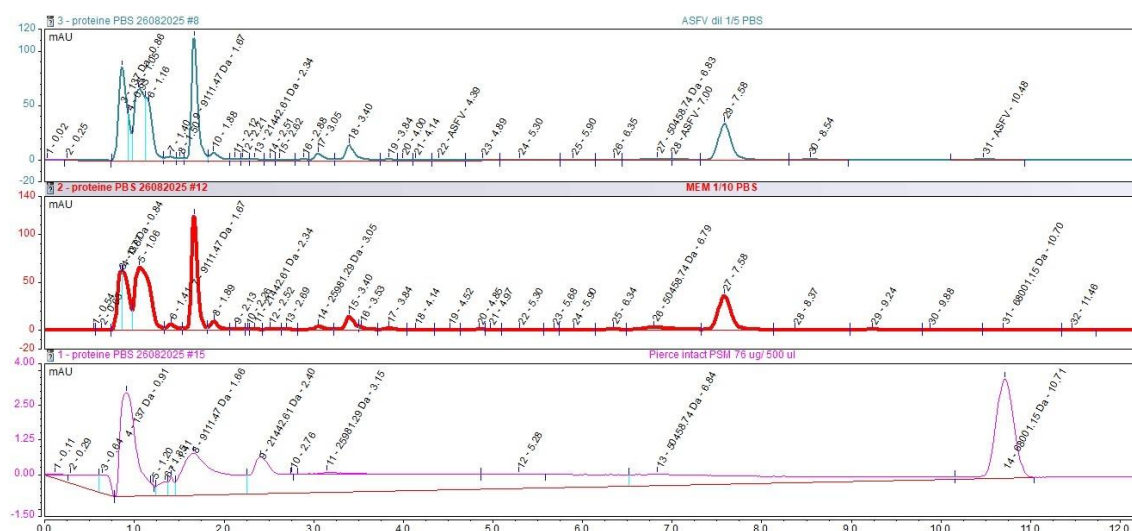


Figure 3. A comparison between chromatographic profile of ASFV suspension and cellular culture medium. The HPLC runs were performed using a reverse phase C18 (blue – ASFV suspension, red – cells culture medium, violet – molecular weight standards; the p72 protein with molecular weight of 68,000–72,000 Da at a retention time RT = 7.00 min; other viral proteins at a retention time RT = 4.39 min and RT = 10.48 min). Graphical representation of peak area (mAU) vs retention time (min); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.

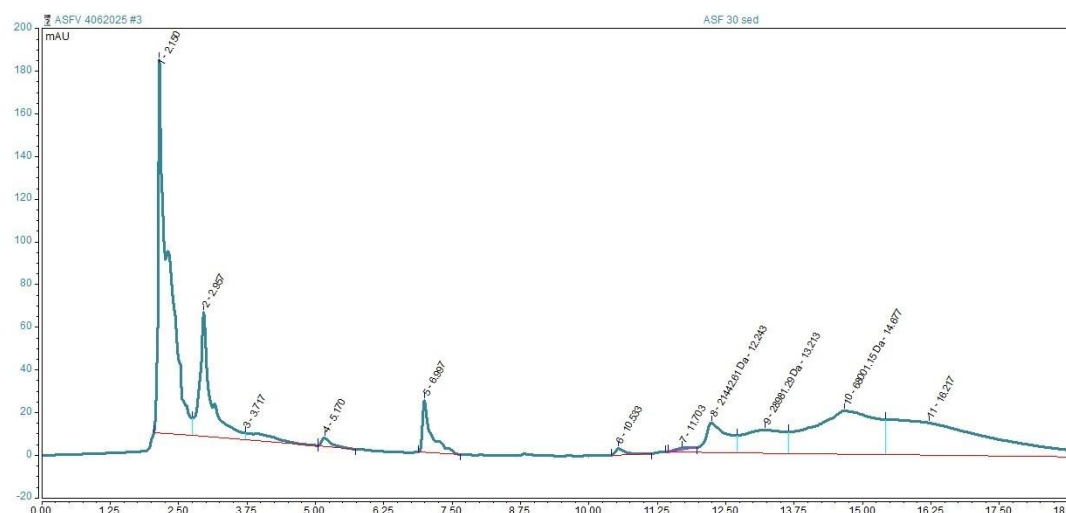


Figure 4. HPLC analysis of ASFV suspension after saline precipitation (30% – 60% $(\text{NH}_4)_2\text{SO}_4$ saturation) (the p72 protein with molecular weight of 68,000–72,000 Da at retention time RT = 14.677 min). Graphical representation of peak area (mAU) vs retention time (min); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.

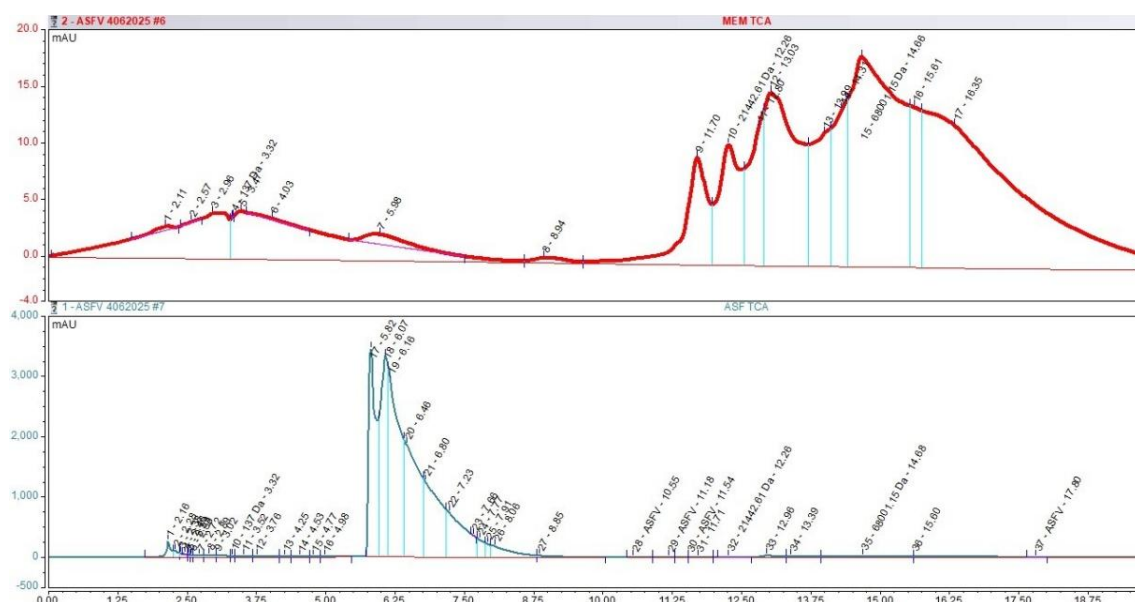
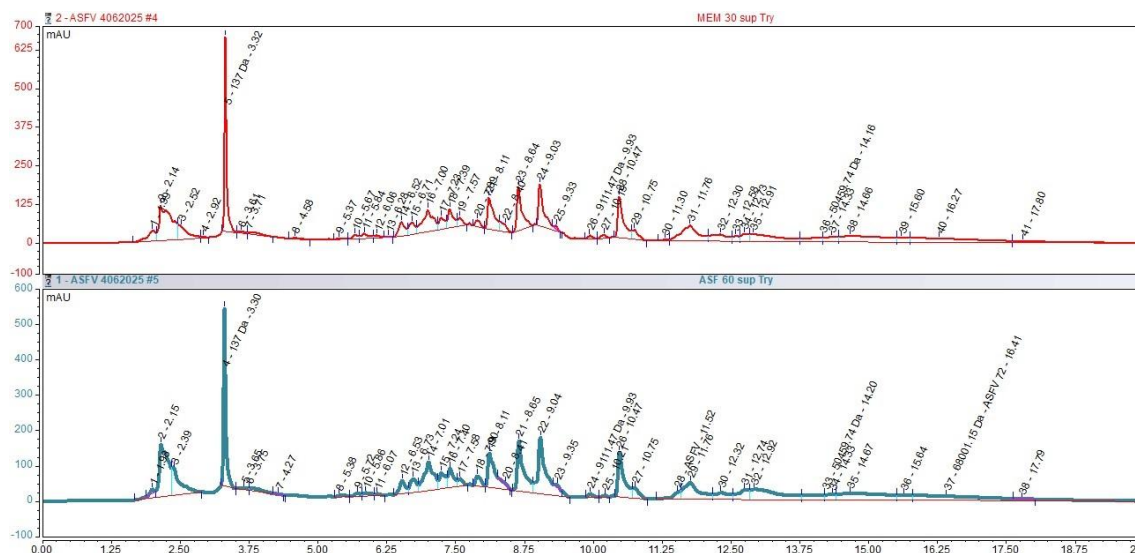


Figure 5. A comparative HPLC analysis of ASFV suspension and cell culture medium after precipitation with 40% CCl_3COOH aqueous solution (blue – ASFV suspension, red – cells culture medium; the p72 protein with molecular weight of 68,000–72,000 Da at a retention time $\text{RT} = 17.80$ min; other viral proteins at a retention time $\text{RT} = 10.55$ min, $\text{RT} = 11.18$ min, and $\text{RT} = 11.54$ min). Graphical representation of peak area (mAU) vs retention time (min); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.



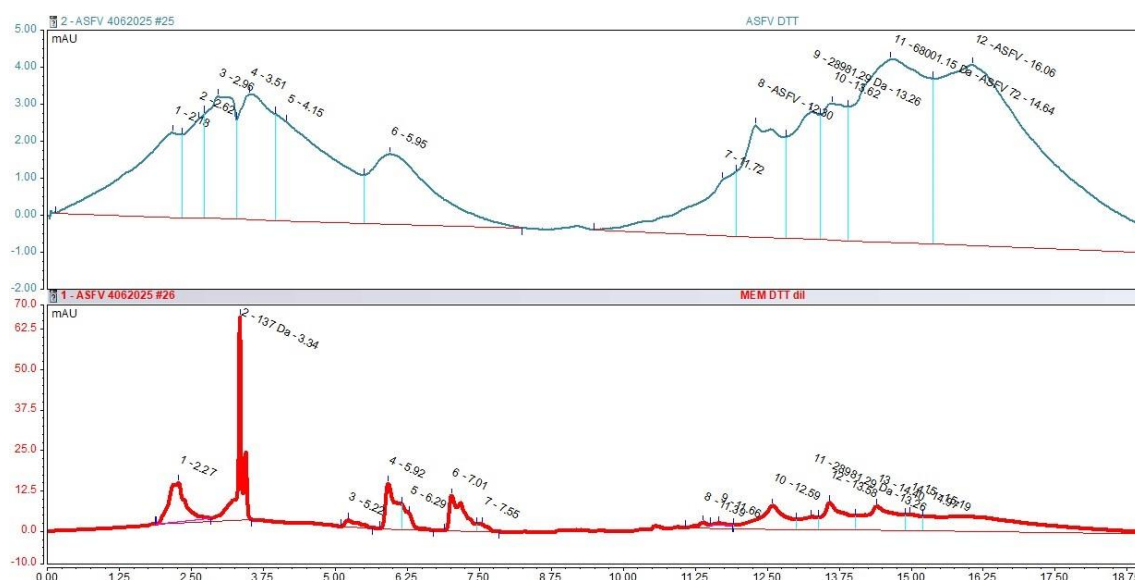


Figure 7. A comparison between chromatographic profile of ASFV suspension and cellular culture medium after treatment with 100 mM $C_4H_{10}O_2S_2$ solution (blue – ASFV suspension, red – cells culture medium; the p72 protein with molecular weight of 68,000–72,000 Da at a retention time $RT = 14.64$ min; other viral proteins at a retention time $RT = 12.30$ min and $RT = 16.06$ min) Graphical representation of peak area (mAU) vs retention time (min.); the peak labels are, as follows: the first number represents the peak number, the last number represents the retention time, the middle number represents the peak name.

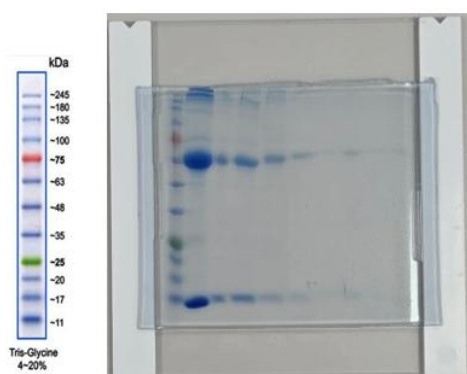


Figure 8. SDS-PAGE analysis of viral suspension

Chromatography is a technique used to separate, identify and purify molecules, for qualitative and quantitative analysis. There are two common approaches for protein separation: size exclusion chromatography and affinity chromatography. Besides them, ion exchange chromatography and reverse phase chromatography are successfully used [24]. The RP-HPLC has successfully provided a means for qualitative and quantitative analysis of ASFV particle proteins. The results presented in this paper show that ASFV can be freed from cellular constituents by different biochemical treatments. The components of cell culture medium are not adsorbed at the surface of stationary phase, and thus, not interfere in protein separation. Our experiment of disruption of ASFV particle shown that up to five proteins can be separated by RP-HPLC, with molecular weight in the range of 10,000–70,000 Da. The results were in concordance with ELISA and SDS – PAGE assay of viral suspension. The SDS – PAGE assay shown the presence of proteins with molecular weight of 11,000–17,000 Da, as well as a protein with molecular weight of 63,000 – 75,000 Da. ELISA test shown the optical density values of viral suspension samples in the range of 0.3–0.7. The electrophoretic profile of viral

suspension shown proteins with molecular weights of 10,000–70,000 Da, as well as proteins with molecular weights above 70,000 Da. By ELISA assay, the presence of viral protein 72 (molecular weight of 72,000 Da) in viral suspension was confirmed. In this study we have shown that a RP-HPLC method could be used for separation of viral proteins. On the basis of the published evidence [25], as well as the results presented in this study, ASFV has a complex protein structure. Our experiments on the disruption of ASFV with $(NH_4)_2SO_4$, CCl_3COOH , $C_4H_{10}O_2S_2$ and trypsin have shown a major protein with molecular weight of 68,000 Da (VP72 protein) and other proteins with molecular weights in the range of 10,000–20,000 Da. The p72 protein has highly antigenic and immunogenic characters and is a major viral component. It is a major capsid protein and is expressed in the later stages of viral infection. In all experiments of protein separation and identification, a protein with molecular weight around 70,000 Da was obtained. The peak area of 70,000 Da protein had the same values in all chromatographic runs of experiment. Our proposed RP-HPLC method has shown the presence of some viral proteins of molecular weights of 10,000–70,000 Da. All experiments were conducted to purify proteins in the range of molecular weight of 10,000–70,000 Da. On the basis of chromatographic analysis of retention times of molecular weight standards, molecular weights of ASFV proteins were identified. The same experiments were done on cell culture medium (saline and acid precipitation of proteins, dithiothreitol and trypsin treatments). The chromatographic profiles of cell culture medium, after each precipitation steps, were totally different from chromatographic profile of viral suspension by HPLC. Proteins with molecular weights of 10,000–20,000 Da, and molecular weight of 70,000 Da, respectively, were not identified. The proposed HPLC method could differentiate between the viral proteins and the proteins and polypeptides of cell culture medium. This fact

demonstrates capacity of method to identify a ASFV proteins from viral suspension. From the analysis of chromatograms, it was found that there is no interference between the viral proteins and the polypeptides and proteins of cells culture medium.

4. Conclusions

The RP-HPLC methods are usually used for separation and quantification polar and nonpolar chemical substances. By using an appropriate combination of mobile and stationary phase, temperature, rate flow, and UV detector we can successfully separate proteins by RP-HPLC. RP-HPLC is more accurate and rapid than affinity, size exclusion or ion exchange HPLC methods. The stationary phase used for protein separation is simple, common in all HPLC laboratories. The mobile phase used in this study is simple to prepared, CH₃CN and aqueous solution of H₃PO₄ are well known reagents used in high performance chromatography techniques. The use of different methods of fragmentation and partial purification of proteins allowed the chromatographic separation and identification of ASFV proteins. Protein precipitation experiments were performed so that proteins with molecular weight in the range of 10,000–70,000 Da were separated. By RP-HPLC analysis of samples, after different methods of protein precipitation, this range of molecular weights was confirmed. The results of protein separation and identification by RP-HPLC were in concordance with SDS-PAGE and ELISA assay of viral suspension. Additional experiments are needed to illustrate the presence of the other viral proteins, although extensive studies have been carried out. We have tried to demonstrate that a simple RP-HPLC method could be used for identification of ASFV proteins. Because a protein with molecular weight around 70,000 Da was separated and identified by the proposed method, it can be used as a subsequent purification of VP72 protein from viral suspension.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

The research work was financed from project ADER 10.1.1. - "Research on the development of immunoprophylactic and biocidal products usable in the control of African swine fever and epidemiological risk factors in Romania".

References

- [1]. A. Esteves, M.I. Marques, J.V. Costa, Two-dimensional analysis of African swine fever virus proteins and protein induced in infected cells, *Virology* 152 (1986) 192-206. [https://doi.org/10.1016/0042-6822\(86\)90384-3](https://doi.org/10.1016/0042-6822(86)90384-3)
- [2]. R.J. Yañez, J.M. Rodriguez, M.L. Nogal, L. Yuste, C. Enriquez, J.F. Rodriguez, E. Vinuela, Analysis of the complete nucleotide sequence of African swine fever virus, *Virology* 208 (1995) 249-278.
- [3]. K. Zhang, S. Li, S. Liu, S. Li, L. Qu, G.F. Gao, Spatiotemporally orchestrated interactions between viral and cellular proteins involved in the entry of African swine fever virus, *Viruses* 13 (2021) 2495. <https://doi.org/10.3390/v13122495>
- [4]. G. Cackett, D. Matelska, M. Sykora, R. Portugal, M. Malecki, J. Bähler, The African swine fever virus Transcriptome, *Journal of Virology* 94 (2020) e00119-20. <https://doi.org/10.1128/jvi.00119-20>
- [5]. G.L. Shimmom., J.Y.K. Hui, T.E. Wileman, C.L. Netherton, Autophagy impairment by African swine fever virus, *Journal of General Virology* 102 (2021) 001637. <https://doi.org/10.1099/jgv.0.001637>
- [6]. G. Wang, M. Xie, W. Wu, Z. Chen, Structures and functional diversities of ASFV proteins, *Viruses* 13 (2021) 2124. <https://doi.org/10.3390/v13112124>
- [7]. A.L. Carrascosa, M. del Val, J.F. Santaren, E. Vinuela, Purification and properties of African swine fever virus, *Journal of Virology* 54 (1985) 337-344.
- [8]. I. Galindo, M.A. Cuesta-Geijo, K. Hlavova, R. Muñoz-Moreno, L. Barrado-Gil, J. Dominiguez, African swine fever virus infects macrophages, the natural host cells, via clathrin- and cholesterol-dependent endocytosis, *Virus Research* 200 (2015) 45-55. <https://doi.org/10.1016/j.virusres.2015.01.022>
- [9]. J. Mercer, M. Schelhaas, A. Helenius, Virus entry by endocytosis, *Annual Reviews of Biochemistry* 79 (2010) 803-833. <https://doi.org/10.1146/annurev-biochem-060208-104626>
- [10]. G. Andres, D. Charro, T. Matamoros, R.S. Dillard, N.G.A. Abrescia, The cryo-EM structure of African swine fever virus unravels a unique architecture comprising two icosahedral protein capsids and two lipoprotein membranes, *Journal of Biological Chemistry* 295 (2020) 1-12. <https://doi.org/10.1074/jbc.AC119.011196>
- [11]. S. Liu, Y. Luo, Y. Wang, S. Li, Z. Zhao, Y. Bi, Cryo-EM structure of the African swine fever virus, *Cell Host Microbe* 26 (2019) 836-843.
- [12]. Z. Wang, Q. Ai, S. Huang, Y. Ou, Y. Gao, T. Tong, Immune escape mechanism and vaccine research progress of African swine fever virus, *Vaccines (Basel)* 10 (2022) 344. <https://doi.org/10.3390/vaccines10030344>
- [13]. J. Polatnick, I.C. Pan, M. Grawell, Protein kinase activity in African swine fever, *Archiv fur die Gesamte Virusforschung* 44 (1974) 156-159.
- [14]. E. Tabares, F.R. Gonzalvo, M.A. Marcotegui, A. Ordas, Growth, purification and fractionation of African swine fever virus, *Commission of the European Communities, Agricultural Research Seminar on Hog Cholera* (1977) 507-531.
- [15]. A.L. Carrascosa, M. del Val, E. Vinuela, Purification and properties of African swine fever

- virus, *Journal of Virology* 54 (1985) 337-344. <https://doi.org/10.1128/jvi.54.2.337-344.1985>
- [16]. D.N. Black, F. Brown, Purification and physicochemical characteristics of African swine fever virus, *Journal of General Virology* 32 (1976) 509-518.
- [17]. D. Iordachescu, I.F. Dumitru, in: *Universitatea Bucuresti (Eds.), Biochimie practica*, 2nd Edition, 1988, pp. 55-57 (in Romanian).
- [18]. D. Iordachescu, I.F. Dumitru, in: *Universitatea Bucuresti (Eds.), Biochimie practica*, 2nd Edition, 1988, pp. 64 (in Romanian).
- [19]. H. Hustoft, H. Malerod, S. Wilson, L. Reubsaet, E. Lundanes, T. Greibrokk, A critical review of trypsin digestion for LC-MS based proteomics, in: Leung HC, Man TK, Flores R (Eds.), *Integrative proteomics* (London: IntechOpen), 2012, pp. 73-88.
- [20]. J. McQueen, P.F. Semple, 21-Angiotensin receptor assay and characterization, *Methods in Neurosciences* 5 (1991) 312 – 330.
- [21]. U.K. Laemli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)* 227 (1970) 680-685.
- [22]. G. Buyukkoroglu, D.D. Dora, F. Ozdemir, C. Hizel, in: *Academic Press (Eds.), Omics Technologies and Bioengineering*, 2018, pp. 317 – 351.
- [23]. F. Fischer, D. Wolters, M. Rogner, A. Poetsch, Toward to complete membrane proteome, *Molecular and Cellular Proteomics* 5 (2006) 444-453.
- [24]. O. Coskun, Separation techniques: Chromatography, pp. 156-160, *North. Clin. Istanbul* (2016).
- [25]. A. Alejo, T. Matamoros., M. Guera, G. Andres, A proteomic atlas of the African swine fever virus particle, *Journal of Virology* 92 (2018) e01293-18. <https://doi.org/10.1128/jvi.01293-18>

Received: 28.11.2025

Received in revised form: 20.03.2026

Accepted: 24.03.2026