DOI: 10.11134/btp.3.2024.4

UDC: 632.938.2

Original Article

IDENTIFICATION AND CHARACTERIZATION OF THE SUPPRESSOR PROTEIN HC-PRO IN PVY-INFECTED POTATO PLANTS OF KAZAKHSTANI SELECTION

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ABSTRACT

Plant viruses pose a serious threat to all agricultural crops, leading to significant yield reduction, deterioration of seed quality, and degradation of cultivars. Potato infection with Potato virus Y (PVY) during the growing season causes severe damage, including leaf curling, leaf spot, and stunted growth due to shortened internodes and ring necrosis of tubers. In recent years, Kazakhstan has seen an increase in aphid populations, the main vectors of plant viruses. Therefore, it is crucial to conduct research on the diagnosis and identification of viral diseases. RNA interference (RNAi) plays a vital role in plant defense against viral infections. Viruses produce suppressor proteins to counteract RNAi, blocking plant defense mechanisms. The structural features of viral suppressors determine their specific functions in efficiently suppressing RNA interference. The HC-Pro protein is one such suppressor, aiding in virus spread and neutralizing plant-synthesized siRNA. In this study, using various methods including visual diagnostics, enzyme-linked immunosorbent assay (ELISA), chromatographic purification of plant extracts infected with PVY, Western blot, and enzymatic reaction with specific HC-Pro antibodies, the suppressor protein HC-Pro encoded by PVY was detected and identified.

Keywords: PVY, FPLC, HC-Pro, PAGE, RNA interference

INTRODUCTION

The low yield of potatoes (*Solanum tuberosum* L.) is often due to infections by viral pathogens, which cause diseases such as striped, wrinkled, and common mosaics, leaf curling and mosaic curling, aucuba mosaic, and others. These diseases are caused by viruses that lack cellular organization and metabolism, and do not synthesize proteins and nucleic acids [1]. Viruses exploit the host plant's protein and nucleic acid synthesis machinery, making them entirely dependent on it; they can only reproduce within living organisms [2].

Plants infected with viruses cannot be treated, and the pathogens are transmitted to subsequent generations, leading to the accumulation of these pathogens in tubers. This degrades the quality of seed material and can lead to crop degeneration. Global warming and rising average winter temperatures contribute to an increase in aphid populations, which in turn increases the spread of viruses [3].

Potato virus Y (PVY) infection during the growing season causes changes in plant habitus, such as leaf curling and mottling, as well as dwarfism due to shortened internodes. In harvested tubers, ring necrotic disease can be observed.

In response to viral invasion, plants initiate an RNA interference – a key molecular mechanism in gene expression regulation and plant virus defense. However, some viruses encode suppressors that can overcome this defense system. The genome of PVY encodes the HC-Pro protein, which suppresses RNA interference. The molecular weight of HC-Pro is 50 kDa, and the weight of a nucleotide pair is 640 Da, so the HC-Pro/siRNA complex is identified in chromatographic fractions with a molecular weight of 60±5 kDa [4].

Creating virus-resistant potato varieties will reduce yield losses and improve seed material quality [5]. This requires studying the effect of the HC-Pro protein on blocking the RNA interference process, which deprives plants of their defense mechanism against the virus [6]. Therefore, it is essential to investigate the structural features and functional characteristics of the viral HC-Pro protein and its complex with siRNA.

MATERIALS AND METHODS

Sample Collection

Leaf material samples were collected from potato plantings grown under field conditions at the «East Kazakhstan Agricultural Experimental Station» (EKAES). Sample collection took into account previously identified disease symptoms on leaf material, plant habitus, and tubers during dynamic digging.

Enzyme-Linked Immunosorbent Assay (ELISA):

The ELISA is based on the principle of complex formation between viral antigens and enzyme-labeled specific antibodies, followed by detection of the enzyme marker with a substrate.

Samples were tested using ELISA for the detection of Potato Virus Y (PVY), Potato Virus M (PVM), Potato Virus L (PVL), Potato Virus S (PVS), and Potato Virus X (PVX) using commercial ELISA diagnostic test systems produced by the «A.G. Lorch Federal Research Center of Potato». The ELISA was performed according to the protocol included in the kit [7].

The first step involved applying antibodies specific to the viruses under study to the wells of the plate, followed by incubation for one hour at 37 °C. Excess antibodies were then washed away, and the samples, as well as positive and negative controls, were added. After a 12-hour incubation at 4-6°C, a second wash was performed, and the specific conjugate was added. Following a wash to remove unbound reaction components and a one-hour incubation at 37°C, an enzymatic reaction with the substrate was carried out, resulting in a color change if viral particles were present in the sample. The results were assessed using a photometer («Multiscan

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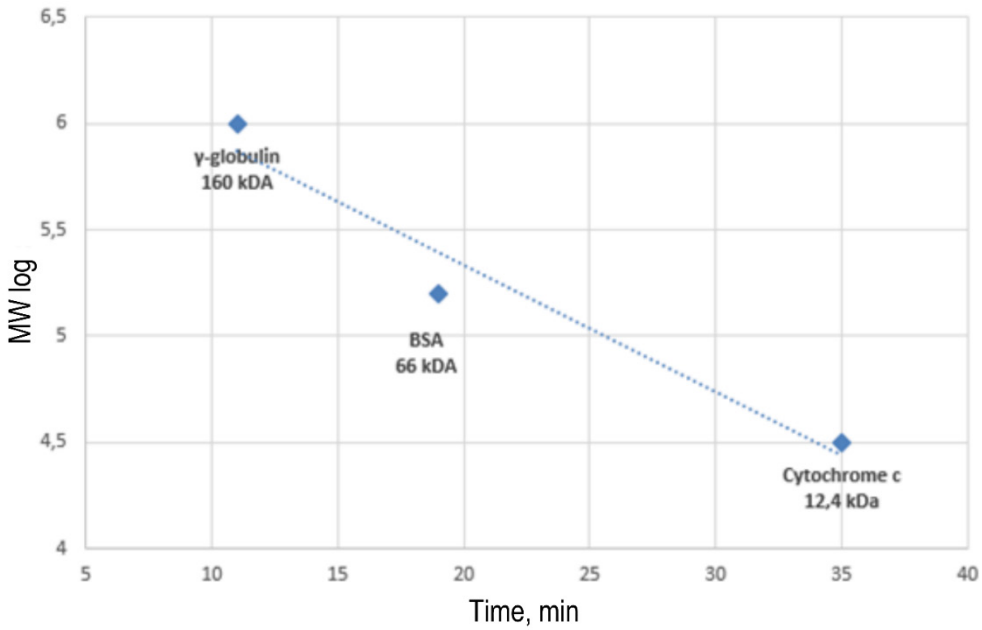

Figure 1. Calibration curve for determining the molecular weight of protein fractions

FC», Thermo Scientific, USA), which measured optical density in the wells at a wavelength of 492 nm. The optical absorption of positive samples had to be three times higher than that of negative samples.

Chromatographic purification of the HC-Pro from PVY-infected plant samples

Extracts from plants confirmed to be infected with PVY were fractionated using a chromatographic system (Pharmacia FPLC system, Sweden). Chromatographic analysis was conducted according to standard methods [8]. A calibration curve was constructed based on calibration results, which was subsequently used to determine the molecular weight of the studied protein complex, as shown in Figure 1.

For system calibration, marker proteins with known molecular weights were used: γ-globulin with a mass of 160 kDa, BSA with a mass of 66 kDa, and Cytochrome C with a mass of 12.4 kDa.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE with the studied proteins was performed in a protein electrophoresis chamber («Mini-PROTEAN Series», Bio-Rad, USA) according to standard methods. Working solutions included: 30% acrylamide; 1.5 M Tris-HCl buffer pH 8.8; 1 M Tris-HCl buffer pH 6.8; 10% SDS (sodium dodecyl sulfate); electrode buffer pH 8.3 [9].

Using these working solutions, the following were prepared: sample dilution buffer, phosphate-saline buffer (PSB), 10% separating gel, 4% stacking gel, Coomassie stain solution, decolorizing solution #1, and decolorizing solution #2.

Precision Plus Protein Kaleidoscope Prestained Protein Standards (well #1) were used as markers for the analysis, and a virus-free sample was placed in the last well (well #10), which was confirmed by ELISA. Electrophoresis power supply parameters were: voltage – 150 V, current – 0.45 A, power – 50 W, time – 60 min. When the proteins in the gel reached the bottom of the gel, the electrophoresis system was turned off.

Western Blotting

Protein fractions from the gel were transferred to a nitrocellulose membrane using Western blotting according to standard methods [10].

The gel was placed on the nitrocellulose membrane, with compressed filter paper and a sponge soaked in buffer on both sides, avoiding bubble formation. The assembly was then loaded into the Western blotting chamber. Parameters were: voltage – 100 V, current – 0.35 A, power – 50 W, time – 60 min.

Next, the membrane was placed in a Petri dish for the detection of specific proteins. To confirm that the proteins on the membrane were the PVY HC-Pro, the membrane was incubated with 15 ml of buffer for samples and conjugates. Anti-HC-Pro conjugate was added to the Petri dishes. Incubation was performed in a thermostat for 1 hour at 37°C. The membrane was then washed with phosphate-saline buffer with added Tween 20 detergent, followed by the addition of substrate. The reaction was stopped with water.

RESULTS

ELISA testing for viral infections and statistical validation of ELISA results

The Enzyme-Linked Immunosorbent Assay (ELISA) results indicated the presence of multiple viruses, including Potato virus Y (PVY), in the collected potato leaf samples from the East Kazakhstan agricultural experimental station. The optical density of positive samples was three times higher than that of negative controls, confirming the assay's high specificity and sensitivity.

To ensure the statistical reliability of the ELISA results, a threshold was established: samples with values below 0.336 were considered negative, those between 0.336 and 0.504 required retesting, and those above 0.504 were deemed positive. The ELISA results are detailed in Table 1.

The ELISA results indicated that all collected samples were infected with PVY. Specifically, Samples #3 and #4 (Colombo (4), Shakird (2)) were co-infected with PVY and PVM; Sample #1 (3/8-1 (2)) was infected with PVY, PVL,

DOI: 10.11134/btp.3.2024.4

Table 1. ELISA results for testing potato varieties for viral infections

No	Sample	PVX	PVS	PVY	PVL	PVM
1	3/8-1 (2)	Negative 0.035	Negative 0.063	**Positive** 0.756±0.039	**Positive** 0.891±0.041	Positive 0.792±0.031
2	Rocco (1)	Negative 0.011	Positive 0.986	Positive 0.691±0.026	Negative 0.09	Positive 0.897±0.032
3	Colombo (4)	Negative 0.012	Negative 0.084	Positive 0.952±0.042	Negative 0.064	Positive 0.698±0.029
4	Shakird (2)	Negative 0.027	Negative 0.049	Positive 1.064±0.045	Negative 0.091	Positive 1.141
5	Colombo (1)	Negative 0.057	Negative 0.086	Positive 1.196±0.047	Negative 0.047	Negative 0.062
6	Colombo (2)	Negative 0.051	Negative 0.082	Positive 0.974±0.035	Negative 0.058	Negative 0.056
7	Alena (1)	Negative 0.069	Negative 0.067	Positive 0.981±0.036	Negative 0.075	Negative 0.072
8	Lilea (1)	Negative 0.048	Negative 0.106	Positive 0.892±0.034	Negative 0.081	Negative 0.065
9	Positive Control	1.878 ± 0.039	2.085±0.032	1.989±0.024	3.021±0.033	2.954 ±0.023
10	Negative Control	0.026 ± 0.019	0.041±0.021	0.039±0.018	0.043±0.014	0.056 ±0.033

and PVM; and Sample #2 (Rocco (1)) was infected with PVS, PVY, and PVM. The remaining samples (#5, 6, 7, and 8) were mono-infected with PVY.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Cell sap from the infected potato samples was subjected to PAGE. The gel was stained with Coomassie dye and destained with two solutions of acetic acid: the first containing 10% acetic acid and the second 7% acetic acid. Identical protein fractions with a molecular mass of 60 ± 5 kDa were detected in Samples 1–8. No such bands were observed in the cell sap from the healthy plant (Sample 9, negative control). The resulting gel is shown in Figure 3.

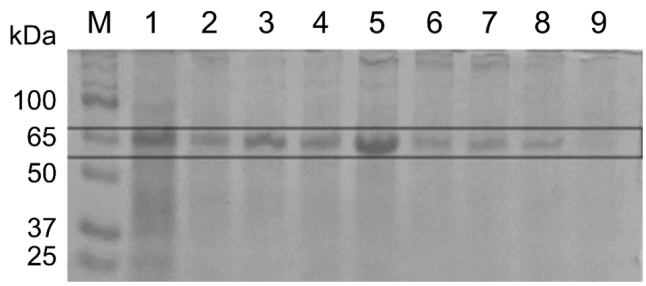

Figure 3. Electrophoretic mobility of proteins in the analyzed samples

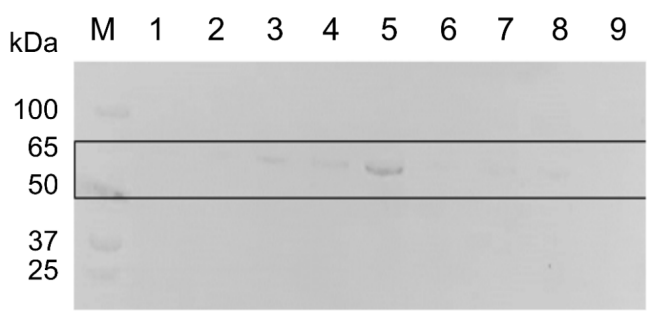

Figure 4. Western Blot with specific anti-HC-Pro rabbit antibodies

The specificity of the SDS-PAGE protein fractions was confirmed by Western blotting using anti-HC-Pro rabbit antibodies. The analysis identified the suppressor protein HC-Pro in Samples #3, 4, and 5, as shown in Figure 4.

Chromatographic Purification of HC-Pro

The extract from the PVY-infected plant with identified HC-Pro (Sample #5, Colombo (1)) was fractionated using a chromatographic system (Pharmacia FPLC system, Sweden). The calibration curve, constructed using marker proteins γ-globulin (160 kDa), BSA (66 kDa), and Cytochrome C (12.4 kDa), was crucial for determining the elution profile of the fraction containing HC-Pro with the corresponding molecular mass of 60 kDa (Figure 1) [11, 12].

The presence of HC-Pro in the chromatographic fractions was also confirmed using specific anti-HC-Pro rabbit antibodies (data not shown). The chromatographic fractions eluted between 15 and 18 minutes and were collected in 5 ml volumes [11, 12]. The chromatogram is presented in Figure 2.

Thus, the combined use of chromatographic and electrophoretic methods successfully identified and characterized HC-Pro protein fractions from PVY-infected plants.

DISCUSSION

Our results demonstrate the efficiency of combined ELISA, chromatographic, and electrophoretic methods in detecting and purifying the HC-Pro from PVY-infected potato plants. The ELISA provided a reliable initial screening for various potato viruses, including PVY. Chromatographic purification allowed for the isolation of the HC-Pro, while PAGE and Western blotting confirmed its presence and molecular weight [4, 6, 9, 11, 12]. Notably, HC-Pro was not detected in all extracts from PVY-infected plants. In samples #1, 2, 3, and 4 (3/8-1 (2), Rocco (1), Colombo (4), and Shakird (2) respectively), where multiple potato viruses were present, the concentration of HC-Pro was negligible, as indicated by the results of Western Blot analysis with specific antibodies. In

DOI: 10.11134/btp.3.2024.4

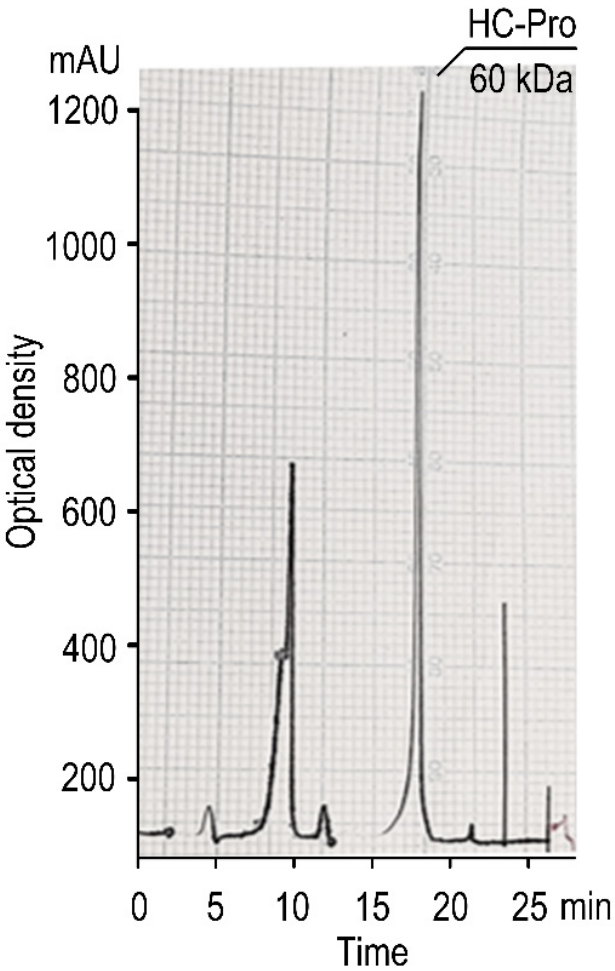

Figure 2. Chromatogram of fractions containing HC-Pro

samples #6, 7, and 8, HC-Pro was also not identified, likely because these plants exhibited mild symptoms due to recent infection and early-stage disease progression. It is probable that during the early stages of PVY infection, the viral protein HC-Pro is synthesized in small amounts, resulting in low concentrations in the plant cell sap. This concentration is sufficient to initiate the mechanism of RNA interference suppression but remains below detectable levels for some time. However, to confirm or refute these assertions, additional molecular studies are necessary.

The identification of the HC-Pro is crucial for understanding the molecular mechanisms of viral suppression in PVY-infected plants. The HC-Pro protein's ability to bind siRNA and suppress RNA interference plays a significant role in the virus's ability to evade the plant's immune response. By elucidating these mechanisms, our research contributes to the development of strategies for enhancing viral resistance in potato plants [4].

Future studies should focus on the detailed structural analysis of the HC-Pro/siRNA complex and its interaction with the plant's RNA interference machinery. Additionally, exploring the potential for genetic modification to enhance resistance to PVY and other viral pathogens could have significant implications for improving crop yields and quality [11].

Overall, our study provides a comprehensive approach to detecting and analyzing the HC-Pro in PVY-infected plants, offering valuable insights into viral pathogenesis and plant immunity.

CONCLUSION

Using a combined method of visual diagnosis and enzyme-linked immunosorbent assay (ELISA), we identified and selected plants infected with the PVY monovirus. This approach allowed us to avoid cross-contamination from other viral infections. Chromatographic purification of extracts from PVY-infected plants revealed fractions containing the suppressor protein HC-Pro complexed with short interfering RNAs (siRNAs) specific to Potato Virus Y (PVY). Results from polyacrylamide gel electrophoresis (PAGE) and Western blotting with specific anti-HC-Pro rabbit antibodies indicate the presence of the HC-Pro in fractions 3, 4, and 5, corresponding to a molecular weight of 60±5 kDa. These findings are supported by the biochemical properties of HC-Pro, including its ability to capture and retain siRNAs on the surface of its subunits. Consequently, we successfully isolated a purified ribonucleoprotein complex HC-Pro/siRNA from PVY-infected plants, which will be used as a source of PVY-specific siRNAs to treat healthy potato plants in vitro, aiming to confer antiviral resistance.

FUNDING

This work was carried out within the framework of the research project «Development and Implementation of Innovative Technology Aimed at Conferring Antiviral Resistance to Agricultural Crop Varieties,» funded by the Ministry of Higher Education and Science of the Republic of Kazakhstan, Individual Registration Number (IRN): AP08052163.

CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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DOI: 10.11134/btp.3.2024.4

УДК: 632.938.2

ИДЕНТИФИКАЦИЯ И ХАРАКТЕРИСТИКА СУПРЕССОРНОГО БЕЛКА HC-PRO В PVY-ИНФИЦИРОВАННЫХ РАСТЕНИЯХ КАРТОФЕЛЯ КАЗАХСТАНСКОЙ СЕЛЕКЦИИ

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АННОТАЦИЯ

Вирусы растений представляют серьезную угрозу для всех сельскохозяйственных культур, приводя к значительному снижению урожая, ухудшению качества семенного материала и деградации сортов. Инфекция картофеля вирусом картофеля Y в период вегетации вызывает серьезные повреждения в виде скручивания и пятнистости листьев, а также карликовость из-за укорачивания междоузлий, кольцевой некроз клубней. В последние годы в Казахстане отмечается увеличение популяции тли, которая является основным переносчиком вирусов. В связи с этим крайне важно проводить исследования по диагностике и идентификации вирусных заболеваний. РНК-интерференция играет важную роль в защите растений от вирусных инфекций. Вирусы для подавления РНК-интерференции вырабатывают супрессорные белки, блокирующие защитные механизмы растений. Структурные особенности вирусных супрессоров определяют их специфические функции в эффективном подавлении РНК-интерференции. Белок HC-Pro является одним из таких супрессоров, способствующих распространению вируса и нейтрализации siRNA, синтезируемых растением. В данной работе, с использованием различных методов, включая визуальную диагностику, иммуноферментный анализ, хроматографическую очистку экстрактов растений, инфицированных PVY, Western blot и ферментативной реакции с использованием специфических антител к HC-Pro, был обнаружен и идентифицирован супрессорный белок HC-Pro, кодируемый PVY.

Ключевые слова: PVY, FPLC, HC-Pro, PAGE, РНК-интерференция.

ӘОК: 632.938.2

ҚАЗАҚСТАНДЫҚ СЕЛЕКЦИЯДАҒЫ PVY-ЖҰҚТЫРҒАН КАРТОП ӨСІМДІКТЕРІНДЕГІ HC-PRO СУПРЕССОРЛЫҚ АҚУЫЗЫН ИДЕНТИФИКАЦИЯЛАУ ЖӘНЕ СИПАТТАУ

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АННОТАЦИЯ

Өсімдік вирустары барлық ауылшаруашылық дақылдарға үлкен қауіп төндіреді, бұл өнімділіктің айтарлықтай төмендеуіне, тұқым сапасының нашарлауына және сорттардың деградациясына әкеледі. Вегетациялық кезеңде картоптың Y вирусы жапырақтардың бұралуы мен дақтары түрінде ауыр зақым келтіреді, сонымен қатар түйнектердің қысқаруына байланысты ергежейлілік, түйнектердің сақиналы некрозын тудырады. Соңғы жылдары Қазақстанда вирустардың негізгі тасымалдаушысы болып табылатын бит популяциясының көбеюі байқалады. Осыған байланысты вирустық ауруларды диагностикалау және анықтау бойынша зерттеулер жүргізу өте маңызды. РНҚ интерференциясы өсімдіктерді вирустық инфекциялардан қорғауда маңызды рөл атқарады. РНҚ интерференциясын басатын вирустар өсімдіктердің қорғаныс механизмдерін блоктайтын супрессорлық ақуыздарды шығарады. Вирустық супрессорлардың құрылымдық ерекшеліктері олардың РНҚ интерференциясын тиімді басудағы ерекше функцияларын анықтайды. HC-Pro протеині - бұл вирустың таралуына және өсімдік синтездейтін siRNA-ны бейтараптандыруға ықпал ететін осындай супрессорлардың бірі. Бұл жұмыста визуалды диагностика, иммуноферменттік талдау, pvy жұқтырған өсімдік сығындыларын хроматографиялық тазарту, Western blot және арнайы HC-Pro антиденелерін қолданатын ферментативті реакция сияқты әртүрлі әдістерді қолдана отырып, pvy кодталған HC-Pro супрессорлық ақуызы табылды және анықталды.

Кілт сөздер: PVY, FPLC, HC-Pro, PAGE, РНҚ-интерференциясы.