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Cleavage of chicken vimentin by the reticuloendotheliosis virus protease

Tong Zhu, Minxun Song, Bing Huang, Yufeng Li and Feng Hu*

Institute of Poultry Science, Shandong Academy of Agricultural sciences, Shandong Key Laboratory of Immunity and Diagnosis of Poultry Diseases, Jinan, Shandong, China

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ABSTRACT

The protease (PR) encoded by reticuloendotheliosis virus (REV) is deeply involved in the lifecycle and infection process of a virus in that it cleaves the viral polyproteins into their mature structural proteins and replication enzymes. Besides this essential role in the lifecycle of a virus, the enzymatic activity of the REV PR targeting chicken vimentin remains unknown. In this study, the recombinant chicken vimentin, fused with a GST-tag, was expressed in the *Escherichia coli* system as a soluble form. The soluble vimentin was purified using Glutathione Sepharose 4B. Then, the *in vitro* cleavage of chicken vimentin by the PR of REV was analyzed. The results showed that chicken vimentin was cleaved by the REV PR. Furthermore, Nano LC-MS/MS analysis showed that the REV PR cleaved chicken vimentin between leucine-239 and glutamine-240, alanine-261 and alanine-262, and histidine-431 and serine-432, respectively. Moreover, the cleavage sites identified in this study were different from other known naturally occurring cleavage sites of other retroviruses. For the first time, we showed that the REV PR cleaved the chicken vimentin at specific sites in chicken vimentin. These results support the possibility that chicken vimentin may serve as a substrate within REV-infected cells, facilitating our understanding of avian retroviruses.

Key words: reticuloendotheliosis virus; protease; vimentin; cleavage

Introduction

Reticuloendotheliosis virus (REV), which belongs to the family *Retroviridae*, is an oncogenic and immunosuppressive virus in multiple avian hosts (SHI et al., 2021). Recently, REV has become distributed worldwide and poses a serious threat to the poultry industry (CALEIRO et al., 2020; CHACON et al., 2022). REV possesses a positive-sense, single-stranded RNA genome, encoding a variety of structural proteins and enzymes necessary for its unique life cycle (CHACON et al., 2022). Similar to other retroviruses, the protease (PR) encoded by REV is responsible for the maturation of viral *gag* and *gag-pol* encoded precursor polyproteins and is required for REV infectivity.

Complex pools of host factors are highly involved in the virus life cycle. Vimentin is a rigid scaffold protein in eukaryotic cells and plays important

*Corresponding author:
Feng Hu, Institute of Poultry Science, Shandong Academy of Agricultural Sciences, 250100, Jinan, Shandong, China. Email address: hufengggg@126.com

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roles in the infection process of many viruses (YU et al., 2022; CHENG et al., 2023). Analysis of cleavage of vimentin has been performed for the human immunodeficiency virus (HIV), the bovine leukemia virus (BLV), the Mason-Pfizer monkey virus (M-PMV), and the myeloblastosis-associated virus (MAV) PR (SNASEL et al., 2000). However, as an important representative of the avian retrovirus, the cleavage properties of the REV PR regarding chicken vimentin have not been documented. In our previous study, the REV PR was expressed in the *Escherichia coli* (*E. coli*) system and purified successfully, the enzyme's excellent cleavage activity was identified in *vitro*, and an optimal enzymatic reaction system was also established (HU et al., 2015).

In the present study, we analyzed the proteolytic cleavage of vimentin mediated by the REV PR. Chicken vimentin was successfully expressed in the *E. coli* system. We are the first to show that chicken vimentin is cleaved by the REV PR at specific cleavage-sites.

Materials and methods

Cells, bacterial strain, antibody. Ten-day-old specific-pathogen-free (SPF) chicken embryos were provided by the Institute of Poultry Science, Shandong Academy of Agricultural Sciences, and were used to prepare primary chicken embryo fibroblast (CEF) cells. CEF cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA). *E. coli* strains DH5α and BL21 (DE3) (TransGen, Beijing, China) were used for gene cloning and expression, respectively. Glutathione Sepharose 4B (Beyotime, Shanghai, China) was used for protein purification. Anti-GST monoclonal antibody (ProteinTech Wuhan, China) was used for identification of the expressed recombinant proteins.

Construction of the recombinant plasmid expressing chicken vimentin. The total RNA was extracted from the CEF cells, and the cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Mix (Takara, Dalian, China). The vimentin gene was amplified using the primers (vim-F: ATGAGCTTCACCAGCAGCAAG, vim-R: TTA CTCCAAGTCATCGTGATGC) and confirmed by sequencing. Then, the nucleotide sequence of the vimentin was codon-optimized and inserted into the pGEX-6p-1 vector to construct the recombinant plasmid pGEX-vimentin. The recombinant plasmid was transformed into competent *E. coli* DH5α cells, extracted and identified by restriction enzyme digestion and sequence analysis to confirm the correct coding sequences.

Expression and purification of the recombinant vimentin. To express the recombinant proteins, the recombinant plasmid pGEX-vimentin was transformed into competent BL21 (DE3) cells. The clone was grown in LB medium with 100 μg/mL ampicillin. The expression of the recombinant proteins was induced with isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.1 mM at 16°C for 12 h. The expressed proteins were identified by 12% SDS-PAGE and immunoblotting with anti-GST antibody. To purify the recombinant proteins, the induced culture was pelleted and disrupted in binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3) with 0.1 mM PMSF by sonication for 10 min (4 s on, 6 s off). After centrifugation at 12,000 × g at 4°C for 10 min, the cell-free extract was collected and stored on ice. The recombinant protein GST-vimentin was purified according to the manual for Glutathione Sepharose 4B. The purified proteins were identified by 12% SDS-PAGE.

Cleavage of vimentin with REV PR. REV PR with enzymatic activity was expressed and purified according to previous studies (HU et al., 2015). To analyze the cleavage of vimentin with the PR in *vitro*, the assay was performed in a 40 μL digestion reaction mixture containing 2.4 μg PR and 17 μg GST-vimentin, as previously described (HU et al., 2015). The reaction was started by addition of the PR, and terminated by the addition of 5× protein loading buffer. To analyze the cleavage of the vimentin by the PR, the samples were separated on 12% polyacrylamide gels and stained with Coomassie blue. The specific cleavage fragments of the vimentin were further analyzed by nano LC-MS/MS analysis.

Identification of cleavage site by N/C-terminal sequencing. GST-vimentin was incubated

with the PR at 37°C for 1 h, separated on 12% polyacrylamide gel, and stained with Coomassie blue. After destaining, the bands of the cleavage products were cut from the gel into 1 mm^3 cubes and subjected to N/C terminal sequencing, based on nano LC-MS/MS analysis using a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Briefly, samples were enzymolyzed by trypsin and chymotrypsin, respectively, and fractionated by the Easy-nLC 1200 system (Thermo Fisher Scientific, Waltham, USA) using an Acclaim PepMap RPLC C18 column (1.9 μm, 100 Å, Dr. Maisch GmbH, Tubingen, Germany). The LC linear gradient run was from 4% to 8% mobile phase B (0.1% formic acid in water -80% acetonitrile) for 2 min, from 8% to 28% B for 43 min, from 28% to 40% B for 10 min, from 40% to 95% B for 1 min and from 95% to 95% B for 10 min at 600 nL/min. The raw MS files were analyzed and searched against the target protein database on the basis of the species of the samples, using Byonic.

Results

Expression and purification of the recombinant proteins in E. coli. In the *E. coli* expression system, the recombinant protein GST-vimentin with a GST tag (approximately 80 kDa) was successfully expressed in soluble form (Fig. 1A). Then, the GST-vimentin was purified using Glutathione Sepharose 4B. The samples of GST-vimentin from different steps in the process of protein expression and purification were analyzed by 12% SDS-PAGE, and are shown in Fig. 1B.

The REV PR exhibits the capacity to cleave vimentin. To analyze the cleavage activity of the PR on vimentin, the recombinant GST-vimentin was incubated with the PR in a time-dependent manner. Specific cleavage of the vimentin was observed in this case. As shown in Fig. 2, two major products of ~50 and ~45 kDa and one minor product of ~25 kDa were observed under the reducing conditions of SDS-PAGE.

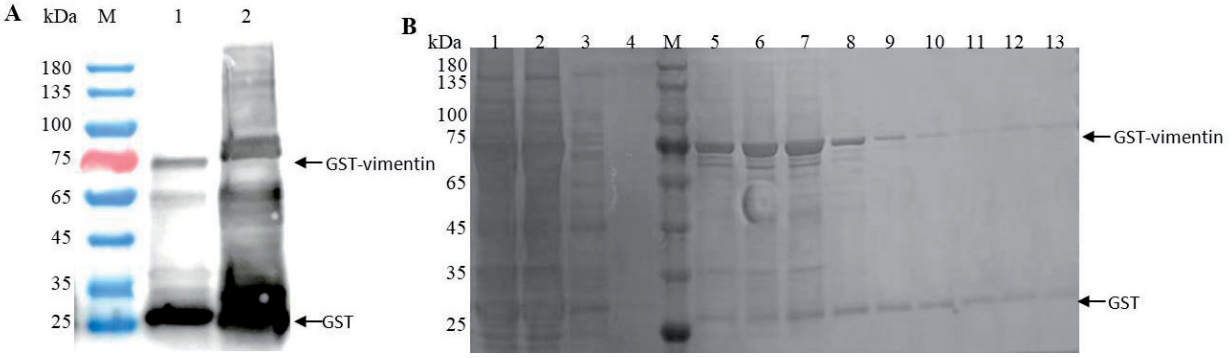

Fig.1. Expression and purification of vimentin in a soluble form as identified by western blotting and SDS–PAGE
(A) Expression of the vimentin identified by western blotting. Lane 1, the induced supernatant of the vimentin; lane 2, the induced supernatant of pGEX-6p-1; (B) Samples from vimentin purification run on 12% SDS–PAGE. Lane 1, the induced supernatant of pGEX-vimentin; lane 2, the flowthrough fraction of the induced supernatant of pGEX-vimentin from Glutathione-Sepharose affinity chromatography; lane 3-4, the eluate from the wash step; lane 5-13, fractions of elution buffer. Lane M, prestained protein markers.

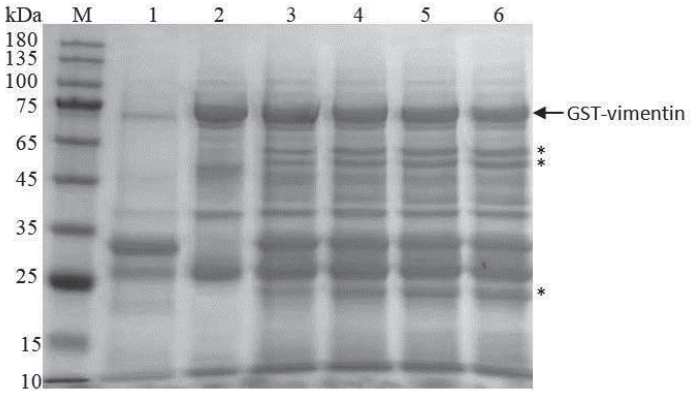

Fig.2. The recombinant vimentin was incubated with REV PR at 37°C for different periods of time and analyzed by SDS-PAGE

Lane 1, REV PR; lane 2, GST-vimentin; lane 3-6, reaction mixtures were incubated for 1, 2, 3, 4 hours, respectively; The cleavage products are indicated with an asterisk (*). Lane M, prestained protein markers.

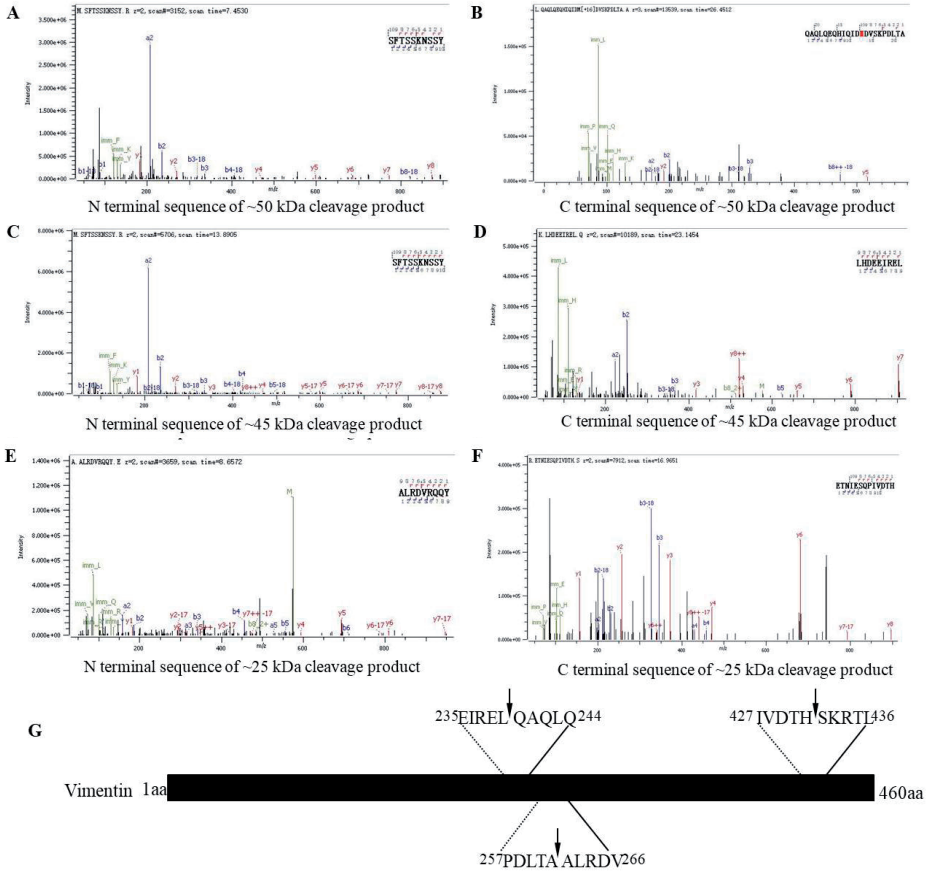

Fig.3. Chicken vimentin is cleaved by the REV PR at specific cleavage-sites

(A-F) The results of nano LC-MS/MS analysis for the N/C terminal sequence of the cleavage products. (G) The vimentin was cleaved by the REV PR in a leucine-glutamine, alanine-alanine or histidine-serine peptide bond. The cleavage sites are indicated with arrows.

Peptide sequence of REV PR cleavage sites. Both the N terminus and the C terminus of the cleavage products of the vimentin were determined by nano LC-MS/MS analysis (Fig. 3). The ~50 and ~45 kDa degradation products shared the same N terminal, the amino acid sequence SFTSSKNSSY, corresponding to the N terminus of vimentin. For the ~50 kDa cleavage product, the C-terminal sequence is QAQLQEQHIQIDMDVSKPDLTA, which corresponds to the sequence between positions 240 and 261 of the vimentin. For the ~45 kDa cleavage product, the C-terminal sequence is LHDEEIREL, which corresponds to the sequence between positions 231 and 239 of the vimentin. For the ~25 kDa cleavage product, the C terminal amino acid sequence is ETNIESQPIVDTH, corresponding to the sequence between positions 419 and 431 of the vimentin, and the N terminal amino acid sequence is ALRDVRQQY, which corresponds to the sequence between positions 262 and 270 of the vimentin. In conclusion, the chicken vimentin was cleaved at three different positions (Fig. 3G), and theoretically there are nine forms of cleaved vimentin, however, we could only observe the ~50, ~45 and ~25 kDa cleavage products, and ~80 kDa full length under the reducing conditions of SDS-PAGE.

Discussion

Similar to other retroviruses, the REV PR can catalyze the hydrolysis of the polyprotein gag-pol to form subsequent p12, p18, p30, p10, PR, RT, and IN in the process of REV maturation (BARBOSA et al., 2007). Furthermore, the currently available antiretroviral therapy against HIV is also based on drugs that include inhibitors of the viral PR (FERREIRO et al., 2022). Therefore, identification of the PR's characteristics may be particularly useful in refining our understanding of the infection mechanism of REV.

Vimentin, as a major component of type III intermediate filaments, is involved in maintaining cell morphology, the mechanical integrity of cells, and cell differentiation (MENG et al., 2023). Recently, it has been suggested that vimentin not only forms a rigid scaffold for determining the shape of cells, but also plays a role in virus infection. A few viruses, such as the African swine fever virus (CHENG et al., 2023), the dengue virus serotype 2 (DV2) (YU et al., 2022), and human parainfluenza virus type 3 (LIU et al., 2022) complete their assembly and form mature viral particles depending on the host protein vimentin. To the best of our knowledge, REV is an important representative of avian retroviruses, however, the relationship between chicken vimentin and the REV PR has not yet been reported.

In this study, chicken vimentin was successfully expressed in soluble form in *E. coli* strain BL21 (DE3). The expressed vimentin could be used for preparation of antibodies and to study its biological functional properties. Although during the purification of the vimentin, the GST protein could not be efficiently eliminated from the purified products (Fig. 1), it did not interfere with the subsequent experiments. Our data indicated that the expressed vimentin could be readily cleaved by the REV PR in *vitro*, which may be a molecular mechanism involved in vimentin's biological function during REV infection because cleavage of cellular proteins by the PR may play an important role in viral replication (Fig. 2). As an important representative of avian retroviruses, we also found that chicken vimentin is cleaved at three different positions by the REV PR (Fig. 3). It would appear that the REV PR has multiple proteolysis sites. Therefore, theoretically there are nine forms of cleaved vimentin, however, we could only observe three cleavage products and full length vimentin under the reducing conditions of SDS-PAGE. This is possible depending on the conditions in which retroviral proteases degrade the host cell proteins *in vitro* (SNASEL et al., 2000). In addition, it was established that the PR protein encoded by several retroviruses could cleave host vimentin proteins at different sites and at different rates (SHOEMAN et al., 1990). Identification of the cleavage site within chicken vimentin provides critical information about the substrate sequence preferences of the REV PR. According to the results of N/C terminal sequencing, the vimentin was cleaved in a leucine-glutamine, alanine-alanine or histidine-serine peptide bond (Fig. 3), which are different from other known naturally occurring cleavage

sites of other retroviruses (SHOEMAN et al., 1990). It seems that analysis of the structure of the interaction between the REV PR and chicken vimentin would facilitate our understanding of the molecular mechanism of interaction between the PR and vimentin. Moreover, whether vimentin within REV-infected cells can be cleaved by the PR needs to be studied further.

Conclusions

In summary, this report describes the first time that chicken vimentin was expressed in a soluble form and cleaved by the REV PR at specific cleavage sites, which is an intriguing finding, although currently of unclear *in vivo* significance. These results may facilitate our understanding of avian retroviruses. Furthermore, vimentin-specific proteases PR could be considered as potential therapeutic candidates for REV.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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SAŽETAK

Proteaza (PR) kodirana virusom retikuloendotelioze (REV) ima važnu ulogu u životnom ciklusu virusa i njegovom zaražavanju ptica tako što cijepa virusne poliproteine u zrele strukturne proteine i replikacijske enzime. Osim navedenoga, enzimska aktivnost REV PR-a usmjerena na pileći vimentin ostaje nepoznata. U ovom je radu rekombinantni pileći vimentin, s oznakom GST, eksprimiran u sustavu *Escherichia coli* u topljivu obliku. Kao takav, vimentin je pročišćen upotrebom glutation-sefaroze 4B. Nakon toga analizirana je razgradnja *in vitro* pilećeg vimentina pomoću proteaze REV-a. Rezultati su pokazali da je REV PR uklonio pileći vimentin. Nadalje, Nano LC-MS/MS analiza pokazala je da REV PR cijepa pileći vimentin na leucin-239 i glutamin-240, alanin-261 i alanin-262 te histidin-431 i kserin-432. Štoviše, mjesta razgradnje identificirana u ovom istraživanju razlikovala su se od poznatih prirodnih mjesta razgradnje drugih retrovirusa. Time je po prvi put pokazano da je REV PR razgradio pileći vimentin na specifičnim mjestima cijepanja. Navedeno upućuje da bi pileći vimentin mogao poslužiti kao supstrat unutar stanica inficiranih REV-om, što bi unaprijedilo razumijevanje avijarnih retrovirusa.

Ključne riječi: virus retikuloendotelioze; proteaza; vimentin; cijepanje