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Post-translational cleavage generates truncated IgY forms in the snake *Elaphe taeniura*

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ABSTRACT

While variable regions of immunoglobulins are extensively diversified by V(D)J recombination and somatic hypermutation in vertebrates, the constant regions of immunoglobulin heavy chains also utilize certain mechanisms to produce diversity, including class switch recombination (CSR), subclass differentiation, and alternative expression of the same gene. Many species of birds, reptiles, and amphibians express a truncated isoform of immunoglobulin Y (IgY), termed IgY(ΔFc), which lacks the υCH3 and υCH4 domains. In Anseriformes, IgY(ΔFc) arises from alternative transcriptional termination sites within the same υ gene, whereas in some turtles, intact IgY and IgY(ΔFc) are encoded by distinct genes. Different from the previously reported IgY(ΔFc) variants, this study identified a truncated IgY in the snake *Elaphe taeniura*, characterized by the loss of only a portion of the CH4 domain. Western blotting and liquid chromatography-tandem mass spectrometry confirmed that this truncated IgY is generated by post-translational cleavage at N338 within the IgY heavy chain constant (CH) region. Furthermore, both human and snake asparaginyl endopeptidase were shown to cleave snake IgY *in vitro*. These findings reveal a novel mechanism for the production of shortened IgY forms, demonstrating that the immunoglobulin CH region undergoes diversification through distinct strategies across vertebrates.

Keywords: Truncated IgY; Post-translational cleavage; Asparaginyl endopeptidase

INTRODUCTION

Immunoglobulin Y (IgY) is the predominant low-molecular-weight antibody in non-mammalian tetrapods, functionally combining characteristics of mammalian IgG and IgE. It plays a role in opsonization and complement fixation while also being capable of mediating anaphylactic reactions (Lundqvist et al., 2006; Magor, 2011). Although IgY functionality varies across species, the υ genes encoding IgY in non-mammalian tetrapods exhibit several conserved features. These genes are typically located at the 3' end of the immunoglobulin heavy chain (IgH) locus and encode four structural domains of the heavy chain constant (CH) region (Sun et al., 2020). Additionally, subclass diversification of the υ gene, akin to mammalian IgG, is widely observed in avian, reptilian, and amphibian species. For instance, two υ gene subclasses have been identified in the IgH loci of ostriches and pigeons (Han et al., 2016; Huang et al., 2018), while genomic analyses have revealed more extensive IgY diversification in reptiles and amphibians (Cheng et al., 2013; Li et al., 2012; Olivieri et al., 2021). In addition to the expression of full-length IgY, many species also produce truncated IgY isoforms that lack the υCH3 and υCH4 domains, termed IgY(ΔFc). This isoform has been identified in diverse taxa, such as ducks (Magor et al., 1994), lizards (Wei et al., 2009), turtles (Li et al., 2012), salamanders, and geese (Huang et al., 2016; Zhu et al., 2014). However, the strategies underlying IgY(ΔFc) generation differ among species. In ducks and geese,

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IgY(ΔFc) is produced through alternative transcriptional termination signals of the same υ gene (Huang et al., 2016; Magor et al., 1992), whereas in turtles, distinct germline-encoded genes independently give rise to both full-length IgY and IgY(ΔFc) (Li et al., 2012).

Our previous research identified two IgY subclasses (IgY1 and IgY2) in the serum of the snake *Elaphe taeniura*, both of which exist as full-length and truncated isoforms (Wang et al., 2012). However, unlike the IgY(ΔFc) isoform reported in ducks and turtles, the shortened IgY in the snake appears to lack only one CH domain based on the molecular weights of the heavy (H) chains. The full-length forms of IgY1 and IgY2 (designated IgY1-L and IgY2-L) possess heavy chains of approximately 70 kDa, while the truncated isoforms (designated IgY1-S and IgY2-S) contain heavy chains of approximately 60 kDa. Further analysis using reverse transcription polymerase chain reaction (RT-PCR) did not detect transcripts encoding the truncated IgY heavy chains, suggesting that IgY1-S and IgY2-S are generated through post-translational protein modifications rather than alternative splicing of the υ gene or the use of differential transcriptional termination sites. Additionally, IgY1 was found to lack a cysteine residue in the CH1 domain, which is necessary for covalent association with light (L) chains. This raises the possibility that the truncated IgY in *E. taeniura* may function as a heavy chain-only antibody or that its heavy chains are associated with light chains through noncovalent bonds.

To identify the mechanism responsible for generating the shortened IgY in *E. taeniura*, antibodies targeting the snake Igλ and the truncated segment of the IgY2 CH4 domain (designated IgY-T) were prepared. A series of experiments, including protein purification, western blotting, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were conducted. Results demonstrated that the molecular weight difference between the two IgY forms in the snake serum was not attributable to glycosylation. The truncated IgY was not a heavy chain-only antibody but was produced through proteolytic cleavage at the N338 position within the CH4 domain. Furthermore, *in vitro* assays revealed that the snake IgY can be cleaved by asparaginyl endopeptidase (AEP). These findings elucidate the mechanism underlying the generation of truncated IgY in the snake *E. taeniura* and provide insights for further exploration into the functional roles of IgY in non-mammalian tetrapods.

MATERIALS AND METHODS

Animals, RNA isolation, and reverse transcription

Adult *E. taeniura* specimens were purchased from a commercial snake farm in Qingzhou, Shandong Province, China, and housed in an animal facility for serum collection and RNA isolation. Total RNA was extracted from spleen tissue using a HiPure Universal RNA Kit (Magen Biotech, China), followed by reverse transcription utilizing M-MLV reverse transcriptase according to the manufacturer's protocols (Invitrogen, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee at Southwest Minzu University (Permit No. 2022MDLS23).

Purification of snake IgY

A total of 5 mL of snake serum was centrifuged at 4 000 r/min for 10 min at 4°C to remove insoluble material. The resulting supernatant was diluted with an equal volume of phosphate-buffered saline (PBS) and subjected to ammonium sulfate precipitation. Saturated ammonium sulfate (10 mL, pH 7.8) was added to achieve a final saturation of 50%, and the mixture was incubated on ice for 30 min. Following centrifugation at 1 500 ×*g* for 10 min at 4°C, the supernatant was discarded, and the resulting pellet was resuspended in ammonium sulfate solutions at decreasing concentrations (45%, 40%, 35%, 30%, and 20%). The snake IgY2 in each precipitation was detected by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The pellet with the highest IgY2 content was resuspended and dialyzed against PBS (pH 7.0) for 24 to 48 h using Millipore dialysis tubes. The final dialyzed solution was further purified using Protein A (HiTrap Protein A HP, GE Healthcare, USA) according to the manufacturer's protocols.

Preparation of snake Igλ- and IgY2-T specific antibodies

Polyclonal rabbit antibodies specific to the snake Igλ and IgY2-T were prepared by Abmart (China). The cDNA encoding the snake Cλ region was cloned into the pET-28a (+) expression vector, fused with a His tag, and expressed in an *Escherichia coli* expression system. Purification was performed using Ni-NTA column chromatography. Additionally, a short peptide (DSFFRYSKLNIP) from the CH4 (385–396) domain of IgY2 was synthesized and conjugated with keyhole limpet hemocyanin (KLH) and virus-like particles (VLPs) as antigens for rabbit immunization. Rabbit serum was collected post-immunization and subjected to protein G purification to obtain Igλ- and IgY2-T-specific polyclonal antibodies.

Western blotting

Serum was obtained by centrifuging whole blood at 3 000 ×*g* for 10 min at 4°C after incubation for 3 h at 4°C to remove the erythrocytes. The denatured serum was treated with DTT and/or PNGase F and endo-α-N-acetylgalactosaminidase (NEB, USA), then separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was then blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat milk for 1 h at room temperature under constant agitation. The membrane was incubated with primary antibodies, including anti-Igλ, anti-IgY1, anti-IgY2, anti-IgY-T (all from Abmart, China), and anti-AEP (R&D Systems, USA), for 1 h at room temperature with agitation. After three washes with TBST, horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Zsbio, China) were added and incubated for 1 h at room temperature. Following five additional TBST washes, protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Thermo, USA).

Two-dimensional electrophoresis (2-DE) and MS analysis

Protein A-purified IgY was precipitated by adding pre-cooled acetone (20× volume), following dissolution in a buffer containing 7 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 65 mmol/L DTT, and 0.8% pH 3–10 IPG buffer. A total of 150 μg of purified protein was loaded onto a hydration tray and overlaid with an immobilized DryStrip (pH 3–10, NL, 7 cm, GE Healthcare, Life Sciences, USA). First-dimension separation was performed by isoelectric focusing (IEF), followed by SDS-PAGE according to established protocols (Cao et al., 2019). Protein spots corresponding to IgY1-L, IgY2-L, IgY1-S, and IgY2-S from 2-DE, as well as an

approximately 10 kDa band from the SDS-PAGE gel, were excised for LC-MS/MS analysis. LC-MS/MS was performed on a nanoLC-LTQ-Orbitrap XL mass spectrometer (Thermo, USA) coupled to an Easy-nLC 1200 HPLC system. The MS data were analyzed using Proteome Discoverer (v.1.4.0.288, Thermo, USA), and the MS2 spectra were searched against the UniProt database using the SEQUEST algorithm with the Snake_contaminants_m.fasta (Accession numbers: JQ305106, JQ305107, JQ305108, PQ226475, PQ226476, PQ226687, PQ226688, PQ226689, PQ226690, PQ226691, PQ226692, PQ226693, PQ226694, PQ226695, PQ226696, PQ226697, and PQ226698). The MS proteomics data were deposited in the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the iProX partner repository under the dataset identifier PXD055127 (Chen et al., 2022; Ma et al., 2019).

***In vitro* cleavage assay by AEP**

The snake serum was diluted 10-fold in a buffer containing 50 mmol/L sodium citrate, 5 mmol/L DTT, 0.1% CHAPS, and 0.5% Triton X-100 (pH 6.0). The diluted serum was incubated at 37℃ for 1 h with human AEP (5 μg/mL, Novo Protein, China) in the presence or absence of the AEP inhibitor AENK (50 μg/mL, Sigma-Aldrich, USA). For the expression of snake AEP, cDNA was synthesized from spleen RNA and used as a template for PCR amplification with the primers AEP-F (5'-ATGGCCTTGAAGGTAGCTGTAC-3') and AEP-R (5'-TCCAAGGCACACTTGATCCA-3'). The amplified product was cloned into the pBudCE4.1 vector and transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen, USA), following the manufacturer's instructions. After 48 h, transfected cells were harvested, washed with PBS, and lysed in a buffer containing 50 mmol/L sodium citrate, 5 mmol/L DTT, 0.1% CHAPS, and 0.5% Triton X-100 (pH 6.0). The resulting cell lysates were incubated with the diluted snake serum at 37°C for 1 h, either in the presence or absence of AENK. Finally, the samples were subjected to SDS-PAGE and analyzed by western blotting.

RESULTS

Detection of IgY1 and IgY2 in serum

We previously detected two IgY subclasses in *E. taeniura* serum, each existing as both full-length and truncated isoforms. Western blotting under nonreducing conditions revealed two distinct bands for each subclass, corresponding to molecular weights of approximately 70 kDa and 60 kDa (Wang et al., 2012). To minimize interference from other serum components and precisely determine IgY molecular weights, IgY was purified using ammonium sulfate precipitation followed by protein A purification. The highest yield of both IgY isoforms was obtained at a 35% ammonium sulfate saturation, with IgY2 detection providing clearer resolution of the two molecular weight variants (Figure 1A).

Post-translational modifications, such as *N*- and *O*-glycosylation, influence immunoglobulin structure, molecular mass, and effector function. Our previous study demonstrated that treatment with PNGase F, which removes N-linked glycans, reduced the molecular weight of both IgY isoforms, confirming the presence of N-glycosylation (Wang et al., 2012). In the current study, digestion with endo-α-N-acetylgalactosaminidase, which cleaves *O*-linked glycans, had no effect on the size of the two IgY2 forms (Figure 1B). These findings suggest that IgY1 and IgY2 are glycosylated by *N*-linked glycans but not by *O*-linked glycans, indicating that glycosylation does not account for the observed molecular weight difference between the two isoforms.

Snake IgY is truncated at the C-terminus and does not exist as a heavy chain-only antibody

Previous sequence analysis revealed that the N-terminus of the IgY1 CH1 domain in *E. taeniura* lacks the conserved cysteine residue required for covalent linkage to light chains. Western blotting analysis showed that the molecular mass of truncated IgY closely resembles that of heavy chain-only antibodies found in camelids (Hamers-Casterman et al., 1993). Given this, we speculated that shortened IgY may function as a heavy chain-only antibody or exist with heavy chains that are non-covalently linked with the light chains. To test this hypothesis, purified IgY was subjected to SDS-PAGE, followed by silver staining, which revealed five distinct bands at approximately 120 kDa, 130 kDa, 140 kDa, 160 kDa, and 180 kDa, respectively (Figure 1C). The observed molecular weights suggested that some bands represented aggregates of two heavy chains. For example, the 120 kDa band likely corresponded to two 60 kDa heavy chains, while the 130 kDa band appeared to consist of one 60 kDa and one 70 kDa heavy chain. However, western blotting demonstrated that these bands were detected independently by IgY2- and Igλ-specific antibodies (Figure 1D, E). Furthermore, when the five bands were excised and subjected to SDS-PAGE under reducing conditions, both the IgY2 heavy chain and λ chain were detected by western blotting (Figure 2A), confirming the presence of Igλ light chains in all bands and excluding the possibility that *E. taeniura* produces heavy chain-only antibodies.

To further investigate whether the snake IgY heavy chains are covalently linked to light chains, serum samples were analyzed by SDS-PAGE under both reducing and non-reducing conditions, followed by western blotting using anti-Igλ antibodies. Results showed that Igλ light chains were detected only under reducing conditions (Figure 2B), indicating that they remain covalently associated with the H chains in their native state. Additionally, when purified IgY was probed with anti-IgY2-T antibodies specific to the peptide derived from the CH4 domain, both the intact heavy chain (~70 kDa) and a smaller band (~10 kDa, termed IgY-T) were detected (Figure 2C). These results suggest that the truncated IgY in *E. taeniura* arises from cleavage at the C-terminus of the full-length IgY.

Snake IgY is truncated at N338 in the CH4 region

To precisely determine the cleavage site responsible for generating truncated IgY in *E. taeniura*, protein A-purified IgY was analyzed using 2-DE. This separation successfully resolved two variants of IgY1 and IgY2 (Figure 3A). Subsequently, the target bands, including IgY1-L, IgY1-S, IgY2-L, and IgY2-S, as well as the 10 kDa IgY-T band excised from SDS-PAGE, were subjected to LC-MS/MS analysis for peptide mapping. LC-MS/MS analysis identified multiple peptides within IgY1-L, including "SKAPIIHVYPPIPGNSR" and "APIIHVYPPIPGNSR" (Supplementary Table S1). LC-MS/MS analysis also showed peptides terminating at N338, including "SKAPIIHVYPPIPGN", in IgY1-S (Figure 3B; Supplementary Table S1). Additionally, IgY-T contained abundant "SRIITCF" peptide (Figure 3C; Supplementary Table S1), which were located immediately downstream of N338 in the IgY1 CH4

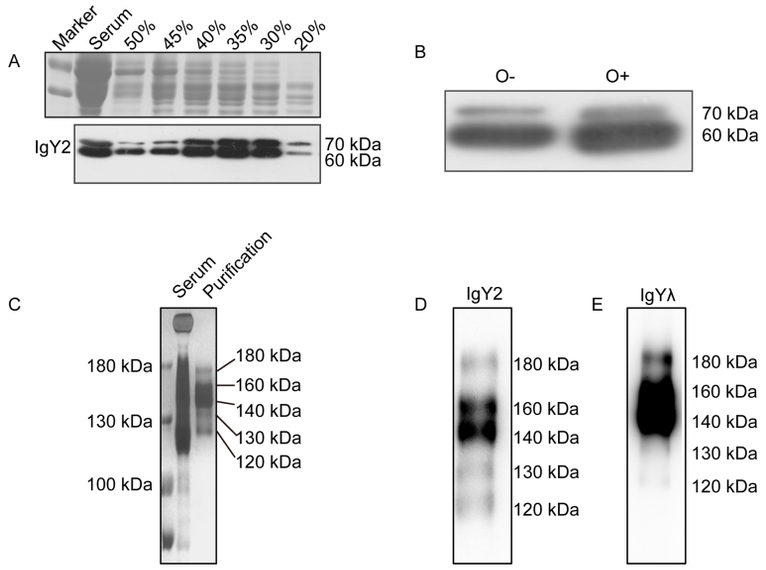

Figure 1 Western blotting detection of IgY2 in serum

A: Detection of IgY2 in purified immunoglobulins. Snake immunoglobulins were purified using ammonium sulfate precipitation at varying concentrations, followed by SDS-PAGE and Coomassie Blue G250 staining. Western blotting was performed under reducing conditions using anti-IgY2 antibodies. B: Detection of glycosylation of IgY2. Lane O-: Serum treated with SDS and DTT. Lane O+: Serum treated with SDS, DTT, and endo-α-N-acetylgalactosaminidase. C: Non-reduced SDS-PAGE gel stained with silver revealed five bands at approximately 120, 130, 140, 160, and 180 kDa. D: Five bands in Figure 1C were detected by western blotting using anti-IgY2 antibody. E: Five bands in Figure 1C were detected by western blotting using anti-Igλ light chain antibody.

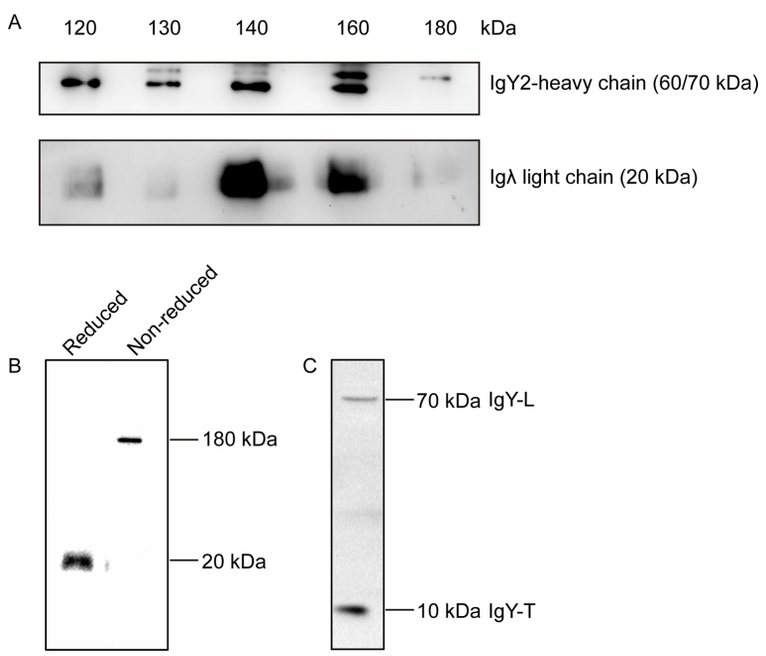

Figure 2 Snake IgY is truncated rather than functioning as a heavy chain-only antibody

A: Non-reducing SDS-PAGE revealed five distinct bands at approximately 120, 130, 140, 160, and 180 kDa, which were excised and analyzed by western blotting using anti-IgY2 and anti-Igλ light chain antibodies. B: Western blotting detection of Igλ light chains under both reducing and non-reducing conditions. C: Identification of the 10 kDa IgY-T fragment in snake serum by western blotting using anti-IgY2-T antibodies.

region but were not detected in IgY1-L. Similarly, IgY2-S contained peptides terminating at N338, including "VFVFPPHRDELN" (Figure 3D; Supplementary Table S1). Furthermore, numerous peptides located after N338, such as "SNHNPLPEDSY", were also detected in IgY2-T (Figure 3E; Supplementary Table S1). These findings provide strong evidence that truncated IgY isoforms in *E. taeniura* are generated via proteolytic cleavage at N338 within the CH4 domain.

Snake IgY is cleaved by AEP *in vitro*, but shows a complex pattern

The above data suggest that truncated IgY forms in *E. taeniura* result from enzymatic cleavage at N338. Also known as legumain, AEP is the only known cysteine protease in mammals that specifically cleaves peptide bonds at asparagine residues (Zhang & Lin, 2021). Given this specificity, we hypothesized that AEP may mediate IgY cleavage in *E. taeniura*. To test this, snake serum was

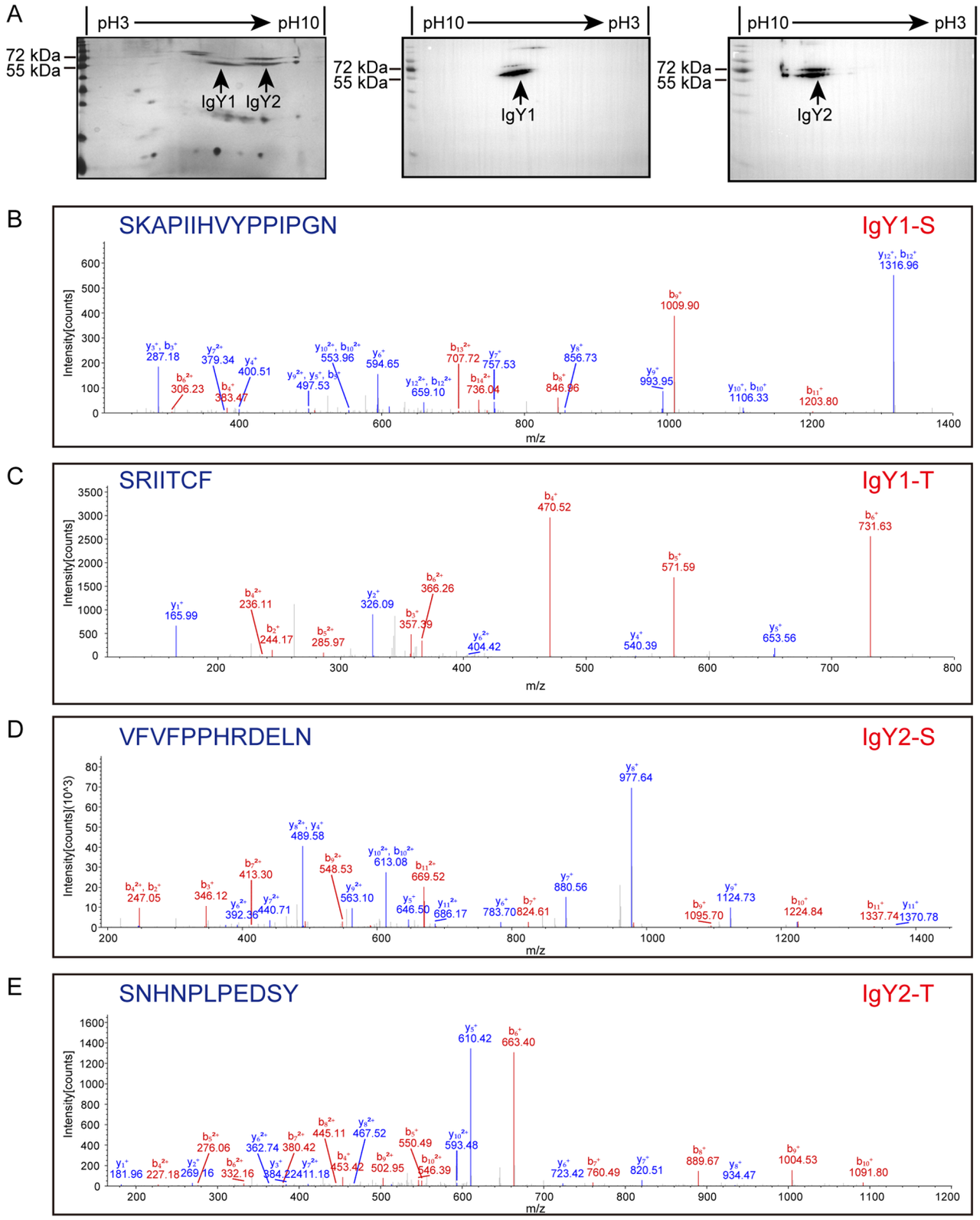

Figure 3 LC-MS/MS analysis of IgY proteolytic fragments

A: Samples were isolated using 2-DE, followed by silver staining (left), and detected by western blotting with anti-IgY1 (middle) and anti-IgY2 (right) antibodies. B: Secondary mass spectra of peptide terminating at N338, detected in IgY1-S. C: Secondary mass spectra of peptide starting at S339, detected in IgY1-T. D: Secondary mass spectra of peptide terminating at N338, detected in IgY2-S. E: Secondary mass spectra of peptide starting at S339, detected in IgY2-T.

incubated with purified human AEP at pH 6.0 and 37°C, followed by SDS-PAGE and western blotting using IgY2- and IgY2-T-specific antibodies. In untreated samples, three bands were detected at approximately 70 kDa, 60 kDa, and 10 kDa, corresponding to the heavy chains of IgY2-L, IgY2-S, and IgY2-T, respectively. Following AEP treatment, the 70 kDa

band disappeared, the 60 kDa band weakened, and two new bands emerged, one slightly larger than 60 kDa and another at approximately 40 kDa. Cleavage was effectively inhibited by the AEP inhibitor AENK (Figure 4A), confirming that AEP can cleave snake IgY *in vitro*. However, the observed cleavage pattern deviated from expectations, suggesting potential differences in cleavage sites between human and snake AEP.

To further investigate, snake AEP was expressed in HEK293T cells, with the resulting lysates incubated with snake serum (Figure 4B). The cleavage pattern mirrored that of human AEP treatment (Figure 4C). Additionally, to evaluate substrate specificity, mouse serum was incubated with human AEP, but no cleavage of mouse IgG was observed (Figure 4A), indicating that AEP exhibits substrate selectivity for asparagine residues. Based on the amino acid sequence of the IgY2 heavy chain, we speculate that multiple AEP cleavage sites exist within the IgY CH region, contributing to the complex cleavage pattern observed (Figure 4D).

DISCUSSION

A diverse antibody repertoire is essential for robust antigen recognition and immune defense. While extensive research has elucidated mechanisms underlying variability in immunoglobulin variable regions, such as V(D)J recombination, gene conversion, and somatic hypermutation (Flajnik, 2002, 2018; Sun et al., 2013), relatively less attention has been devoted to diversity of CH regions, primarily focused on class switch recombination (CSR) (Pan-Hammarström et al., 2007; Stavnezer et al., 2008). However, beyond CSR, additional diversification of CH regions occurs through subclass differentiation and the alternative expression of a single gene (Sun et al., 2020). IgY and its truncated variant, IgY(ΔFc), have been reported across a range of species, with distinct mechanisms governing their generation. In Anseriformes, IgY(ΔFc) arises from alternative transcriptional termination signals within the Cυ2-Cυ3 intron (Magor et al., 1994). In contrast, lizards and salamanders generate IgY(ΔFc) through alternative splicing, with the 3'-untranslated region of IgY(ΔFc) transcripts located immediately downstream of the Cυ2 exon (Wei et al., 2009; Zhu et al., 2014). In turtles, IgY and IgY(ΔFc) are encoded by separately distinct genes (Li et al., 2012). In this study, we identified a previously unreported mechanism for the production of truncated IgY in *E. taeniura*. Unlike transcriptional or genomic alterations seen in other species, truncated IgY in this snake species results from post-translational proteolytic cleavage at N338 within the Cυ region. This distinct mechanism expands our understanding of immunoglobulin diversity, highlighting an alternative strategy for modulating IgY structure and function across vertebrates.

Truncated antibody isoforms have also been observed across diverse taxa, including both primitive fish and mammals. In neonatal nurse sharks, a naturally occurring truncated form of IgM, encoded by germline genes, lacks the CH2 domain (Rumfelt et al., 2001). Truncated IgM in mice arises through a distinct, non-enzymatic cleavage mechanism, in which the μ heavy chain is cleaved, resulting in the loss of

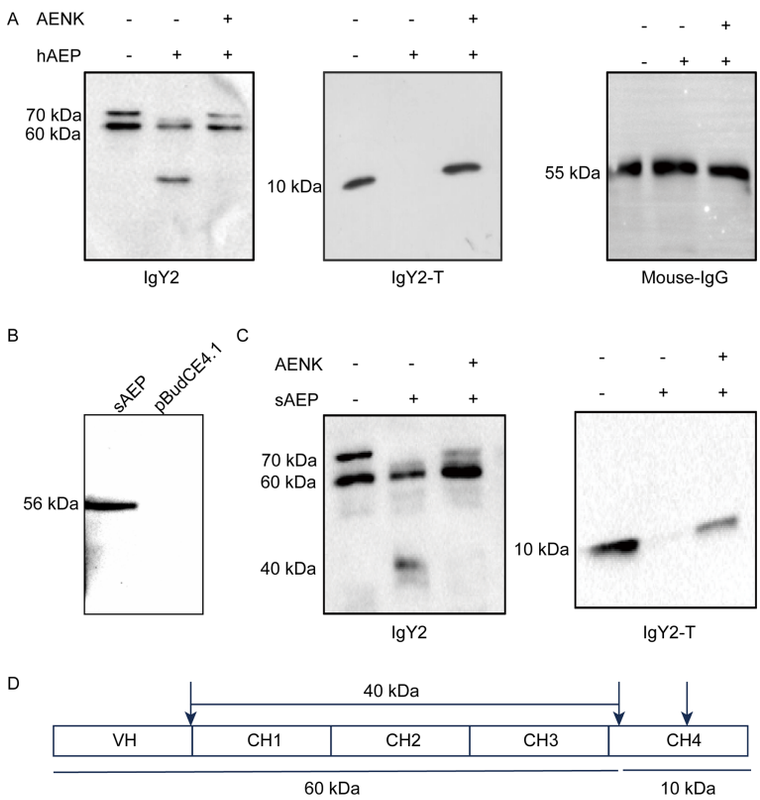

Figure 4 AEP-mediated cleavage of snake IgY

A: Snake or mouse serum was incubated with recombinant human AEP (hAEP), with or without the AEP inhibitor AENK, for 1 h at 37°C and pH 6.0. Cleavage pattern was detected using anti-IgY2, anti-IgY2-T, and anti-mouse IgG antibodies. B: Western blotting analysis of expressed snake AEP (sAEP) in HEK293T cells using anti-His tag antibodies. C: HEK293T cells expressing snake AEP were lysed with pH 6.0 lysis buffer. Lysates were incubated with snake serum, with or without the AEP inhibitor AENK, for 1 h at 37°C. Cleavage pattern was detected using anti-IgY2 and anti-IgY2-T antibodies. D: Predicted cleavage sites of IgY heavy chain by AEP *in vitro*.

its variable region. This cleavage is dependent on the presence of specific proteins and is influenced by pH levels (Klaus et al., 2018). These findings suggest that multiple vertebrate lineages have independently evolved diverse strategies for producing truncated antibodies, which may play functional roles in shaping immune responses.

Truncated antibodies not only exhibit reduced molecular weight but also lack critical functional domains, which can significantly influence immune responses. Understanding the immunological implications of these truncated antibody forms is essential for elucidating their potential advantages for the host. In reptiles and Anseriformes, IgYΔ(Fc) lacks the Fc region, impairing macrophage-mediated clearance of opsonized pathogens. However, this truncation also prevents the binding of antibody-covered virions to cellular Fc receptors and complement component C1q, thus inhibiting antibody-dependent enhancement (ADE) and restricting viral entry into host cells (Meddings et al., 2014). Notably, duck serum contains a higher proportion of IgYΔ(Fc), which may contribute to enhanced viral neutralization while reducing inflammatory activation. This immunoglobulin profile may partially explain why ducks serve as natural reservoirs for certain viruses (Magor, 2011). In mammals, the primary binding site for IgG to FcγR resides at the N-terminus of the Cγ2 domain (Shields et al., 2001), whereas in chickens, mutagenesis studies indicate that the IgY Fc segment interacts with its receptor at the Cυ3/Cυ4 interface (Taylor et al., 2010). Considering the phylogenetic relationship between avian and reptile IgY, the absence of the CH4 domain in truncated snake IgY is likely to affect its interaction with Fc receptors, potentially altering its immunological function.

Our study demonstrated that truncated IgY in *E. taeniura* results from proteolytic cleavage at N338 within the CH region. AEP is an endolysosomal cysteine protease broadly expressed across cell types, known for its ability to cleave protein substrates at the C-terminus of asparagine residues (Dall & Brandstetter, 2013). Similar to other members of the papain family of lysosomal cysteine proteases, AEP activation is triggered by an acidic pH environment (Li et al., 2003; Zhao et al., 2014). Consistent with this, our cleavage experiments were conducted under acidic conditions. Both human and snake AEP exhibited consistent cleavage patterns when incubated with snake serum IgY (Figure 4A, C). Sequence alignment revealed 76% amino acid identity between the core domains of human and snake AEP, indicating conservation of AEP across species. Notably, AEP failed to cleave mouse IgG *in vitro* (Figure 4A), suggesting that AEP specifically targets snake IgY *in vitro*. However, treatment of the snake serum with AEP *in vitro* resulted in the loss of the 70 kDa of full-length IgY band and the emergence of two new bands, one slightly larger than 60 kDa and another approximately 40 kDa in size (Figure 4A, C). These findings suggest that AEP cleaves IgY at multiple sites under *in vitro* conditions. Based on the molecular weights of these bands, we hypothesize that, in addition to cleavage at N338 within the CH region, AEP may also target sites between the VH and CH1 domains and within the CH4 region (Figure 4D). Despite these *in vitro* findings, an important question remains: why does the AEP-mediated cleavage pattern observed *in vitro* differ from the truncated IgY isoforms detected *in vivo*? One possibility is that AEP does not act alone under physiological conditions but instead functions in concert with other proteases or regulatory factors to ensure site-specific cleavage *in vivo*. In conclusion, this study identified a novel post-translational mechanism for generating truncated IgY isoforms in *E. taeniura*, expanding our understanding of immunoglobulin diversity and the evolutionary adaptability of the immune system.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

T.H. and Y.F.Z.: writing–review & editing; M.Z. and T.H.: writing-original draft; M.Z., L.H., T.Y.X., T.W., L.J.D., D.Y., H.W.C., J.C.Z., G.S.C., X.D., and H.T.H.: investigation and analysis. All authors read and approved the final version of the manuscript.

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