Received: 30 October 2021 | Revised: 26 June 2022 | Accepted: 28 July 2022

DOI: 10.1002/jez.b.23173

METHOD AND PROTOCOL

JEZ-B MOLECULAR AND DEVELOPMENTAL EVOLUTION WILEY

An evaluation of the effect of hydrofluoric acid (HF) treatment on keratins

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Funding information

the Strategic Priority Research Program of Chinese Academy of Sciences, Grant/Award Number: XDB26000000; National Natural Science Foundation of China, Grant/Award Numbers: 41902013, 41922011, 41872016, 42288201; the Fundamental Research Funds for the Central Universities, Grant/Award Number: No. 0206-14380137

Abstract

Hydrofluoric acid (HF) is commonly used in geological and paleontological research to extract organic fossils for morphological and chemical studies. However, during HF treatment, organic matter can also be altered, which raises concerns that HF-treated organic matter may not be representative of the original organic matter. To provide reference data for protein studies on fossils, herein, we use Fourier transform infrared (FTIR) spectroscopy to investigate the effect of HF (21.3 M) treatment on keratins, with treatment durations ranging from 2 to 48 h. Results show that the FTIR spectra of HF-treated samples are overall similar to that of the untreated sample, while curve fitting shows that HF treatment has led to alteration of the secondary structure in all the HF-treated samples and the effect is time-dependent. The 2- and 4-h treatment mainly reduced the content of the random coils, α-helix, and intermolecular β-sheet. From 8h onwards, the content of random coils greatly increased at the expense of other structures. Our results imply that for protein detection in fossils using FTIR spectroscopy, the negative effect of HF treatment is not substantial, as the bands characteristic of proteins, that is, amide A, amide B, amide I, amide II, and amide III, are still present after the 48-h treatment. If the target is a secondary structure, the effect of HF treatment should be considered. When HF treatment is necessary, limiting the treatment duration to less than 4h may be a choice.

KEYWORDS

evolution, FTIR, HF treatment, keratin, secondary structure

1 | INTRODUCTION

Integrating data from evolutionary developmental biology and paleontology is important to understand the evolution of organisms (Chipman & Edgecombe, 2019; Hall, 2002; Wu, Yan, et al., 2018). Data from both fields contribute to the building of a sound phylogenetic framework within which we can infer the evolution of specific characters. Paleontology can calibrate the timescale of evolution and provide evidence of extinct forms and the sequence of evolutionary events, while evolutionary developmental biology can provide insight into the mechanisms underlying characters' evolution (Chipman & Edgecombe, 2019; Hall, 2002; Wu, Yan, et al., 2018). Moreover, mounting evidence suggests that fossils can provide molecular information in addition to morphological information about extinct organisms (Briggs & Summons, 2014).

However, the small amount of the preserved organic matter and the interference of minerals challenge the analysis of the molecular information preserved in fossils (Dallongeville et al., 2016). Consequently, isolation procedures have often been used before applying analytical techniques (Delarue et al., 2021). Hydrofluoric acid (HF) is commonly used as a reagent to remove silicate minerals (Craig & Collins, 2000; Oonk et al., 2012; Riding & Kyffin-Hughes, 2004).

However, there is evidence that HF can alter the organic matter during demineralization (Delarue et al., 2021). Studies from soil science showed that treatment of soil samples with HF can lead to the loss of C and N, and suggested that proteinaceous material may be lost (Gonçalves et al., 2003; Rumpel et al., 2006; Sanderman et al., 2017; Schmidt & Gleixner, 2005). Understanding the effect of HF treatment on proteins with experiments is crucial to determine whether to use HF treatment in studies and the optimal treatment duration.

Among biomolecules, DNA and RNA have the lowest preservation potential in fossils (Briggs & Summons, 2014). By contrast, proteins, also carrying phylogenetic information, are more stable (Briggs & Summons, 2014; Demarchi et al., 2016). Molecular and developmental studies have proposed many hypotheses on the evolution of structures present in living organisms, which remain to be tested with direct evidence from fossils, for example, hypotheses on the relationship among reptilian scales, avian scales, and avian feathers (e.g., Alibardi & Sawyer, 2002; Dhouailly, 2009; Wu, Lai, et al., 2018).

The abundant fossil feathers discovered in the last several decades have provided valuable information on the early evolution of feathers, powered flight, and sexual selection (e.g., Clarke, 2013; Feo et al., 2015; Wang et al., 2021; Xu & Guo, 2009; Xu et al., 2014; Zheng et al., 2013). Modern feathers are predominately constructed of β-keratins (also referred to as corneous β-proteins), with a small amount of α-keratins (Alibardi, 2013, 2017). β-Keratins are characterized by a central β-sheet region, while α-keratins have a central α-helical domain (Alibardi, 2017; Calvaresi et al., 2016; Fraser & Parry, 2009, 2014). Recent studies indicated that keratins may persist into deep time, largely owing to the extensive cross-linking by disulfide bonds and the numerous hydrophobic residues (Pan et al., 2016, 2019; Schweitzer et al., 2018; Yin et al., 2013). Nevertheless, several studies challenged the preservation of keratins in deep time, arguing that the immunological methods used therein to identify keratins in fossils are prone to false positives (Saitta & Vinther, 2019; Saitta et al., 2017, 2018).

Here, we chose keratins as a case study to evaluate the effect of HF treatment on proteins. We used white turkey feathers in the experiments. We characterized the alteration of keratins using Fourier transform infrared (FTIR) spectroscopy, a well-established technique for determining the secondary structure of peptides and proteins (Barth, 2007; Byler & Susi, 1986; Kong & Yu, 2007), which has been recently applied to characterize proteins in fossils (e.g., Boatman et al., 2019; Jiang et al., 2017; Lee et al., 2017; Lindgren et al., 2011; Manning et al., 2009).

2 | MATERIALS AND METHODS

Five small pieces of samples (about 1 cm^2) were cut from a white turkey flight feather, and each was immersed in 1 ml of 21.3 M HF. The treatment durations were 2, 4, 8, 24, and 48 h, respectively. After treatment, the samples were rinsed three times with deionized water. These samples are referred to as HF2h, HF4h, HF8h, HF24h, and HF48h, respectively. Before FTIR analysis, the samples were dried overnight at 45°C. An untreated piece from the same feather was used as a control.

FTIR analysis was performed using a Nicolet iS50 FTIR spectrometer with a Continuμm microscope at the Nanjing University. The instrumental setup included an aperture size of 80 × 80 μm^2, a spectral resolution of 4 cm^{-1}, a wavenumber range of 7000–650 cm^{-1}, and 128 scans. Two to three spectra were collected for each sample and the averaged spectrum was used in subsequent analyses.

To evaluate the changes in the secondary structure of keratins, curve fitting of the amide I region (1700–1600 cm^{-1}) was performed. Before curve fitting, a straight baseline passing the ordinates at 1700 and 1600 cm^{-1} was subtracted. The curve fitting was performed using the Fityk software (Wojdyr, 2010). Gaussian functions were used to fit the data, with initial parameters of the functions set using the peak-detection algorithm in Fityk.

3 | RESULTS

3.1 | Comparison of FTIR spectra

The FTIR spectra of the untreated and HF-treated white turkey feather samples are overall similar (Figure 1), and show bands characteristic of proteins, that is, amide A at around 3288 cm^{-1} (NH stretching), amide B at around 3077 cm^{-1} (NH stretching, an overtone of amide II), amide I at around 1637 cm^{-1} (C = O stretching, CN stretching, CCN deformation, NH bending), amide II at around 1537 cm^{-1} (NH bending, CN stretching, CO bending, CC stretching, NC stretching), and amide III at 1231 cm^{-1} (NH bending, CN stretching, CO bending, CC stretching) (Barth, 2007; Kong & Yu, 2007; H. Yang et al., 2015).

The bands at 2961, 2931, 2875, and 2851 cm^{-1} are assignable to CH asymmetric stretching of $-CH_3$, CH asymmetric stretching of $-CH_2$, CH symmetric stretching of $-CH_3$, and CH symmetric stretching of $-CH_2$, respectively (Dumas & Miller, 2003). They can result from lipids in the feathers and amino acid side chain vibrations (Esparza et al., 2017; Forgács et al., 2013).

3.2 | Curve fitting of the amide I region (1700–1600 cm^{-1})

The amide I region can be well fit by six Gaussian functions (Figure 2 and Table 1). In the untreated sample, the six functions are centered at 1619, 1637, 1658, 1673, 1686, and 1694 cm^{-1}, respectively. The bands at 1637 and 1694 cm^{-1} can be assigned to β-sheet (Dong et al., 1992; Goormaghtigh et al., 1994a). The bands at 1673 and 1684 cm^{-1} are assignable to β-turn (Dong et al., 1990; Goormaghtigh et al., 1994b). The band at 1619 cm^{-1} is assigned to an intermolecular β-sheet resulting from aggregation (Hu et al., 2006; Quiquampoix et al., 1995). The band at 1658 cm^{-1} can be due to random coils and α-helix (Goormaghtigh et al., 1994b). As feathers

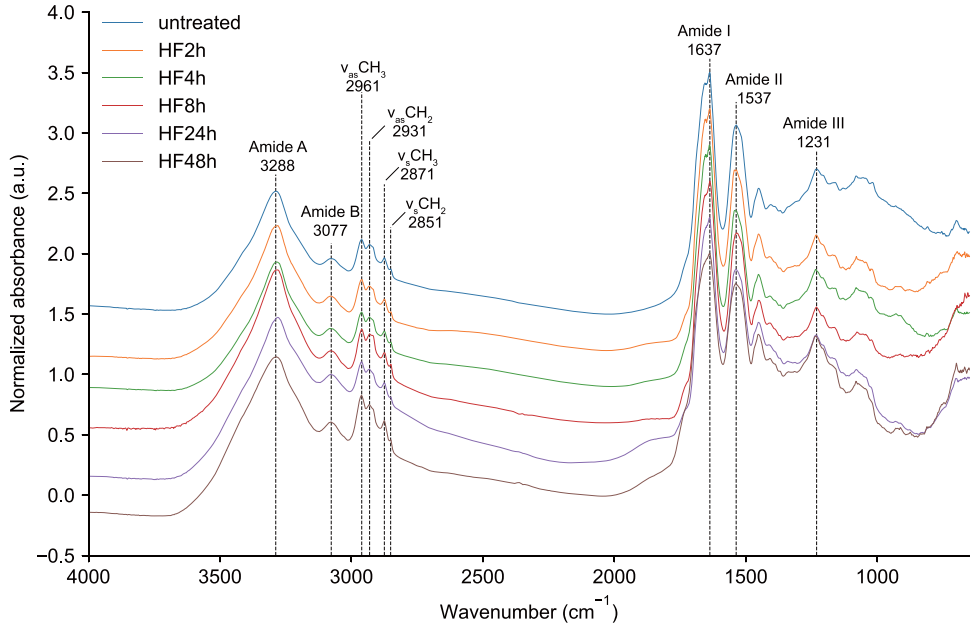

FIGURE 1 Comparison of the FTIR spectra of untreated and HF-treated feather samples. v, stretching vibration; as, asymmetric; FTIR, Fourier transform infrared; HF, hydrofluoric acid; s, symmetric.

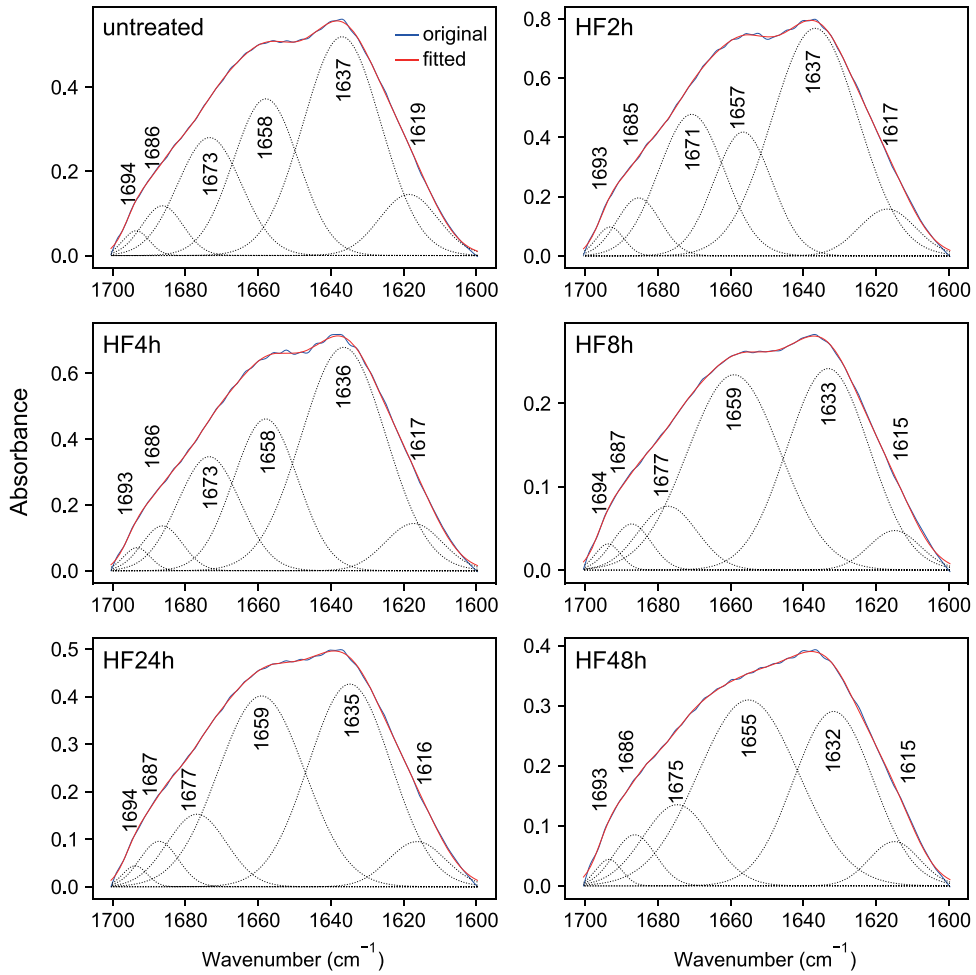

FIGURE 2 Curve fitting of the amide I region of the FTIR spectra of untreated and HF-treated white turkey feather samples. FTIR, Fourier transform infrared; HF, hydrofluoric acid.

TABLE 1 Assignment of the components of the amide I band to secondary structure

Untreated	HF2h	HF4h	HF8h	HF24h	HF48h	Assignment
1619	1617	1617	1615	1616	1615	Intermolecular β-sheet
1637	1637	1636	1633	1635	1632	β-Sheet
1658	1657	1658	1659	1659	1655	Random coils + α-helix
1673	1671	1673	1677	1677	1675	β-Turn
1686	1685	1686	1687	1687	1686	β-Turn
1694	1693	1693	1694	1694	1693	β-Sheet

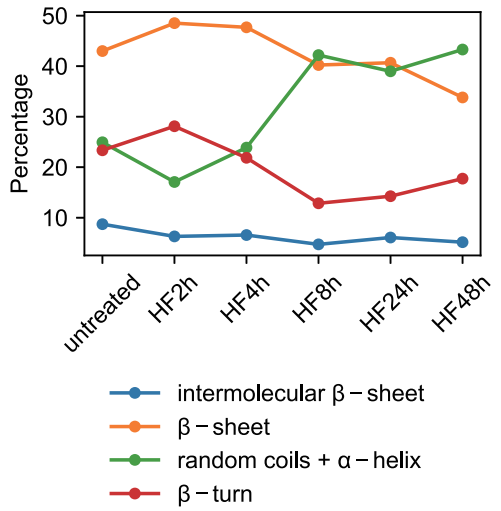

FIGURE 3 Changes in the secondary structure of keratins in the white turkey feather samples with the duration of HF treatment.

contain only a small amount of α-keratins (Alibardi, 2013), this band is likely due mainly to random coils.

The positions of these bands did not change much (within 2 cm^{-1}) in the samples HF2h and HF4h (Table 1). In sample HF8h, the low component of β-sheet (1637 cm^{-1} in the untreated sample) downshifted to 1633 cm^{-1}. The intermolecular β-sheet downshifted to 1615 cm^{-1}. The low component of β-turn upshifted to 1677 cm^{-1}. The same trend was also observed in HF24h and HF48h.

The intensities of these bands were more variable than their positions, which reflects the changes in the secondary structure of keratins (Figure 3). The content of random coils decreased in HF2h and HF4h, but greatly increased in the other HF-treated samples. This trend indicates that HF treatment first led to the breakage of original random coils and α-helix, but with the increase of treatment duration, new random coils formed. The content of the intermolecular β-sheet slightly decreased in the HF-treated samples, indicating the aggregated structure was partly dissolved. The content of β-sheet is increased in HF2h and HF4h, but decreased in the other samples. The content of β-turn is increased in HF2h, but decreased in the other samples. The increases in the contents of β-sheet and β-turn are likely because these structures are more stable than other structures. That is, the relative decreases of random coils, α-helix, and intermolecular β-sheet led to the relative increases of β-sheet and β-turn in these samples.

4 | DISCUSSION

4.1 | Alteration of keratins following HF treatment

Our results show HF treatment led to the alteration of the secondary structure of keratins in all the HF-treated samples, and the effect of HF treatment is time-dependent. The 2- and 4-h treatment appears to have mainly affected the random coils, α-helix, and intermolecular β-sheet. From 8 h onwards, the content of random coils greatly increased at the expense of other structures. The alteration of the secondary structure indicates that HF treatment partially denatured the keratins. Nevertheless, the HF treatment did not affect the identification of the amide bands characteristic of proteins. The methods applied to protein remain in paleontology from detection to identification (Dallongeville et al., 2016), almost all target the amides of proteins. Therefore, we can conclude that as long as the target of further analysis is the amides of proteins, the negative effect of HF treatment could be beneath discussion.

4.2 | Implications for HF treatment on fossils

To date, one major application of FTIR spectroscopy in protein study on fossils is to detect proteins based on the presence of amide bands, especially the amide I and amide II bands (Bobroff et al., 2016; Jiang et al., 2017; Lee et al., 2017; Manning et al., 2009; Reisz et al., 2013; Z. Yang et al., 2019). Our results indicate that the negative effect of HF treatment on this application is negligible.

The secondary structure of keratins is important to understand the molecular evolution of skin appendages, for example, scales and feathers. Evo-Devo studies showed that avian reticulate scales on the plantar surface of toes consist only of α-keratins, while other avian scales—including scutate scales, scutellate scales, and interstitial scales—and reptilian scales consist of both α-keratins and β-keratins (Sawyer et al., 1986; Toni et al., 2007). Moreover, reptilian scales and avian scales, except for reticulate scales, develop from an anatomical placode, which suggests that avian reticulate scales may be derived structures. Scales resembling avian reticulate scales in morphology have been found in multiple non-avian dinosaurs and early birds (e.g., Cuesta et al., 2015; Godefroit et al., 2020; Hendrickx et al., 2022; Xing et al., 2017). To characterize the secondary structure of keratins preserved in fossil scales can help answer when avian reticulate scales evolved their peculiar composition.

Based on the development of modern feathers, Prum (1999) proposed a five-staged feather evolution model. Paleontological studies on feathers and feather-like integumentary structures associated with theropods identified not only structures consistent with these five

stages but also structures that cannot be interpreted using extant feather morphogenesis, which greatly improved our understanding of the morphological evolution of feathers (Lin et al., 2020; Xu & Guo, 2009; Xu et al., 2014). Recently, pennaceous feathers of the Jurassic paravian dinosaur *Anchiornis* were found to be dominated by α-keratins rather than by β-keratins as in modern feathers, which suggested that the molecular composition continued to evolve after the appearance of pennaceous feathers to obtain the physical and mechanical properties for flight (Pan et al., 2019). However, this hypothesis has been challenged by recent studies arguing that the immunological methods used therein to identify keratins in fossils are prone to false positives (Saitta & Vinther, 2019; Saitta et al., 2017, 2018; but see Schweitzer et al., 2019). FTIR spectroscopy could be a viable technique to independently test this hypothesis by characterizing the secondary structure of keratins preserved in fossil feathers.

Despite the potential to address such evolutionary questions, the application of FTIR spectroscopy in determining the secondary structure of protein remains in fossils is still limited (Lindgren et al., 2011), largely due to the poor ratio between organic and inorganic matter in fossils. To determine the secondary structure of proteins using FTIR spectroscopy, data treatment methods, such as curve-fitting and Fourier self-deconvolution, are needed, which requires that the target matter is not at the trace level (Bobroff et al., 2016). Isolating organic matter may be a means to solve the problem. Unfortunately, our results indicate that HF treatment will likely alter the secondary structure of proteins if preserved, although it can concentrate the organic matter. Nevertheless, the results on the alteration of the secondary structure with treatment duration suggest that when HF treatment is necessary, limiting the treatment duration to less than 4h may be a choice.

Our results also have implications for protein identification based on immunological methods, as alteration of the secondary structure can damage some conformational epitopes (Goding, 1996). In the Digestion and Capture Immunoassay, HF (4M) is used to digest the minerals and release mineral-bound proteins (Craig & Collins, 2000). In this protocol, the digestion was conducted at a low temperature (4°C) to limit the damage on the epitopes, but the signal deteriorated when being digested for more than 15 h (Craig & Collins, 2000). The application of in situ immunohistochemistry involves the preparation of ultrathin sections (Lindgren et al., 2018; Pan et al., 2016, 2019; Schweitzer et al., 2016). Without demineralization, the cut ultrathin sections may easily disintegrate. But with the increase in the duration of HF treatment, more and more conformational epitopes will likely be damaged. Hence, it is also necessary to limit the duration of the HF treatment when applying in situ immunohistochemistry.

5 | CONCLUSIONS

Our results show that the FTIR spectra of all HF-treated white turkey feather samples are overall similar to that of the untreated sample, with the identification of amide bands characteristic of proteins not affected by HF treatment. Detailed comparison using curve fitting shows that alteration of the secondary structure has occurred to all the HF-treated samples, with major alterations occurring when treatment duration exceeds 4 h, as indicated by the abrupt increase of the content of the random coils. Our results suggest that when using FTIR spectroscopy to detect protein residues based on the presence of amide bands, the negative effect of HF treatment could be little. But if the target is to address the molecular evolution of specific structures—for example, feathers and scales—based on the secondary structure of proteins preserved in fossils, the damage to the secondary structure by HF treatment should be considered. The trend over treatment duration of the alteration suggests when HF treatment is necessary, limiting the treatment duration to less than 4h may be a choice. Further paleontological studies combining HF treatment with analytical techniques such as FTIR spectroscopy and mass spectrometry will help to resolve the debates on the preservation of proteins in deep time and provide more information on the evolution of organisms.

ACKNOWLEDGMENTS

We thank Zhengfan Lin for assistance during HF treatment, and Yongjiang Xu during FTIR analysis. The research was supported by the National Natural Science Foundation of China (Grant Nos. 41902013, 41922011, 41872016, and 42288201), the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB26000000), and the Fundamental Research Funds for the Central Universities (Grant No. 0206-14380137).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/jez.b.23173

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How to cite this article: Zhao, T., & Pan, Y. (2022). An evaluation of the effect of hydrofluoric acid (HF) treatment on keratins. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 1–8. https://doi.org/10.1002/jez.b.23173