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Independent Determination of Cystine in Keratin Proteins

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Abstract: The role of cystine residues in a protein is well recognized, providing the disulfide bonds for the structural integrity of a wide range of proteins. Hence, the determination of cystine in proteins is critical in understanding the structural functionality of proteins. The amino acid analysis (AAA) is a popular method to determine amino acid residue compositions in proteins. In practice, oxidation of or chemical modification to cystine is often performed prior to AAA. However, these pretreatments are indiscriminate towards cystine and cysteine. Hence, it is difficult to distinguish cystine from cysteine in protein AA composition analyses, especially for cystine-rich proteins such as keratin. In this report, we demonstrate that it is possible to determine cystine residues in protein selectively independent from cysteine, using the conventional AAA, without pretreatments. Our experimental results have shown that cystine did not transform into cysteic acid during acid hydrolysis, as has been reported previously. Our results also showed a part of L-cystine transformed to D-cystine. Finally, we applied the same AAA to determine the cystine residue levels in feather and human hair samples successfully and compared those with the results obtained from AAA using the pretreatment by oxidation.

Keywords: L-/D-cystine, D-cysteine, L-cysteine, Keratin, Amino Acid Analysis

1. Introduction

Disulfide bonds play a crucial role in the folding/unfolding and structural stabilization of many proteins. The disruptions in disulfide bonds are also strongly associated with loss of protein function and activity. An increasing number of neurodegenerative diseases have been linked to the misfolding of a specific protein for which the rearrangement of disulfide bonds is believed to be involved [1]. One example is transmissible spongiform encephalopathies resulting from the conversion of prion protein from its normal cellular isoform to an abnormal scrapie isoform through the rearrangement of disulfide bonds [2]. 21% of the ~90,000 protein structures of the Protein Data Bank contain at least one disulfide bond, and this incidence is considerably higher, especially in many structural and extracellular matrix proteins and receptors [3].

Cystine is the single most important amino acid (AA) residue to provide the disulfide bond for numerous structural proteins. An example includes the keratin intermediate filament (IF) protein network where the disulfide bonds function as intra- and inter-crosslinks for the protein molecules involved in the keratin fibril network. Keratin has cystine residue counts higher than any other protein. Keratin typically contains 4~12 cystine residues per 100 AA residues, whereas collagen, another structural protein, typically contains less than 1 cystine residue per 100 AA residues. Accordingly, the determination of cystine in protein is a key to understanding the structural functionality of keratin, and proteins in general. Furthermore, the determination of the number of cystine and cysteine residues independently is crucial for determining the AA compositions of unknown proteins.

Amino acid composition analysis, or simply amino acid analysis (AAA), is a classical protein analytical method, which finds a wide application in medical and food science research and is indispensable for protein quantification. It is a complex technique, comprising two steps, hydrolysis of a protein sample and chromatographic separation and detection of the residues. For the chromatographic separation, ion-exchange chromatography (IEC) is often used. Hydrolysis

is the first step to breaking down a protein into individual AAs for IEC. The conditions of the hydrolysis have been described in detail elsewhere [4]. The most common hydrolysis is acid hydrolysis [5]. We refer to this method as the conventional AAA. In practice, however, the determination of cystine in a protein by IEC is often preceded by chemical modification of cystine to protect cystine from being oxidized [6–9]. One example includes reducing the disulfide bonds to give cysteine residues which are subsequently subjected to a reaction by sodium iodoacetate to convert the thiol group of cysteine to the S-carboxymethyl group. The result is a protein with the original cystine residues converted to S-carboxymethyl cysteine (SCMC) residues [10]. SCMC does not undergo any chemical transformation during hydrolysis. By quantifying SCMC by AAA, the original composition of cystine and cysteine in the keratin protein sample can be determined. However, this approach does not distinguish between cystine and cysteine since once cystine is reduced to cysteine, there is no distinction between cysteine residues converted from cystine and the original cysteine residues. The result provides only the summation of both cystine and cysteine residues. Furthermore, as has been pointed out before, this approach may be inadequate for the characterization of intermediates isolated during the disulfide-coupled folding of cystine-containing proteins such as keratin [11]. Another popular process is to oxidize cystine to cysteic acid prior to AAA.[8] Cysteic acid is stable during hydrolysis. These procedures are practiced based on a belief that cystine, and also L-cysteine, is oxidized to L-cysteic acid during hydrolysis for AAA [6, 7, 12, 13].

None of the approaches cited here can separately quantify cystine and cysteine independently in the original protein since either cystine is first converted to cysteine or both cystine and cysteine are oxidized. The conversions of cystine to other derivatives have been also proposed [14–16]. Neither approach provided the cystine residue content in the proteins independently from the cysteine residue amount. For example, no keratin protein deposited in the Human Intermediate Filament Database contains cystine residues [17]. This is because of the pretreatments which convert cystine to either cysteine or cysteic acid. Hence, the original composition of cystine and cysteine residues is not known from keratin IF proteins.

The commercial interest in keratin has been increasing rapidly, given its unique biological characteristics which have been suggested to include anti-aging, biocompatibility, cell recognition, and cell adhesion [18–21]. These characteristics have spurred, for example, commercial applications in fields such as cosmetics, wound dressing, dermatology agents, and scaffold components for tissue engineering. As a result, extraction and recovery of keratin from keratinous animal body parts (KABPs) such as wool, hog hair, nails, hooves, and others have become an active industry. In the process of extraction and recovery of keratin, the cleavage of the disulfide bonds is the central step, given the keratin protein is tightly integrated into the IF composed of several keratin protein dimers which are bonded through the disulfide bonds. Once the disulfide bonds are cleaved, cystine is reduced to two highly reactive cysteine molecules. Accordingly, cysteine is often chemically modified to a more stable form for AAA.

However, as is pointed out above, these procedures are indiscriminate towards cystine and cysteine. Accordingly, any information on how much cystine is preserved after the extraction of keratin is lost using these pretreatments. Further, when a protein has both cystine and cysteine residues mixed in its composition, their composition is unknown with usual pretreatments. For example, cystine residues in all keratin proteins registered in Protein Data Bank are expressed as ½Cys, i.e., cysteine. It is impossible to identify which cysteine residue was the original native cystine. When using pretreatments before AAA, cystine in extracted keratin is typically reported as ½Cys in the literature, often by analyzing the composition of cysteic acid [8, 9, 22–29]. This situation makes it difficult to characterize the effects of preserving native cystine residues from keratin IFs on the performance of keratin-based commercial products such as haircare products, wound dressings, and other important biomedical applications. The significance of cystine residues has been stressed in keratin-based haircare and wound dressing products [30–32]. However, the effect of the cystine residue on the efficacy of those products is still not well-understood. Therefore, it is desirable to determine cystine residues selectively, independent from cysteine, so that the relationship between the number of cystine residues in keratin used in keratin-based products and their efficacy may be established. There is an urgent need to simultaneously quantify cystine and cysteine with a non-destructive method that will permit the quantification of cystine residues separately from cysteine residues on keratin samples that were not possible with previous methods.

We found two reports observing that cystine underwent racemization during hydrolysis [33, 34]. The racemization of amino acids by acid hydrolysis has been known [35, 36]. Hence, there is a conflict in how cystine behaves during hydrolysis: whether it results in oxidation to cysteic acid or racemization of cystine. Furthermore, there is very little quantitative data on cystine during its hydrolysis for AAA in the literature. Friedman et al. have presented no data for the fate of cystine during hydrolysis, while Jacobson et al. focused more on what was involved in the racemization of cystine than what happened after hydrolysis [33, 34].

Our objective in this work is to demonstrate that cystine residues mostly survive during hydrolysis for AAA, contrary to what has been reported previously [6-9, 11-15, 22-29]. To achieve this objective, we used free AAs including L- and D-cystine, L-cysteine, and cysteic acid to examine their behaviors during hydrolysis without any pretreatments mentioned above. We observed the fate of cystine before and after hydrolysis by monitoring the peak position in the chromatogram. We also monitored the changes in the peaks of cystine and cysteine by AAA with and without oxidation as the pretreatment. Separately, we performed circular dichroism to study the racemization of L-cystine after hydrolysis. D-cystine was clearly found. These results should provide ~~the~~ important information on the fate of cystine not only during

hydrolysis, but also with and without oxidation as the pretreatment. Finally, we apply the same hydrolysis to feather and human hair to determine their amino acid compositions for comparison with previous studies.

2. Materials and Methods

2.1. Materials

L-cystine (≧99.7%), D-cystine (≧98 %), L-cysteine (≧98.5%), and L-cysteic acid monohydrate (≧99.0%) were purchased from Sigma Aldrich (St. Louis, USA) and used without further purification. The concentration of each chemical for AAA was 2.45 mg/L in distilled water to which 0.1 N HCl was added. Feather samples were supplied by Pilgrim's Pride. They were first washed in water by stirring and then dried afterward. Subsequently, they were finely cut with scissors and ground by a grinder to prepare a powder for AAA. Human-cropped hair samples were supplied by Green Circle Solans who collects cropped hairs from hair salons. The human hair powder was preprepared in the same way as the feather powder. Table 1 summarizes the chemical compositions of both powders.

Table 1. *Chemical Compositions of The Keratin Powders.*

Sample	Crude Protein %	Crude Fat %	Ash %	Cd ppm	Pb ppm	As ppm
Feather	97.2	1.74	1.01	< 0.05	0.52	0.12
Human Hair	85.6	2.75	1.58	< 0.02	0.11	0.09

The crude protein was determined by analyzing the total nitrogen by the Kjeldahl method, while the crude fat was measured by the Soxhlet extraction followed by weighing the dried leftover mass. These methods have the detection limits of 0.1% and 0.25%, respectively. The ash was analyzed by heating the sample at 600°C for two hrs. and weighing the leftover mass. This method has a detection limit of 0.01%. The heavy metals were determined by the inductively-coupled plasma emission spectrometer with a detection limit of 0.1 ppm. The missing component in the human hair composition was moisture due to insufficient drying. Given the low contents of crude fat, the effects of protein-lipid interactions on results from AAA were not expected to be significant.

2.2. Amino Acid Analysis

First, the free AAs have been analyzed. L-cystine was analyzed before and after acid hydrolysis, while L-cysteine, L-cysteic acid, and D-cystine were measured without hydrolysis. The condition of the acid hydrolysis was 6 N HCl aq. solution for 24 hrs. at 110°C. This is a very similar condition to the hydrolysis by which cystine has been believed to be oxidized to cysteic acid [8, 9, 22–29]. Separately, L-cystine was subjected to oxidation prior to AAA by adding performic acid to the sample solution which was kept at 2°C overnight to complete the oxidation. The performic acid was prepared fresh by mixing 30% hydrogen peroxide with 88% formic acid just prior to use. It was added to the samples and left overnight at 2°C to complete the oxidation. AAA was performed by the Hitachi L-8800 AA analyzer (Tokyo, Japan). IEC was used to separate each AA followed by a post-column ninhydrin reaction detection system. L-norleucine (Norleu) was used as the standard. The error was within 2% in terms of the retention time and the peak area in the chromatogram. Subsequently, two KABP samples were analyzed: a feather sample and a human hair sample by the same technique without any treatment.

3. Results and Discussion

3.1. Free Amino Acids Analysis

We first examined the transformation of the free AAs after hydrolysis. All the measurements were performed in triplicate. Figure 1 shows some of the column chromatograms of the free AAs and cysteic acid. Norleu was detected at the retention time (RT) of 34.10 min. as it was spiked in as a standard. NH_3 present in the environment readily reacted with ninhydrin and appeared as a small peak at the right end, with other non-integrated changes in the baseline due to buffer changes in the method. Table 2 summarizes RT and the concentration in mole L^{-1} of the compounds: L-cystine and L-cysteine before hydrolysis (BH), after acid hydrolysis (AH), and after oxidation (Ox), and D-cystine and L-cysteic acid for BH only.

Table 2. *The Retention Time (RT) and the Concentration (mole L^{-1}) of Free AAs and L-Cysteic Acid.*

	L-cystine		D-cystine		L-cysteine		L-cysteic acid	
	RT min	mole L^{-1} x 10^{-3}	RT min	mole L^{-1} x 10^{-3}	RT min	mole L^{-1} x 10^{-3}	RT min	mole L^{-1} x 10^{-3}
BH[a]	22.74	9.75	22.71	9.77	14.92	19.74	3.22[e]	14.12[e]
AH[b]	22.75[d]	9.28			3.22	19.73		
Ox[c]	3.22	19.36			3.22	19.73		

[a]No hydrolysis was performed before AAA.
[b]The hydrolysis was performed before AAA. See the text for the procedure.
[c]The oxidation was performed before AAA. See the text for the procedure.
[d]The mixture of L- and D-cystine.
[e]L-cysteic acid before hydrolysis.

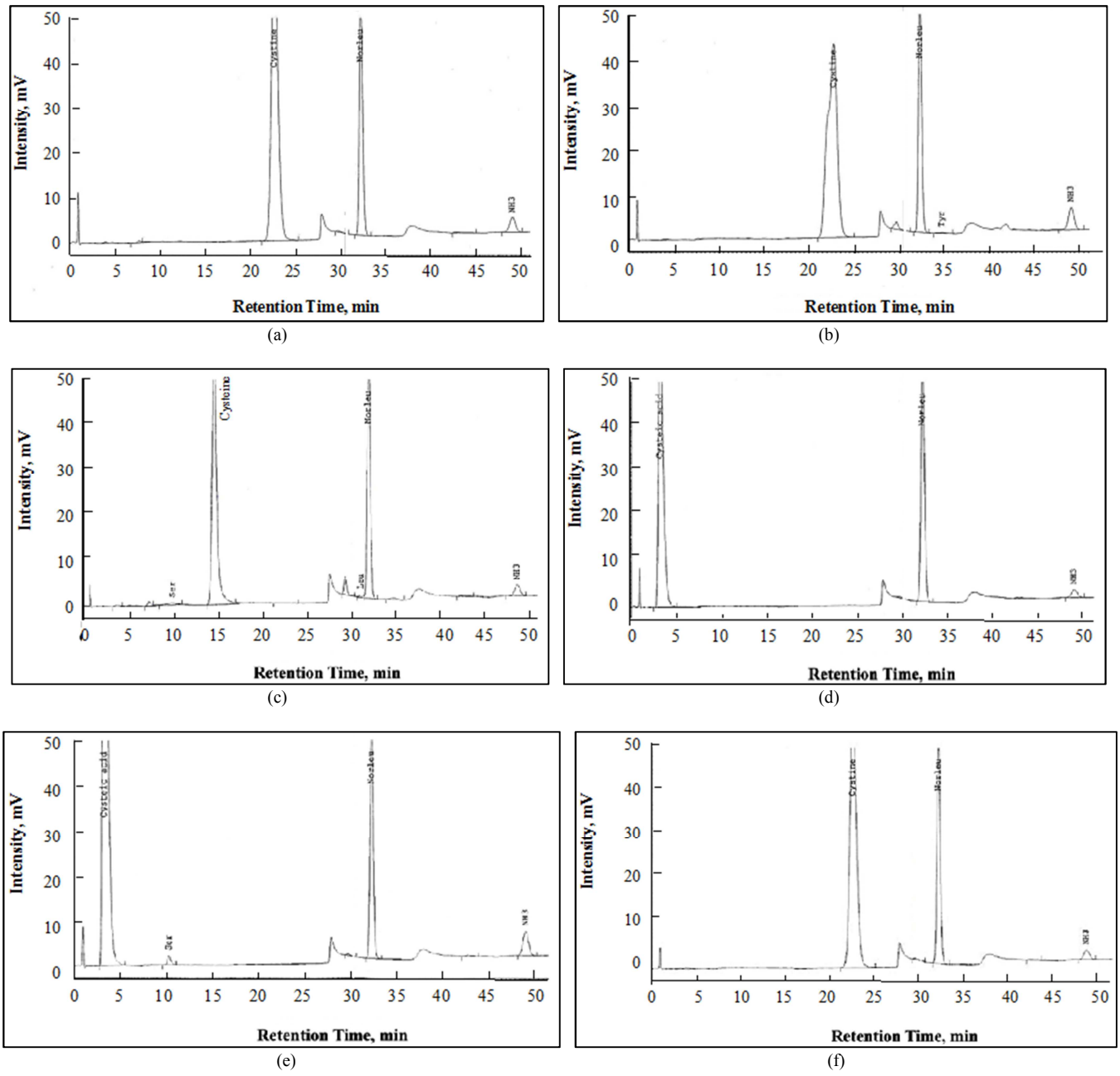


Figure 1. *The ion-exchange column chromatogram of L-cystine (a) before and (b) after hydrolysis, (c) that of L-cysteine without hydrolysis, (d) that of L-cysteic acid without hydrolysis, (e) that of L-cystine after oxidation, and (f) that of D-cystine without hydrolysis. The peak at the RT of 32.30 min in each chromatogram is due to L-norleucine which was used as the internal standard for AAA.*

Figure 1 (a) and (b) display the chromatograms of L-cystine before and after hydrolysis, respectively. A sharp peak appears at the retention time (RT) of 22.74 min. before hydrolysis, while the peak changed its shape by having a shoulder on the left side, but still at nearly the same RT of 22.75 min. after hydrolysis. Based on the peak areas, we estimated that the concentrations of L-cystine before and after the hydrolysis were 9.75 x 10^{-3} mole L^{-1} and 9.28 x 10^{-3} mole L^{-1}, respectively. The difference in the concentration before and after hydrolysis was somewhat larger than the margin of error, ~ ±0.2 x 10^{-3} mole L^{-1}. A small fraction of L-cystine may have been lost during hydrolysis. That loss amounts to 4.8 % of the original concentration, according to Table 2, not a significant loss. The value was based on the peak areas which had an error of less than 2% determined from triplicate experiments. Losses of some AAs during acid hydrolysis have been reported before and a correction has been proposed [37]. We examine this shoulder further later.

Figure 1 (c) displays the peak of L-cysteine appearing at 14.92 min. without hydrolysis in a separate analysis of L-cysteine. Hence, it is clear that the peak at 22.75 min in Figure 1 (b) was not due to L-cysteine. L-cystine is also suggested to be oxidized to L-cysteic acid during acidic hydrolysis [9, 21–29]. Figure 1 (d) exhibits the peak of L-cysteic acid at 3.22 min without hydrolysis or oxidation. This peak is absent in Figure 1 (b). These observations suggest

that hydrolysis of L-cystine does not convert L-cystine to L-cysteic acid as has been suggested in the past [9, 22-30]. Next, L-cystine was subjected to oxidation and analyzed by IEC. Figure 1 (e) shows the peak at RT of 3.22 min. which is likely due to L-cysteic acid. Separately, oxidation was applied to L-cysteine as well before AAA. The peak appeared at 3.22 min. in the chromatogram, not shown here. This result suggests that L-cysteine is also converted to L-cysteic acid by oxidation. This observation is in line with the previous reports [22–30]. Based on these results, it appears that hydrolysis and oxidation have different effects on L-cystine, whereas they all convert L-cysteine to L-cysteic acid. Accordingly, our results reveal that L-cystine and L-cysteine behave differently in hydrolysis and oxidation. Based on the peak area in Figure 1 (e), the concentration of L-cysteic acid was 19.36 x 10^{-3} mole/L. Hence, the conversion of L-cystine to L-cysteic acid through oxidation was nearly stoichiometric: 9.75 x 10^{-3} mole/L to 19.36 x 10^{-3} mole/L, according to the following reaction:

$$\text{Cys-Cys} \rightarrow 2\ \text{Ala-SO}_3\text{H} \qquad (1)$$

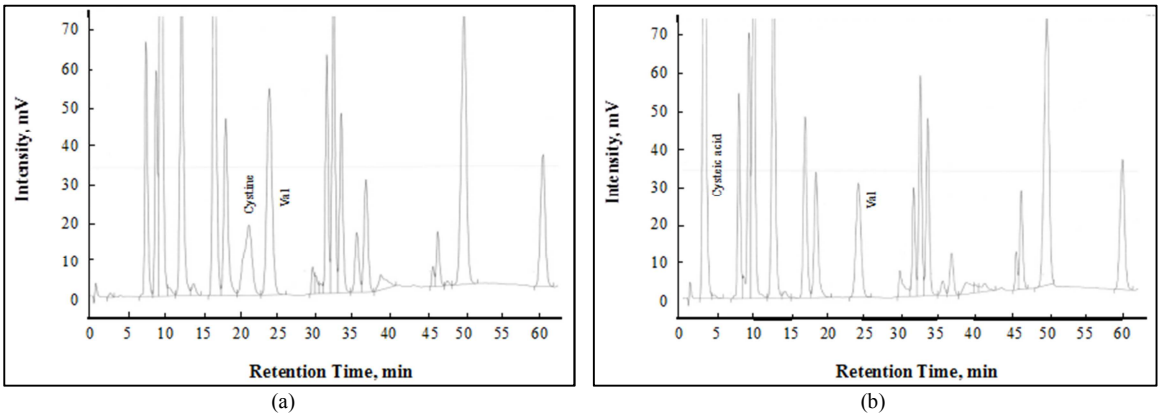
Our results also confirm that the oxidation of cystine to cysteic acid prior to AAA ensures a quantitative analysis of cystine residues. However, this approach does not distinguish between cystine and cysteine, since cysteine can be also oxidized to cysteic acid.

Racemization of L-cystine during hydrolysis has been reported in the past [33, 34]. There are three possible stereoisomers of cystine: meso-cystine, D, D-cystine (D-cystine), and L, L-cystine (L-cystine). These isomers have been reported earlier [34]. In meso-cystine, only one a-carbon of the two amino acid groups undergoes racemization. In D-cystine, the α-carbon of each amino acid group undergoes racemization. Finally, L-cystine is the mirror image of L-cystine and the meso-cystine before racemization. Structurally, meso-cystine is closer to L-cystine cystine, given that one of the amino acid groups in meso-cystine can be superimposed to that of L-cystine, whereas L- and D-cystine cannot be superimposed on one another.

In order to examine the left shoulder of the peak in Figure 2 (b), D-cystine was analyzed by IEC without hydrolysis as one possible stereoisomer as a result of the racemization. Figure 2 (f) shows the chromatogram of D-cystine with the peak appearing at 22.71 min. which is very close to the left shoulder of the peak in Figure 2 (b). We infer that the peak around 22 min in Figure 2 (b) was a convolution of peaks for the stereoisomers involved in the racemization of L-cystine. We suspect that the peak for meso-cystine was somewhere between the two peaks, if it was one of the isomers after hydrolysis: one for D-cystine at 22.71 min. and another for L-cystine at 22.75 min. Meso-isomer has been identified as one of the isomers as a result of racemization of L-cystine [33]. The examination of the deconvolution of this peak to all possible isomers through further instrumental work is warranted.

3.2. Amino Acid Compositions of Feather and Human Hair Samples

We have analyzed the AA compositions of the feather and human hair powders by AAA with and without oxidation as the pretreatment following hydrolysis. The condition for the oxidation was described under Methods. Figure 2 (a) and (b) show the column chromatograms of the feather powder without and with oxidation, respectively. Similar chromatograms were obtained for the human hair powder, hence not shown here. The most striking difference between the charts in Figure 2 (a) and (b) with and without oxidation is that the peak at 22.76 min, due to cystine, without oxidation completely disappeared when oxidation was performed as the pretreatment and instead, a new, large peak at 3.18 min, due to cysteic acid, appeared. The peak at 22.76 min. with the shoulder on the left at 22.72 in Figure 2 (a) which is almost identical to the peak for L-cystine after hydrolysis shown in Figure 2 (a). Both figures suggest that cystine was converted to cysteic acid by oxidation. Figure 2 (a) also reveals a negligible amount of cysteine in the keratinous sample. Based on Table 2, the peak around 3.2 min. should appear if there were any appreciable cysteine residues present in the sample. Very few studies have been reported on the separate compositions of cystine and cysteine residues in keratinous samples. Figure 2 (a) also demonstrates that cystine in the keratin sample survived hydrolysis prior to AAA.



(a) (b)

Figure 2. *The ion exchange column chromatogram of the feather powder (a) without and (b) with oxidation.*

Table 3. *Amino Acid Composition Analyzed by This Study in Comparison to Previous Studies.*

(a) Feather

AA	mole%			
	No Ox[a]	Ox[b]	Ref. 24	Ref.23
Asx[c]	6.04	5.91	6.00	3.4
Thr	4.74	4.94	4.55	3.9
Ser	13.49	13.67	8.94	15.0
Glx[d]	9.05	8.44	6.50	6.8
Pro	11.36	11.44	11.40	10.5
Gly	12.24	11.19	14.40	13.9
Ala	6.38	5.11	6.67	8.6
Val	8.15	7.70	8.58	8.0
Ile	4.39	4.74	4.38	3.2
Leu	7.88	6.80	9.36	7.8
Tyr	1.81	0.78	2.55	1.2
Phe	3.65	3.42	2.30	3.3
His	0.41	0.42	0.87	0.0
Lys	1.25	1.05	0.67	0.1
Arg	4.93	4.82	3.65	3.6
Met	0.29	0.94	0.97	0.0
Cystine	3.93	0.00	—	—
Cysteic Acid	0.00	8.66	8.22	—
SCMC[e]	—		—	8.2

[c]Aspatic acid and asparagine.
[d]Glutamic acid and glutamine.
[e]S-carboxymethylcysteine.

(b) Human Hair

AA	mole %			
	No Ox	Ox	Ref.38	Ref. 37
Asx[c]	6.07	6.80	5.18	5.06
Thr	7.77	7.89	7.65	6.61
Ser	11.85	11.13	12.41	10.38
Glx[d]	14.14	12.03	11.65	10.80
Pro	8.47	7.80	7.68	9.19
Gly	6.21	6.10	5.59	6.25
Ala	4.92	4.74	4.31	4.21
Val	6.11	5.51	5.75	5.98
Ile	2.96	2.82	2.87	2.59
Leu	6.88	6.06	5.94	5.66
Tyr	2.22	2.04	2.12	1.54
Phe	1.76	1.60	1.63	1.74
His	0.85	0.90	0.81	0.75
Lys	2.67	2.38	2.50	2.17
Arg	7.26	6.25	6.09	9.72
Met	0.37	0.51	0.61	0.00
Cystine	8.95	0.00	—	—
½ Cys	0.00	16.70[e]	16.84	17.35

[c]Aspatic acid and asparagine.
[d]Glutamic acid and glutamine.
[e]Cysteic acid.

Tables 3 (a) and (b) list the mole % of AAs for the feather and human hair powder samples determined by AAA with and without oxidation, respectively, including the results from earlier reports [23, 24, 38, 39]. Our AAA without any pretreatment clearly shows a high content of cystine residues which is well-known and expected in KABP such as feathers and human hair. The differences between the mole % of cysteic acid after oxidation and what was expected from the stoichiometric conversion to cysteic acid from cystine before oxidation are within the experimental error for both samples. Hence, our experiment found that the conversion from cystine to cysteic acid the oxidation was stoichiometric for both samples without loss. For the other amino acids measured, the concentrations between AAA with and without oxidation were similar and within the error, except for a few AAs. For example, tyrosine showed considerable losses after oxidation which is well documented [40–42]. Our results demonstrate that the conventional AAA without pretreatment can be applied, at least to these keratinous samples, to determine cystine independently from cysteine in a single run.

A tiny peak for cysteine appeared in the charts for both keratinous samples from AAA without oxidation. This could be a result of cysteine having been oxidized during hydrolysis. In fact, a small peak appeared around 3.2 min. which could be cysteic acid in Figure 2 (a). Still, the small peak area relative to that of cystine, which suggests the original keratinous sample had very few cysteine residues. This observation would have been unlikely, using pretreatments convert both cystine and cysteine to cysteic acid, SCMC, or some other functional group [23, 24, 38, 39]. Once such treatments are performed, neither the cysteic acid level nor the SCMC level provides any information on either cystine or cysteine residue level separately in the original sample by AAA. It is of interest to note that the cysteic acid level in Ref. A, the SCMC level in Ref. B, and the ½ Cys levels in Refs. C and D were almost twice the cystine levels in our study for both samples without oxidation. This observation suggests that the cysteic acid level, the SCMC level, and the ½ Cys level in the previous reports may have been all due to cystine. They were unlikely due to cysteine for both samples, as we found in this work, using AAA with and without oxidation.

4. Conclusion

We have analyzed cystine by conventional AAA under typical acid hydrolysis conditions (6N HCl for 24 hrs.) and unambiguously demonstrated that cystine largely survived during acid hydrolysis. Our detailed analysis included free AAs and through chromatography their identities were determined with and without acid hydrolysis. This observation is opposed to the conventionally held view that cystine is oxidized during hydrolysis. From this belief, a variety of pretreatments have been proposed to protect cystine, though these methods only gave the result as the sum of cysteine and half cystine. Based on our new finding, it may be possible to determine L-cystine independently from cysteine through the conventional AAA without pretreatments, as we have shown using human hair and feather samples. This finding has rarely been reported in the literature. In the limited instances, both amino acids were reported separately under an unusual hydrolysis condition that is not common and not reproducible, given a lack of information [43, 44]. Thorley-Lawson et al. have reported cystine separately from cysteine, but they used a ^{14}C labeling to cystine which adds extra instrumentation and additional

work, not suitable for a routine amino acid analysis [45].

Our work suggests that the conventional AAA can be used for a direct, selective determination of cystine without chemical modification or separate processes to estimate the cystine content prior to hydrolysis. Based on the results of AAA for the free amino acids, we have analyzed the AA compositions of the feather and human hair powders by AAA described in this work. For each analysis, the data shows cystine residue content of 3.93 mole % and 8.95 mole % for the feather and the human hair samples, respectively. These numbers were compared with the results from AAA including the oxidation pretreatment. After oxidation, the peak at 22.7 min disappeared, and a peak at 3.22 min appeared, as anticipated when cystine is oxidized to cysteic acid. This observation shows that cystine was not oxidized during acid hydrolysis, unless being first oxidized as a pretreatment.

It should be noted, however, that the oxidation or the modification of cystine prior to hydrolysis can provide accurate data on the sum of cystine and cysteine residues for a give protein. On the other hand, a small fraction of cystine residues can be lost during hydrolysis by our approach, as is mentioned above. Hence, our work warrants further study to examine the accuracy in comparison to other currently used pretreatments to discern between cystine and cysteine residues. While our technique was proven in unpurified human hair and feather samples as examples, it should be applied to other proteins to validate this approach.

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