Analytical Methods

DOI: 10.1002/ange.200602144

Direct Identification of Tryptophan in a Mixture of Amino Acids by the Naked Eye**

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Tryptophan, an essential amino acid, plays a crucial part in biological processes such as protein biosynthesis, animal growth, and plant development.[1] Tryptophan is also one of the key factors that determines protein activity, hydrophobicity, and diversity.[2] The detection of tryptophan is thus of great importance for its biochemical study.[3,4] However, amino acids often coexist and have similar properties, and thus the detection of an individual one suffers from serious interference and usually has to be carried out through an efficient separation technique such as HPLC.[3] Over the past several decades, much effort has been devoted to seeking a simple and reliable method by which tryptophan could be identified in a mixture of amino acids directly by the naked eye.[3–5] Such a method is extremely desirable for amino acid production and the fast screening of tryptophan in proteolysates, but unfortunately it is still lacking.[2a,4]

It is known that a reversible formylation at the 1-position of tryptophan may occur upon treatment with anhydrous formic acid saturated with gaseous HCl.[6] In an effort to expand our research on developing site-specific labeling methods for proteins,[7] we were therefore interested in trying to introduce the formyl group at the N1 position of tryptophan. When we attempted to improve the formylation reaction by treating tryptophan in a mixed aqueous solution of formic acid and hydrochloric acid, a violet-blue color was surprisingly produced. More interestingly, this reaction exhibits a high selectivity toward tryptophan only, with no color observed with the other amino acids. We envision that this new color-generating reaction could be used as a specific identification method for tryptophan.

Figure 1 shows the absorption spectra of tryptophan in different media. It can be seen that water or formic acid has little effect on the absorption of tryptophan in the visible region, whereas hydrochloric acid causes a broad but rather weak absorption band, and the mixed aqueous solution of HCOOH and HCl leads to the emergence of two new bands at about 364 and 560 nm. It is noted that the addition of formic acid can induce the appearance of the violet-blue color only in HCl media, but not in other acids such as H_2SO_4 and H_3PO_4,[8] thus revealing the catalytic effect of HCl.[6a] Moreover, the violet-blue color becomes more intense with increasing temperature and time, and at a higher temperature (such as 70 °C) water-insoluble brown products can be partly formed, thus indicating a possible decomposition.

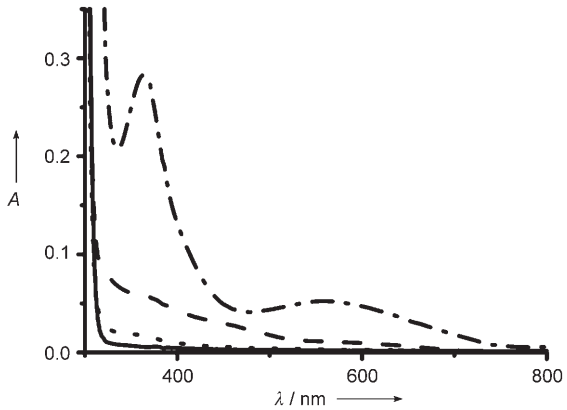

Figure 1. Absorption spectra of tryptophan (5 mM) in water (——), 18% (w/w) formic acid solution (••••), 6 M HCl (– – –), and the mixed solution of 18% formic acid and 6 M HCl (–•–•). See the Experimental Section for more details.

Several factors were examined in detail to enable the reaction conditions to be optimized. These studies showed that the violet-blue color is related to the acidity, the presence of oxygen, the reaction time, and the temperature, but independent of light. On the basis of this finding, a mixed solution of 6 M HCl and 18% formic acid with a heating time of 5 h at 50 °C were chosen for the present reaction, since under these conditions no apparent degradation of tryptophan was detected[8] and a distinct color change could be observed by the naked eye. The color reaction can also occur at room temperature, but takes a long time (usually completed within 4–7 days).

To assess the specificity of the reaction, 20 standard amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) were tested in parallel under the same conditions. The results showed that, with the exception of tryptophan, none of the other amino acids resulted in the generation of any color.[8] This unique property of tryptaphan enables it to be distinguished easily from the other amino acids directly by the naked eye. Figure 2 shows the results of such a preliminary study. As can be seen, tryptophan, whether present alone (C) or in mixed amino acids (D), exhibits the predicted violet-blue color, whereas the other amino acids do not produce any color (B). The method may also be applied to solving the question as to whether a peptide or protein contains tryptophan residues, as demonstrated in Figure 2 through the use of reduced glutathione (E) and egg white albumin (F) as examples. Moreover, by monitoring the absorbance at about 364 or 560 nm,[8] the

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[**] This work was supported by the National Science Fund for Distinguished Young Scholars (no. 20525517), the NSF of China (no. 20435030), and the Chinese Academy of Sciences.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

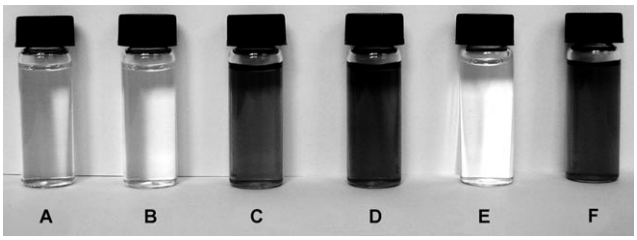


Figure 2. Photograph of solutions of HCOOH/HCl containing different compounds. A) Reagent blank (reaction solution of 18% formic acid and 6 M HCl); B) a mixture of 9 representative amino acids (L-alanine, L-arginine, L-glutamine, L-histidine, DL-methionine, L-phenylalanine, L-proline, DL-serine, and L-tyrosine) with various side-chain features; C) tryptophan; D) amino acid mixture (B) in the presence of tryptophan; E) reduced glutathione; F) egg-white albumin. The concentration of each of the amino acids as well as the reduced glutathione was 20 mM, while that of the egg-white albumin was 0.5 mM. The color reactions (A–D) were performed at 50 °C for 5 h and the reaction solutions were then allowed to stand at room temperature (25 °C) for 4 days, whereas those of E and F were conducted at room temperature for 7 days.

tryptophan content in samples could be estimated, although the sensitivity of the method is not high (detection limit: ca. 0.1 mM, signal/noise = 3:1).

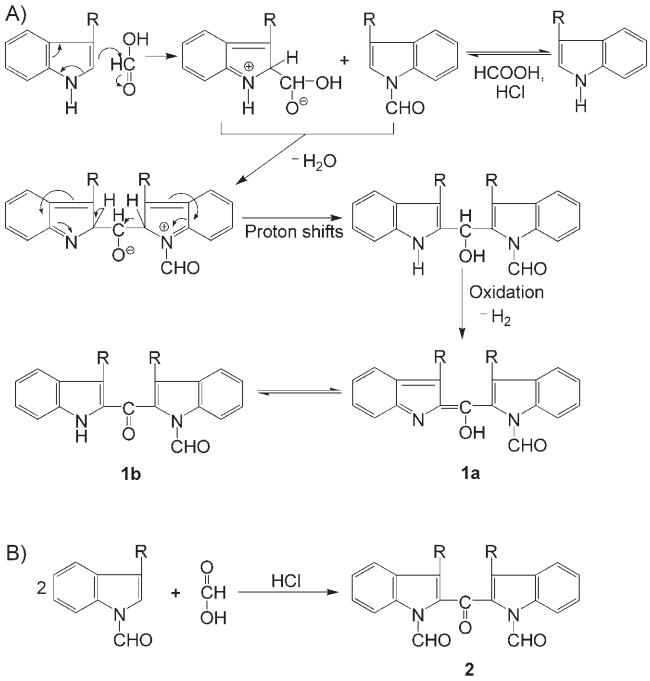
To explore the reaction mechanism, the reactions of tryptophan and its reference compounds indole and indole-3-propanoic acid (IPA) were compared in the HCOOH/HCl system. It was found that all three compounds can react but give different colors: IPA, similar to tryptophan, produces a violet-blue color, while indole yields an orange-red color with an absorption peak at 484 nm.[8] Furthermore, the rate and intensity of the color reactions are increased in the following order: indole > IPA > tryptophan, clearly indicating that the indole skeleton is an essential moiety for the color reactions, and the steric hindrance of the substituent at the 3-position on the indole ring determines the reaction rate and intensity. It is known that carbolines can not be generated in HCOOH media,[9] and in this system the color interference from the corresponding carbolines could therefore be excluded. However, both indole and IPA, which do not have an α-amino group, produced color, which suggests that the α-amino group of tryptophan may not take part in the reaction process.

Isolation of the violet-blue pigment from the reaction solution was first attempted by column chromatography and extraction with organic solvents such as petroleum ether, chloroform, and ethyl acetate, but was unsuccessful. Subsequently, HPLC was used to isolate the pigment, and two chromatographic peaks appearing at 6.03 and 7.12 min[8] were characterized as tryptophan (m/z 205 $[M+H]^+$) and 1-formyltryptophan (m/z 233 $[M+H]^+$) by MS analysis.[8] However, the expected blue pigment that should have a large conjugated structure was not found, possibly because of its instability under the present test conditions. Therefore, the reaction solution was then subjected directly to electrospray ionization (ESI) MS analysis. Besides tryptophan and 1-formyltryptophan, two more products with higher molecular weights (m/z 463 and 491) were detected.[8] These high-molecular-weight ions, which have not been reported previously,[4,10] may contain at least two tryptophyl units, and be responsible for the violet-blue color.

The violet-blue color is quite stable in strong acid media (at least a week), but will disappear in neutral and basic media: the absorption maximum at about 560 nm gradually decreases and eventually vanishes as the pH value is increased. Conversely, acidification of the reaction solutions back to a strongly acidic pH results in the absorption spectra of the solutions being restored.[8] Moreover, the extent to which the absorption spectra is recovered depends on the time and pH value: the higher the pH value of the solutions, the slower and less complete is the recovery.

The peak indicative of 1-formyltryptophan in the HPLC chromatograms also changes with pH, and the trend of the change is consistent with that of the solution color: when the solution pH changes from a low to high value, the solution color fades away, and is concomitant with the gradual disappearance of the chromatographic peak corresponding to 1-formyltryptophan. Hence, 1-formyltryptophan may be a precursor of the colored derivative, and the reaction in which the violet-blue color is generated undoubtedly involves a quasireversible acid–base equilibrium. On the other hand, the recovery behaviors of the absorption spectra of indole and IPA resemble that of tryptophan (see the Supporting Information), but indole displays the fastest recovery, which can be attributed to the reduced steric hindrance at the 3-position of the indole ring.

Although the colored derivative has not been isolated, according to ESI-MS analysis it might be a rearranged compound (m/z 463 $[M+H]^+$ for compound **1**, or m/z 491 $[M+H]^+$ for compound **2**) of 1-formyltryptophan, whose mechanism of formation is shown in Scheme 1. Current



Scheme 1. Possible mechanism for the reaction of tryptophan in HCOOH/HCl media which generates the violet-blue color. R = $CH_2CH(NH_2)COOH$.

evidence does not allow confirmation as to whether compound **1** (derived from 1 mol of tryptophan and 1 mol of 1-formyltryptophan) with a possible enolate tautomerization, compound **2** (derived from 2 mol of 1-formyltryptophan), or both result in the violet-blue color. It cannot be ruled out that a dimer formed directly by the coupling of 1-formyltryptophan through its 2-position makes a contribution to the violet-blue color, but this contribution, if it exists, would be a minor one, since such a dimer is difficult to form because of the large steric hindrance.

A comparative study of the current method was made with other known approaches,[5,11] in particular with the acid-ninhydrin method, in which both HCOOH and HCl are also involved.[11a] The reaction color in the acid-ninhydrin system is yellow, which may be attributed to the reaction of the α-amino group of tryptophan with ninhydrin to generate a yellow carboline product.[4b,12] In our system without ninhydrin, the resulting violet-blue color may be ascribed to the formation of compound **1** or/and compound **2**, as discussed above. The acid-ninhydrin method is fast and sensitive, but is inconvenient and unselective. In particular, cysteine and tyrosine interfere with the determination of tryptophan.[8] The reaction of tryptophan with *p*-dimethylaminobenzaldehyde and sodium nitrite in sulfuric acid has been widely used for the detection of tryptophan.[3,4a,11b] However, this method is also inconvenient because the reaction needs to be conducted in the dark because of the influence of light,[11b] and the accuracy is limited because of various interferences.[4a] A recently reported approach based on a diazo coupling reaction with *N*-(1-naphthyl)ethylenediamine is fast, but is subject to the interference of methionine and aromatic primary amines.[5] Thus, high selectivity and simplicity are the most noticeable advantages of the proposed method over the others discussed above.

In conclusion, the violet-blue color reaction of tryptophan in HCOOH/HCl has been characterized as a valuable method for identifying tryptophan in a mixture of amino acids, and for corroborating the existence of tryptophyl residues in peptides or proteins. The reaction investigated here may also provide a useful clue for a better understanding of the diseases blue diaper syndrome and purple urine bag syndrome which are caused by a metabolic disorder of tryptophan.[13]

Experimental Section

General procedure: In a typical experiment, tryptophan (25 mM, 1.0 mL) was added to a solution containing concentrated hydrochloric acid (37%, w/w, 2.5 mL) and formic acid (88%, w/w, 1.0 mL), and the final volume of the reaction solution was diluted to 5.0 mL with water. After the mixture was heated at 50 °C for 5 h in a water bath, and then cooled to room temperature, the absorption spectra were recorded in 1-cm quartz cells, with water used as the reference. When necessary, the pH value of the reaction solution was adjusted with NaOH or HCl. For comparison, indole and IPA were treated under the same conditions.

Received: May 30, 2006
Revised: August 7, 2006
Published online: September 19, 2006

Keywords: amino acids · analytical methods · tautomerism · tryptophan · UV/Vis spectroscopy

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