pH Response of Colored Copolypeptide Hydrogel Containing Tryptophan Treated with Nitric Acid

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ABSTRACT: Coloration of L-tryptophan (Trp) treated with nitric acid was observed under varying pH, *i.e.*, between yellow (below pH 10.5) and red (above pH 12.5). To elucidate the coloration mechanism of Trp by treatment with nitric acid, the colored compound was fractionated and investigated spectroscopically. Trp formed a yellow compound consisting of a tricyclic structure, and nitro groups were introduced into the indole ring. The color change with the variation of pH is due to the dissociation of nitrogen atoms in the indole ring. Colored copolypeptide hydrogel containing Trp residues was also prepared by aminolysis of copoly(γ -benzyl L-glutamate-co-Trp) membrane in the presence of crosslinking agent and by immersion of the membrane in nitric acid. Color change was also observed for the hydrogel. The pH dependence of such membrane properties as degree of swelling and permeability coefficient of styrene glycol was investigated. These properties changed drastically in the pH region of color change. This can be explained by the dissociation of Trp residues.

KEY WORDS Tryptophan / Color Change / Copolypeptide Hydrogel / Treatment with Nitric Acid / pH Response /

Poly(*N*-hydroxyalkyl L-glutamine), in hydrogels or vital tissue, features a peptide bond and is expected to be applicable to biomaterial production due to its flexibility, inactivation of antigen–antibody reaction,¹ biodegradability,^{2–6} non-toxicity of degradation products,⁷ and so on.^{8–12} We previously reported that conformations of peptide chains in hydrogels can be controlled and membrane properties, such as degree of swelling, solute permeability, enzymatic degradability, and mechanical properties, can be regulated by altering the hydrophobicity of the side chains.^{13–15} This paper reports polypeptide hydrogels containing L-tryptophan (Trp) residues, which contain an indole ring and have the largest hydrophobicity among natural amino acids. Trp is oxidized easily under an acidic condition and changes into various structures due to the high electronic density of the indole ring. For example, when the pyrindole compound is brought into contact with sulfuric acid and a trace of oxidizing agent is added, blue color develops which slowly is transformed to dirty green and finally a yellow,¹⁶ and perchloric acid converts Trp to a greenish-yellow compound and subsequent addition of iron chloride changes the greenish-yellow Trp to a reddish-orange color.¹⁷ This method is used for detection of Trp within proteins. Trp also shows coloration by condensation reaction between the carbonyl group of various aldehyde compounds and a carbon atom at the 2-position of the indole ring under acidic conditions. This reaction can be classified as Hopkins–Cole (fatty aldehyde) or Neuberger–Rhode reaction (aromatic aldehyde). The products show various colors, and the structure of each has been clarified. These procedures are used in qualitative and quantitative analysis of peptides including Trp. Since pink colored by-products were produced by the accidental oxidation of the indole ring of Trp during peptide synthesis, attempts have been

made to depress these formations.^{18,19} With the exception of a report on the reaction of Trp and aldehyde, no reports have been made on the coloration of Trp.

We previously found that Trp is colored when treated with strong acids, such as nitric acid, trifluoroacetic acid (TFA) or dichloroacetic acid (DCA), and shows reversible color change with variation of pH.²⁰ Trp treated with TFA forms a tricyclic structure and shows color change from red (below pH 4.0) to yellow (above pH 5.5) by the dissociation of indole ring.²¹ Trp treated with nitric acid showed opposite color change against pH, that is, from yellow (in acidic pH) to red (in alkaline pH). The present study clarifies the coloration mechanism of Trp by treatment with nitric acid. First, *N*-acetyl Trp ethyl ester (ATE) was treated with nitric acid. The compound having the target color was fractionated by column chromatography, and its structure was investigated by spectroscopic measurements. Structural change accompanied by color change with the variation of pH was also proposed. A colored copolypeptide hydrogel including Trp residues was prepared by treatment with nitric acid, and the pH dependence of physical properties of the membrane was investigated.

EXPERIMENTAL

Materials

Preparation and Purification of N-acetyl Trp Ethyl Ester Treated with Nitric Acid. ATE was dissolved in nitric acid with irradiating ultra violet (UV) (wavelength 365 nm, intensity 300 $\mu\text{W cm}^{-2}$) for 72 h. Crude ATE treated with nitric acid (ATE-N) that shows reversible color change with variation of pH was obtained by adding *n*-hexane and diethyl ether to acid solution. ATE-N was purified by column chromatography using ODS gel (Fuji Silisia Chromatex DM1020T, 100–200 mesh) as packing to obtain the compound with the target color. The purity of ATE-N after purification was

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checked by HPLC (TOSOH CCPE, UV-8000, ODS-80TM) using methanol–water (2:1, v/v) as developing solvent, and peak detection was carried out at 254 nm.

Synthesis of Random Copolymer

The starting copolymer (GT) consisting of γ -benzyl L-glutamate (γ -BLG) and Trp was synthesized using *N*-carboxyanhydride (NCA) method. γ -BLG NCA and Trp NCA were prepared by reaction with bis(trichloromethyl) carbonate (triphosgene),^{22–24} and purified by recrystallization from ethyl acetate solution with petroleum ether. These γ -BLG NCA and Trp NCA, at a mole ratio of 9 to 1, were dissolved in a 1:1 (v/v) mixture of dioxane and dichloromethane. Polymerization was initiated with triethylamine (TEA) at an NCA-to-TEA molar ratio of 50 to 1. The starting copolymer was precipitated in a large amount of cold methanol and dried *in vacuo*. All solvents used were distilled twice. The composition of GT was estimated by ¹H NMR measurement and elementary analysis.

Preparation of the Hydrophilic Membrane

The hydrophilic copolymer membrane, poly(*N*-hydroxyethyl L-glutamine-co-Trp) (EGT), was prepared by aminolysis of GT membrane cast from chloroform solution.²⁵ GT membrane was immersed in 2-amino-1-ethanol (EA) with 10 mol% of crosslinking agent, 1,8-octamethylenediamine (OMDA), at 58°C for 72 h. Debenzylation of GT was confirmed by the disappearance of absorption due to ester groups at 1740 cm⁻¹ in IR spectrum. Figure 1 presents a schematic diagram of the preparation of EGT membrane. Linear water soluble copolymer without crosslinking was also prepared according to the same method as for the hydrophilic membrane. The water soluble copolymer was obtained by dialysis and lyophilized.

Preparation of Colored Membrane

Yellow-colored, nitric acid-treated membrane, EGT-N, was prepared by a method similar to that for ATE. The treatment of the membrane with nitric acid was carried out for 1 h to prevent degradation of the main chain. The EGT-N membrane was washed repeatedly with methanol and water to remove remaining nitric acid and dried *in vacuo* for 72 h.

Measurements

Methods. The structure of purified ATE-N was estimated by ¹H NMR (Varian Associates Model XL-200 spectrometer) and IR (Shimadzu Model FT-IR 4200 spectrophotometer) measurements. Chemical shifts in ¹H NMR spectra are reported as δ values (ppm) relative to tetramethylsilane (TMS) as the internal standard. IR spectra were measured by KBr in the region of 4000–400 cm⁻¹. UV-VIS absorption spectra of EGT-N membrane and ATE-N were measured by a JASCO Model V-520 spectrophotometer with a quartz cell having a path length of 1 cm. pH was adjusted by 5*N* NaOH and HCl to control the change of concentration at a minimum. Nonaqueous titration with thymol blue (TB) as an indicator was carried out to elucidate the existence of negatively charged ATE-N. Titration was performed by dissolving ATE-N in dimethylfor-

Figure 1. Schematic diagram of the preparation of the EGT membrane.

mamide (DMF) and then 0.1*N* sodium methoxid/benzene solution added dropwise to the ATE-N solution in an atmosphere of nitrogen. The end point of TB was taken as the point where the color of the solution changed from pale yellow to blue.

Degree of Swelling of the EGT-N Membrane

The degree of swelling Q_w (%) was determined by equilibrating the membrane in various buffer solutions at 25°C. Q_w is given by,

$$Q_w = \frac{W_w - W_d}{W_d} \times 100$$

where W_w and W_d are the weight of the hydrogel and xerogel, respectively.

Solute Permeability of the EGT-N Membrane

To examine the pH dependence of solute permeability of EGT-N membrane, solute permeation of styrene glycol throughout the membrane was measured with membrane-separated glass cells at 25°C. The permeability coefficient (P_s) is given by,

$$J_s = \frac{dC_0}{dt} \times \frac{V}{S} \quad P_s = \frac{J_s \times \Delta x}{\Delta c}$$

where J_s is the flow rate, dC_0/dt is the increment rate of

Figure 2. Results of HPLC before and after purification: (a) ATE-N unpurified and (b) ATE-N 72.

solute concentration in the side having lower concentration, V is the volume of the lower concentration side, S is the effective permeation area, P_s is the permeability coefficient, Δx is the thickness of the swelled membrane and Δc is the difference of initial concentration. The increment rate of styrene glycol was estimated from the absorption intensity at 256 nm in UV measurement. Initial concentration in the higher concentration side was $2.5 \times 10^{-2} \text{ mol l}^{-1}$.

RESULTS AND DISCUSSION

Color Change of ATE-N

The solution of ATE-N was colored yellow below pH 10.5 to absorb the wavelength (around 400 nm) that appears bluish-purple to purple, and became red as it absorbed the wavelength (around 500 nm) that appears as blue above pH 12.5. ATE-N exhibits absorption around 370 nm in UV spectrum. Many aromatic nitro compounds, such as dinitro aniline and nitro aniline, show yellow color to absorb the light around 300 nm to 400 nm.²⁶ From these results, we conclude that ATE-N shows reversible color change in the pH region of 10.5 to 12.5 and nitro group is apparently introduced into the indole ring when treated with nitric acid.

Color Change of Purified ATE-N

Figure 2 shows the results of HPLC of ATE-N before and after purification measured using a methanol–water (2:1, v/v) mixture as developing solvent. ATE-N indicates four peaks at 20–30 mm, at 62 mm, at 72 mm, and at 84 mm, respectively. By the area summation method, ATE-N consists of 13% of the compound that shows a peak at 62 mm, 14% of the compound that shows a peak at 72 mm, and 16% of the compound that shows a peak at 84 mm. From the results of HPLC after

Figure 3. IR spectra of ATE and ATE-N 72.

purification, the compounds that show a peak at 72 mm (ATE-N 72) could be separated.

ATE-N 72 shows reversible color change as well as unpurified ATE-N in the pH region of pH 10.5 to 12.5. Both compounds that showed the peaks at 62 mm and at 84 mm also change in color between yellow and red in the same pH region, though these compounds could not be separated completely. This suggests that ATE-N consists of several components that have similar structure. The other peaks could not be separated since they were in close proximity.

Estimation of Structure of ATE-N 72

Figure 3 illustrates IR spectra of ATE and ATE-N 72. By comparison of their IR spectrum, ATE-N 72 exhibits the absorption due to C=O stretching vibration of amide bond at 1660 cm^{-1} , versus ATE exhibits it at 1638 cm^{-1} . Although ATE also indicates absorption due to N–H deformation vibration at 1557 cm^{-1} , the absorption of ATE-N 72 cannot be observed. Hino *et al.* reported that the protonation of Trp derivatives occurs at the 3-position of indole to generate indolenium cation in acidic conditions, and that cyclization took place owing to the fact that an α -nitrogen atom retains enough nucleophilicity to react with the 2-position of the protonated form.²⁷ If ATE forms the cyclic structure through treatment with nitric acid, variation of IR spectrum is reasonable. Nitration of aromatic compounds such as indole and pyrrole, which has high electronic density and reactivity, has been reported.²⁸ ATE-N 72 indicates strong absorption due to C–NO₂ stretching vibration at 1529 cm^{-1} and at 1321 cm^{-1} in IR spectrum. ATE-N 72 is thus introduced nitro group into indole ring by the treatment with nitric acid and seems to change the structure shown in Figure 4.

¹H NMR spectrum of ATE-N 72 in CDCl₃ is shown in Figure 5 together with that of ATE. The peaks observed in the ¹H NMR spectrum of ATE were assigned to as follows.

δ 1.2–1.3 CH₃ of ester (3H), δ 1.95 CH₃ of acetyl (3H), δ 3.3 CH₂ bonded at the 3-position of indole (2H),

Figure 4. Cyclization of ATE by treatment with nitric acid.

Figure 5. ^1H NMR spectra of ATE and ATE-N 72 in CDCl_3 : (a) ATE and (b) ATE-N 72.

δ 4.1–4.2 CH_2 of ester (2H), δ 4.9–5.0 CH of amino acid (1H), δ 6.0–6.1 NH of amino acid (1H), δ 6.9–7.6 indole (5H), δ 8.35 NH of indole (1H).

Based on the above, the peaks of ATE-N 72 were assigned to as follows.

δ 1.3–1.4 CH_3 of ester (3H), δ 1.9–2.0 CH_3 of acetyl (3H), δ 4.2–4.3 CH_2 of ester (2H), δ 4.4 CH of amino acid (1H).

In ^1H NMR spectrum of ATE-N 72, the peak at δ 8.5 disappeared by addition of D_2O , and was assigned to NH of indole. In the case of nitration occurred at the indole ring, the electron density of the indole ring is reduced by electron-attractive of nitro group, and the peaks of proton of indole seem to shift to low magnetic field. Two singlet peaks observed at δ 8.4 and at δ 8.8 shifted about 1.5 ppm to low magnetic field compared with that of ATE. Although ATE has five protons in the indole ring, ATE-N 72 exhibits only two singlet peaks in its spectrum. These results support nitration of the indole ring. Split peak at δ 2.8–3.1 was attributed to CH_2 bonded at the 3-position of indole through steric difference causes on protons of the CH_2 which was taken in a part of the ring structure. The peak at δ 6.0 attributed to NH of amino acid disappeared by the treatment with

Figure 6. Dissociation form of ATE-N 72.

nitric acid. This agrees with the results of IR measurement. ATE-N 72 is thus a dinitro compound substituted by nitro groups either at the 5- and 7-positions or at the 4- and 6-positions of the indole ring, and form a cyclic structure as shown in Figure 4.

Coloration Mechanism of ATE-N 72

ATE-N 72 shows reversible color change in the pH region of 10.5 to 12.5, due possibly to a negative charge. From the results of nonaqueous titration using tymol blue as an indicator, ATE-N 72 has negative charge in alkaline pH. Although the lone-pair of nitrogen of indole ring is employed to maintain the aromaticity, the acidity of indole ring of ATE is not so strong as to dissociate negatively. The nitro group introduced into an aromatic ring lowers electronic density of the ring and increases acidity by the strong electronic attractive effect. The nitro group also makes the acidity of aromatic ring increase owing to the distribution of a lone-pair reaches to nitro groups as *p*-nitroaniline and *p*-nitrophenol, and non-localized energy becomes large by negative resonance effect.²⁹ The acidity of ATE-N 72 appears to increase similarly by introduced nitro group, and ATE-N 72 colored red through the conjugation becomes long by dissociation of indole ring as shown in Figure 6.

The ATE-N 72 thus forms the tricyclic structure which introduced two nitro groups into the indole ring by the treatment with nitric acid. Color change of ATE-N 72 occurs by the dissociation of indole ring, attributable to the strong electron attractive effect of the nitro group.

Color Change of the EGT-N Membrane

The EGT-N membrane shows a yellow color in acidic pH and becomes red as well as in ATE treated with nitric acid. Figure 7 shows the pH dependence of VIS spectra of EGT-N membrane. The membrane was colored yellow below pH 10.5 to absorb the wavelength (around 400 nm) that appears bluish-purple to purple, and became red as it absorbed the wavelength (around 500 nm) that appears as blue above pH 12.5. EGT-N shows a color change from yellow to red in the pH region of 10.5 to 12.5, and this pH region is similar to that of ATE-N 72.

Figure 7. pH dependence of VIS spectra of EGT-N membrane.

Figure 8. pH dependence of the degree of swelling (Q_w) of EGT-N membrane.

Degree of Swelling of the EGT-N Membrane

The pH dependence of the degree of swelling (Q_w) is illustrated in Figure 8. As can be seen from Figure 8, Q_w of EGT-N membrane increased above pH 10.5. This pH region agrees with the pH region where the change in color of both ATE-N 72 and EGT-N membrane was observed. The variation of Q_w is reversible against pH. Although it is thought about transition of molecular conformation as a reason why the value of Q_w increase against pH, the conformation of EGT-N exists in random conformation in whole pH region. This exhibits that Q_w increases because the Trp residues within copolypeptide change to the tricyclic structure as well as in the case of ATE treated with nitric acid, and have a negative charge in an alkaline pH.

Solute Permeability of the EGT-N Membrane

The solute permeability of the EGT-N membrane as well as Q_w varies in the pH region where color change

Figure 9. pH dependence of permeability coefficient of EGT-N membrane.

is observed. Figure 9 shows the pH dependence of the permeability coefficient (P_s) of styrene glycol of the membrane. P_s and Q_w increased above pH 10.5. This increment of P_s seems to result from extension of permeation pathway of the membrane by repulsion of dissociating Trp residues. The degree of swelling and solute permeability of EGT-N membrane varied in the region where color change takes place.

Thus, new pH response hydrogel accompanying color change can be prepared by the treatment with strong acid and is expected to be applicable as material that can monitor interactions of Trp with cationic substances, *e.g.*, drug and metal ions, by color change.

The major conclusions of this investigation are as follows.

(1) *N*-acetyl Trp ethyl ester treated with nitric acid (ATE-N) shows reversible color change from yellow (below pH 10.5) to red (above pH 12.5).

(2) ATE-N was purified by column chromatography, and the structure of colored compound (ATE-N 72) was identified by ^1H NMR and IR measurement.

- ATE-N 72 forms a tricyclic structure by the nucleophilic reaction of α -nitrogen atom of ATE and the 2-position of indole ring, and is substituted by nitro group.

- ATE-N 72 is colored yellow by introduction of nitro group, and becomes red owing to the elongation of conjugated system accompanied by dissociation of indole ring above pH 10.5.

(3) The pH response material, EGT-N, can be prepared by the treatment of copoly(*N*-hydroxyethyl L-glutamine-co-Trp) hydrogel, EGT, with nitric acid.

- EGT-N membrane shows reversible color change as well as in ATE treated with nitric acid, and its physical properties vary drastically in the pH region where the color change is observed.

- The results of Q_w and P_s reflect the results of ATE-N 72 which dissociates above pH 10.5.

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