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pH response of coloured copolypeptide hydrogel containing tryptophan treated with trifluoroacetic acid

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The coloration phenomenon of L-tryptophan (Trp) treated with trifluoroacetic acid (TFA) was observed under varying pH, i.e., from yellow (above pH 5.5) to red (below pH 4.0). In order to elucidate the coloration mechanism, the coloured compounds were fractionated and investigated spectroscopically. It was found that Trp forms a yellow compound consisting of a tricyclic structure, and that the change in colour with the variation of pH is due to the dissociation of the nitrogen atom in an indole ring. Coloured copolypeptide hydrogel containing Trp residues was also prepared by a method similar to that for Trp. The colour change was also observed for the hydrogel. The pH dependence of such membrane properties as degree of swelling, solute permeability, and the mechanical property was investigated. These properties changed drastically in the pH region of the colour change. This can be explained by the dissociation of Trp residues. © 1998 Elsevier Science Ltd. All rights reserved.

(Keywords: tryptophan; coloration; pH response)

INTRODUCTION

Poly(*N*-hydroxyalkyl L-glutamine), in either hydrogels or vital tissue, features a peptide backbone and is expected to be applicable to biomaterials production for reason of its flexibility, substance permeability, inactivation of antigen–antibody reaction[1], biodegradability[2–6], non-toxicity of degradation products[7] and so on[8–12]. We have previously reported that conformation of peptide chains in hydrogels can be controlled and that the membrane properties, such as degree of swelling, solute permeability, enzymatic degradability and mechanical properties, can be regulated by altering the hydrophobicity of side chains[13–15].

In this paper, we report on polypeptide hydrogels containing L-tryptophan (Trp) residues, the residues of which contain indole rings, which are known to have the largest hydrophobicity among amino acids.

Trp is oxidized easily under acidic conditions and changes into various structures due to the high electronic density of the indole ring. For example, it is known that when the pyrindole compound is brought into contact with sulfuric acid and a trace of oxidizing agent is added, a blue colour develops, which is slowly transformed to dirty green and finally a yellow[16], and that perchloric acid converts Trp to a yellowish green compound, and subsequent addition of iron chloride changes the greenish yellow Trp to a reddish orange colour[17]. This method is used for detection of Trp within proteins. Trp also shows coloration by the 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 either a Hopkins–Cole reaction (fatty aldehyde) or a Neuberger–Rhode reaction (aromatic aldehyde). The products show various colours, and the structure of each has been clarified. These procedures are used in both qualitative and quantitative analyses of peptides including Trp. Furthermore, since pink coloured 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, however, no reports have been made on the coloration of Trp.

We previously found that Trp is coloured when treated with strong acids, such as trifluoroacetic acid (TFA), dichloroacetic acid (DCA) or nitric acid, and that it shows reversible colour change with variation of pH[20]. The aim of the present study is to clarify the coloration mechanism of Trp by treatment with TFA. First, *N*-acetyl L-tryptophan ethyl ester (ATE) was treated with TFA. Then the compound having the target colour was fractionated by column chromatography, and its structure was investigated by various spectroscopic measurements. The structural change accompanied by colour change with the variation of pH was also proposed. In addition, a coloured copolypeptide hydrogel including Trp residues was prepared by the treatment with TFA, and the pH dependence of the various physical properties of the coloured hydrogel was investigated.

EXPERIMENTAL

Materials

Preparation and purification of N-acetyl L*-Trp ethyl ester treated with TFA.* *N*-Acetyl L-Trp ethyl ester (ATE) was treated by dissolving in TFA with irradiating u.v. (wavelength 365 nm, intensity 300 μW/cm^2) for 72 h. Crude ATE treated with TFA (ATE-T) that shows reversible colour change with variation of pH was obtained by adding

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n-hexane and diethyl ether to an acid solution. ATE-T was purified by column chromatography using ODS gel (Fuji Silisia Chromatrex DM1020T, 100–200 mesh) as packing to obtain the compound of target colour. The purity of ATE-T after purification was checked by h.p.l.c. (Tosoh CCPE, UV-8000, ODS-80TM) using methanol/water (4/1, v/v) as a developing solvent, and the peak detection was carried out at 254 nm.

Synthesis of random copolymer. The starting copolymer (GT) consisted of γ-benzyl L-glutamate (γ-BLG), and Trp was synthesized using the *N*-carboxyanhydride (NCA) method. γ-BLG NCA and Trp NCA were prepared according to the method reported by Blout and Karlson[21], and purified by recrystallization from ethyl acetate solution with petroleum ether. These γ-BLG NCA and Trp NCA, in a mole ratio of 9:1, were dissolved in a 1:1 (v/v) mixture of dioxane and dichloromethane. The polymerization was initiated with triethylamine (TEA) at an NCA:TEA molar ratio of 50. The starting copolymer was precipitated in a large amount of cold methanol and dried in vacuo. All the solvents used in the synthesis were distilled twice. The composition of GT was estimated by ^{1}H n.m.r. 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[22]. The 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 i.r. spectrum. *Figure 1* denotes a schematic diagram of the preparation of the EGT membrane. Linear water-soluble copolymer without crosslinking was also prepared according to the same method as the hydrophilic membrane. The water-soluble copolymer was obtained by dialysis and lyophilized.

Preparation of coloured membrane. Red-coloured, TFA-treated membrane, EGT-T, was prepared by a method similar to that for Trp derivative (ATE). The EGT-T membrane was washed repeatedly with methanol to remove remaining TFA and dried in vacuo for 72 h.

Measurements

Methods. The structure of purified Trp derivative (ATE) treated with TFA, ATE-T was estimated by ^{1}H n.m.r. (Varian Associates Model XL-200 spectrometer) and i.r. (Nicolet Instruments Model IMPACT 400 FT-IR spectrophotometer) measurements. Chemical shifts in ^{1}H n.m.r. spectra were reported as δ values (ppm) relative to tetramethylsilane (TMS) as an internal standard. I.r. spectra were measured by the KBr method in the region of 4000–400 cm^{-1}. U.v./vis absorption spectra of ATE-T and EGT-T membrane were measured by a Jasco Model V-520 spectrophotometer with a quartz cell having a path length of 1 cm. The pH was adjusted by 1 N HCl and 1 N NaOH. Fluorescence spectra of ATE-T in methanol/water mixture were measured in a Shimadzu Model RF-540 spectrophotofluorometer. An excitation wavelength of 292 nm was employed for the measurements, since Trp was excited at this wavelength. To elucidate the existence of positively charged ATE-T, nonaqueous titration with crystal violet (CV) as an indicator was carried out by dissolving ATE-T in glacial acetic acid with 1% CV/acetic acid solution and then 0.1 N perchloric acid/acetic acid solution added dropwise to the ATE-T solution. The blue end point of CV was taken as the point where the colour of the solution changed from purple to crystal blue.

Degree of swelling of the EGT-T 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 hydrogel and xerogel, respectively.

Solute permeability of the EGT-T membrane. To examine the pH dependence of solute permeability of EGTT 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 solute concentration in the side having lower concentration,

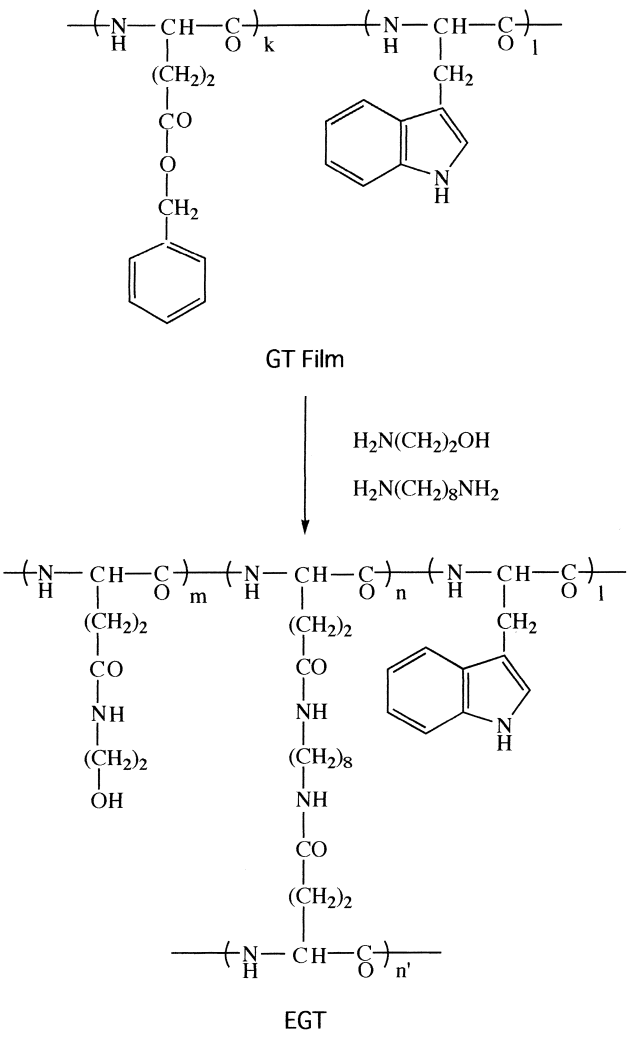


Figure 1 Schematic diagram of the preparation of EGT membrane

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 u.v. measurement. Initial concentration in the higher concentration side was 2.5×10^{-2} mol/l.

Tensile property of the EGT-T membrane. The tensile modulus of the EGT-T membrane was measured in various buffers at 25°C using an Orientec Co. Tensilon UTM-4LH equipped with a 1 kg loadcell. A wrapping film was used at chuck parts to prevent the membrane from breaking or from slippage at the chuck. The membrane was tested at a strain rate of 30% per minute. The Young's modulus E was estimated from a stress–strain curve.

RESULTS AND DISCUSSION

Colour change of ATE-T

The solution of ATE-T turned red below pH 4.0 as it absorbed the wavelength (around 490 nm) that appears as blue, and became yellow as it absorbed the wavelength (around 400–450 nm) that appears as bluish–purple to purple above pH 5.5. U.v. spectra of ATE in TFA solution also showed that the absorption at 360 nm increased with decreasing of the absorption at 286 nm, which is the characteristic absorption of the indole ring. Moreover, when excited at 292 nm, ATE-T showed a maximum fluorescence at 384 nm, *versus* a maximum at 354 nm for ATE. Generally, u.v. absorption and fluorescence maxima vary with structural change such as increment of conjugated system, and aromatic heterocycles and aromatic polycycles such as acridine absorb the wavelength around 340–360 nm[23]. From these results, we conclude that ATE-T changes from red to yellow in the pH region of 4.0–5.5 and seems to form a polycyclic structure when treated with TFA.

Colour change of purified ATE-T

Figure 2 shows the results of h.p.l.c. before and after purification of ATE-T measured using a methanol/water mixture as developing solvent. ATE-T indicated mainly three sharp peaks, and contained unreacted ATE. By the area summation method, it was determined that ATE-T consisted of 27.6% of the compound that showed a peak at 41 mm, 20.9% of the compound that showed a peak at 62 mm, and 21.6% of unreacted ATE. From the results of h.p.l.c. after purification, it was apparent that separation was possible between the compound that showed a peak at 41 mm (ATE-T 41) and the compound that showed a peak at 62 mm (ATE-T 62). The other peaks could not separate since they were in close proximity or described small areas. ATE-T 41 was colourless and did not show a colour change, while both ATE-T 62 and unpurified ATE-T changed in colour from red to yellow in the pH region of 4.0–5.5. These results demonstrate that, generally, colour-change can be regarded as occurring by ATE-T 62.

Estimation of structure of ATE-T 62

The structure of ATE-T 62 was estimated by various spectroscopic analyses. The i.r. spectrum of ATE-T 62 is shown in *Figure 3* together with that of ATE. In the spectrum of ATE-T 62, the absorption due to N–H deformation vibration cannot be observed, *versus* that which can be observed at 1557 cm^{-1} in ATE spectrum.

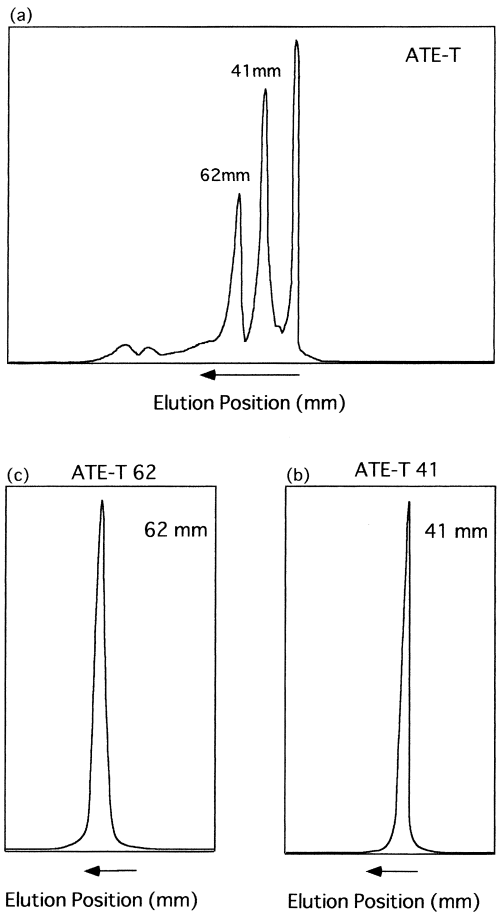


Figure 2 The results of h.p.l.c. before and after purification: (a) ATET unpurified, (b) ATE-T 41, and (c) ATE-T 62

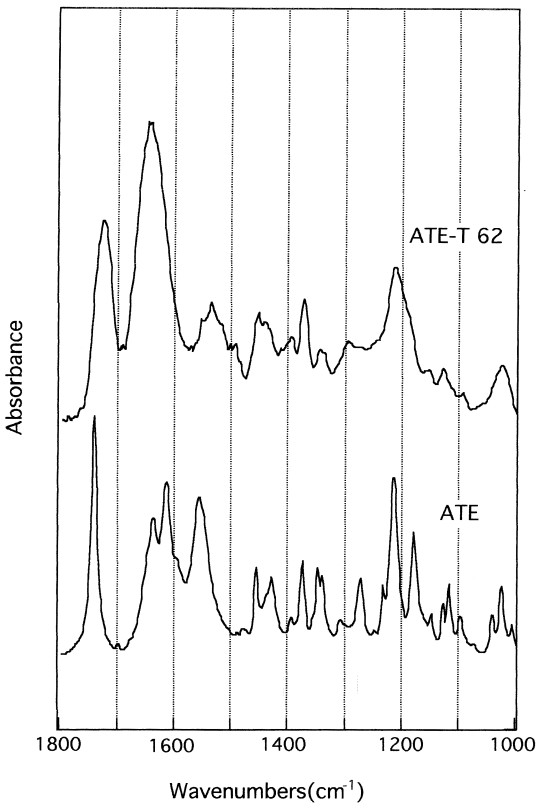


Figure 3 I.r. spectra of ATE and ATE-T62

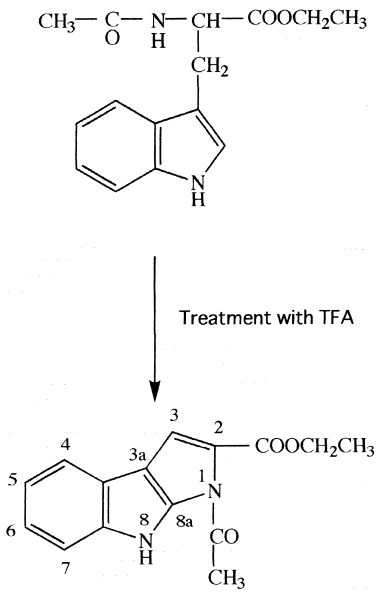


Figure 4 Cyclization of ATE by treatment with TFA

Although ATE also shows the peak due to ester at 1743 cm^{-1}, the peak of ATE-T 62 is shifted to the lower wavenumber (observed at 1726 cm^{-1}). Uphaus *et al*. have reported that the reaction of *N*-acetyl L-Trp derivatives and TFA yields β-carboline derivatives[24]. However, ATE-T 62 shows the peak due to C=O stretching vibration of amide bond at 1650 cm^{-1} in the i.r. spectrum, although the amide bond does not exist in β-carboline derivatives. Therefore the structure of ATE-T 62 cannot be regarded as β-carboline derivatives. Taniguchi and Hino 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 bonded to an acetyl group retains sufficient nucleophilicity to react with the 2-position of the protonated form[25]. If ATE forms the cyclic structure through treatment with TFA, the variation of i.r. spectrum is reasonable. It would appear that ATE changed to the structure shown in *Figure 4*.

^{1}H n.m.r. spectrum of ATE-T 62 in DMSO-d6 is shown in *Figure 5* together with that of ATE. The peaks observed in the ^{1}H n.m.r. spectrum of ATE were assigned to as follows: δ 1.0–1.1 CH_3 of ester (3H), δ 1.75–1.9 CH_3 of acetyl (3H), δ 2.9–3.2 CH_2 bonded at the 3-position of indole (2H), δ 3.9–4.1 CH_2 of ester (2H), δ 4.4–4.6 CH of amino acid (1H), δ 6.9–7.5 indole (5H), δ 8.2–8.4 NH of amino acid (1H), δ 10.8–10.9 NH of indole (1H).

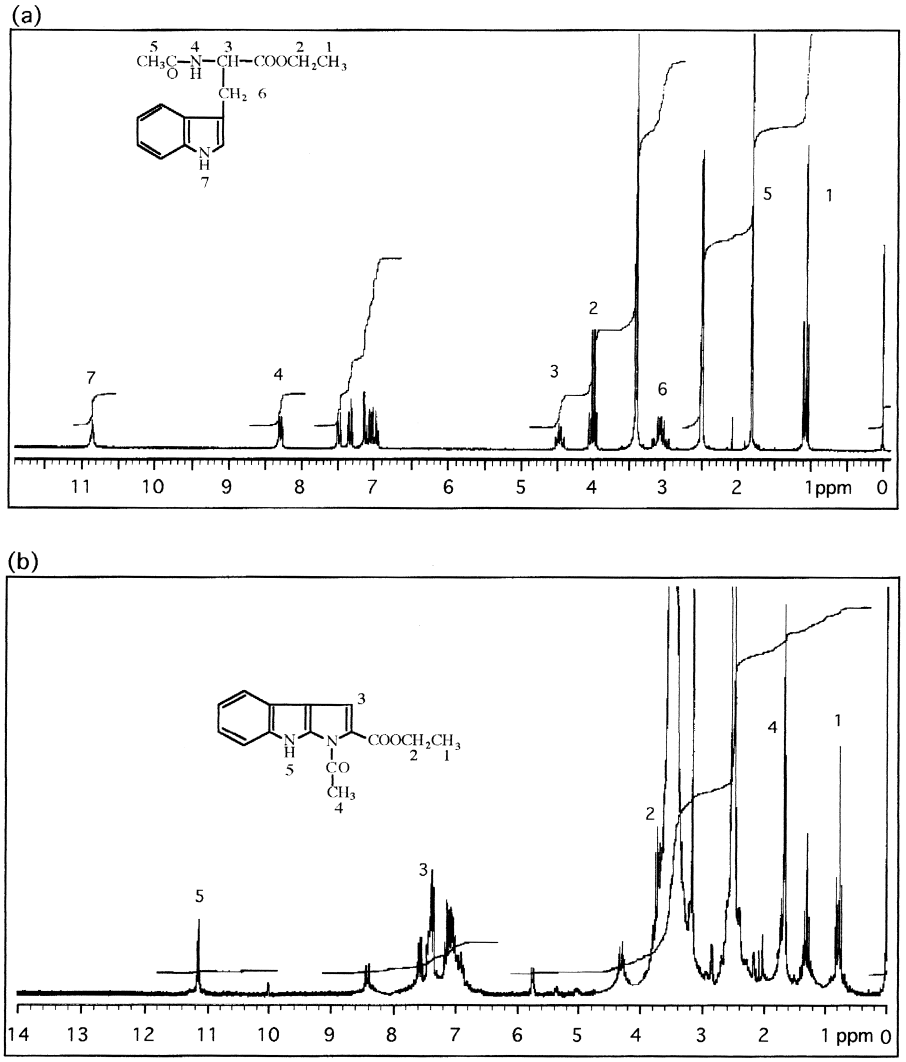


Figure 5 ^{1}H n.m.r. spectra of ATE and ATE-T 62 in DMSO-d_6: (a) ATE, and (b) ATE-T 62

On the other hand, in the spectrum of ATE-T 62, there were four sharp peaks, two of them due to CH_3 of ester (observed at δ 0.9–1.3), and the rest attributed to CH_3 of acetyl (observed at δ 1.7–1.9). It would thus appear that ATE-T 62 consists of two components. However, the results for h.p.l.c., even though measured in different conditions, showed a single peak. Also, n.m.r. spectra measurements using $CDCl_3$ and CD_3OD result in a number of peaks, the intensities of which varied by the kind of solvent. ATE-T 62 may cause isomerization (tautomerization) under the conditions of n.m.r. measurements. Therefore, the structure of ATE-T 62 in DMSO-d_6 was estimated because the exchange rate of proton is slow and the identification of the peak is easy in the solvent. The peaks of ATE-T 62 were assigned as follows from the value of the integral curve and the intensity ratio: δ 0.9–1.0 CH_3 of ester, δ 1.9CH_3 of acetyl, δ 3.7–3.8 CH_2 of ester, δ 7.4– 7.5 H at the 3-position of indole, δ 11.1–11.2 NH of indole.

Moreover, it is thought that ATE-T 62 isomerizes (tautomerizes) as shown in *Figure 6*; the peaks of the tautomer were attributed to as follows: δ 1.3–1.4 CH_3 of ester, δ 1.65 CH_3 of acetyl, δ 4.25–4.35 CH_2 of ester, and δ 5.7–5.8 H at the 3a-position of tautomer.

The peak at δ 8.4–8.5, which indicates the same intensity as a proton at the 3a-position was attributed to a proton at the 3-position of the tautomer. From the results of i.r. and n.m.r. measurements, it is considered that the compound shown in *Figure 5* was produced by the treatment with TFA.

Figure 7 shows fluorescence spectra of ATE-T 62 against pH. It has been reported that the physical properties and electronic absorption and fluorescence of indole and its common derivatives, such as β-carboline, were affected by acidity and basicity of solvent, and generated cation or dication in acidic solvent[26]. Although ATE-T 62 exhibited a fluorescence maximum at 380 nm in pH 6.2, another fluorescence maximum was observed at 430 nm in pH 2.2. Moreover, it becomes apparent that ATE-T 62 is about 45% dissociated from nonaqueous titration, as shown by use of crystal violet (CV) as an indicator. It was proved that ATE-T 62 has a positive charge in acidic pH. The indole ring of ATE has weak basicity and it cannot dissociate, because the lone-pair of nitrogen is employed to hold the aromaticity. On the other hand, the 10 π-electrons needed to form an aromatic ring exist in ATE-T 62, except for the lone-pair of nitrogen, so that ATE-T 62 has a strong basicity compared with ATE, and is regarded as an aromatic amine like aniline. Aniline dissociates around pH 5.0 through the lone-pair of nitrogen nonlocalized on the whole of the ring. ATE-T 62 also seems to dissociate in a similar pH, and it is supposed that the colour change is due to the variation of the electronic state of the aromatic ring by reversible dissociation, as shown in *Figure 8*.

From the results described above, it becomes apparent that ATE-T 62 forms the cyclic structure through treatment with TFA, and the variation of the electronic states accompanied by dissociation of the indole ring causes the colour change.

Colour change of the hydrophilic membrane treated with TFA. EGT-T membrane shows a red colour in acidic pH, and indicates a yellow colour in alkaline pH as well as in

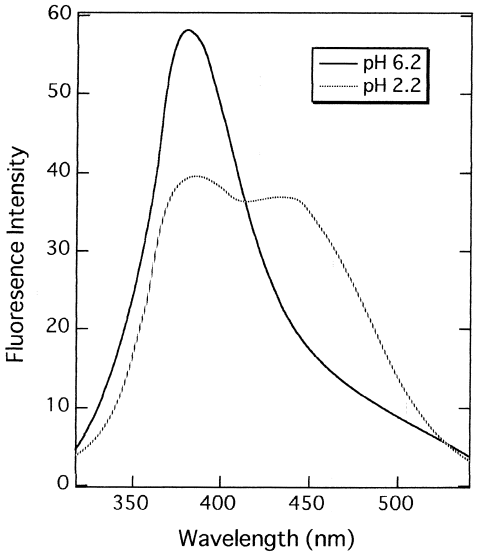


Figure 7 pH dependence of fluorescence spectra of ATE-T 62

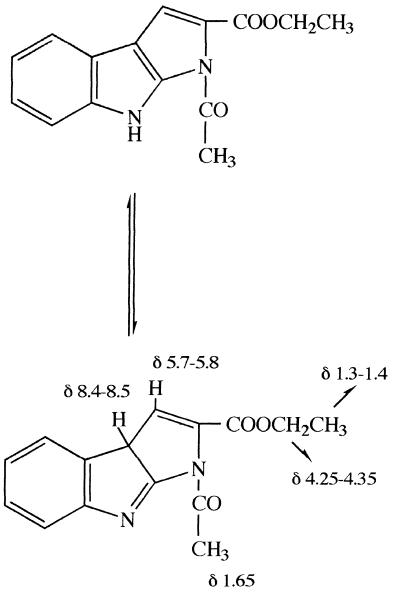


Figure 6 Isomerized (tautomerized) form of ATE-T 62

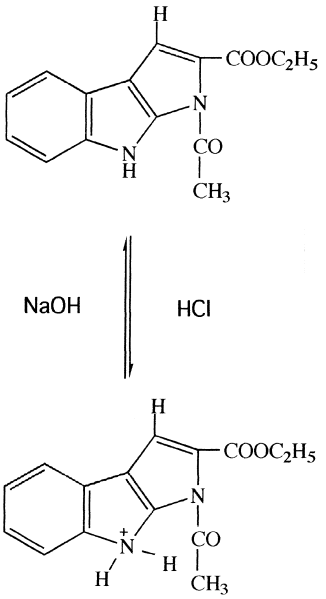


Figure 8 Dissociation form of ATE-T 62

ATE treated with TFA. *Figure 9* shows the pH dependence of Vis spectra for the EGT-T membrane. The membrane indicates a red colour below pH 4.0, and becomes a yellow colour above pH 5.5. It was ascertained that the membrane shows a colour change from red to yellow in the pH region of 4.0–5.5, and that this pH region is similar to that in which ATE-T 62 changes colour.

Degree of swelling of the EGT-T membrane. The degree of swelling (Q_w) of the EGT-T membrane in various pH buffer solutions is shown in *Figure 10*. Though the Q_w of the untreated EGT membrane was 658% and did not change with pH, the Q_w of the EGT-T membrane increased largely in the pH region of 4.0–5.5. This pH region agrees with the pH region where the ATE-T 62 and EGT-T membrane changed in colour from red to yellow. Although it is thought that transition of molecular conformation is a reason for the value of Q_w increasing against pH, the conformation of EGT-T exists in random conformation, and does not vary even though measured at different pH values. This shows that Q_w increases progressively, because the Trp residues within a copolypeptide change to a tricyclic structure, as well as the case of ATE treated with TFA, and has a positive charge in an acidic pH.

Solute permeability of the EGT-T membrane. It is considered that the solute permeability of EGT-T membrane varies in the pH region where colour change takes place as well as Q_w. *Figure 11* shows the pH dependence of permeability coefficient (P_s) of styrene glycol of the membrane. Both the P_s and Q_w increased below pH 5.5. This increment of P_s seems to have resulted from the extension of the permeation pathway of the membrane by repulsion of dissociating Trp residues.

The degree of swelling and solute permeability of the EGT-T membrane varied in the region where colour change takes place, and reflected the result of ATE-T 62 which dissociates below pH 5.5.

Mechanical properties of the EGT-T membrane. The stress–strain (S–S) curves of many synthetic polymer membranes usually show typical elastomeric behaviours and exhibit an inflection point in the low strain region[27]. On the other hand, it is known that polypeptide hydrogels show behaviour characteristic of human skin, with low modulus at low strain and high mechanical strength at high strain[15]. The tensile properties of the hydrogels are highly dependent on the degree of swelling.

The S–S curves of EGT, EGT-T and poly(*N*-hydroxyethyl L-glutamine) (PHEG) membranes in buffer solution at pH 7.4 are shown in *Figure 12*, together with the values of Young's modulus E and Q_w. In a comparison of E values, EGT membrane shows higher E than that of PHEG membrane. This is regarded as the polymer density per unit area increase with decreasing the value of Q_w from 820% to 658% by introduction of a hydrophobic Trp residue. Although it is thought that the mechanical strength of the EGT-T membrane is decreased by the treatment with TFA, the elongation of EGT-T membrane was only reduced by a little, and maintains the mechanical property of hydrogel. Furthermore, the value of E depended on pH, and decreased in the pH region where colour change of EGT-T

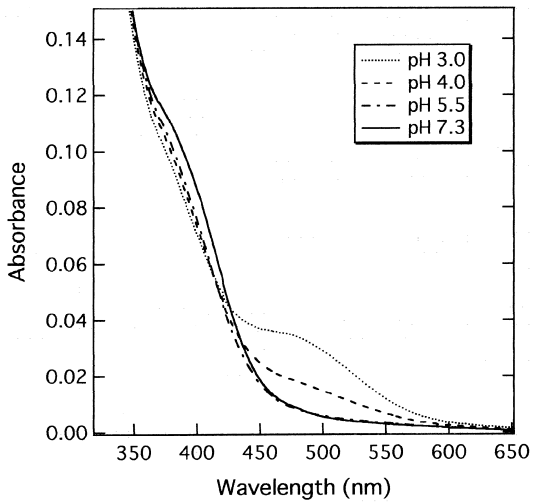


Figure 9 pH dependence of Vis spectra of EGT-T membrane

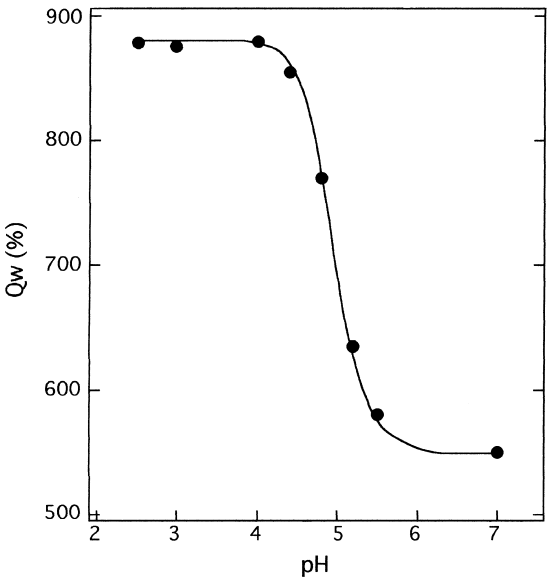


Figure 10 pH dependence of the degree of swelling (Q_w) of EGT-T membrane

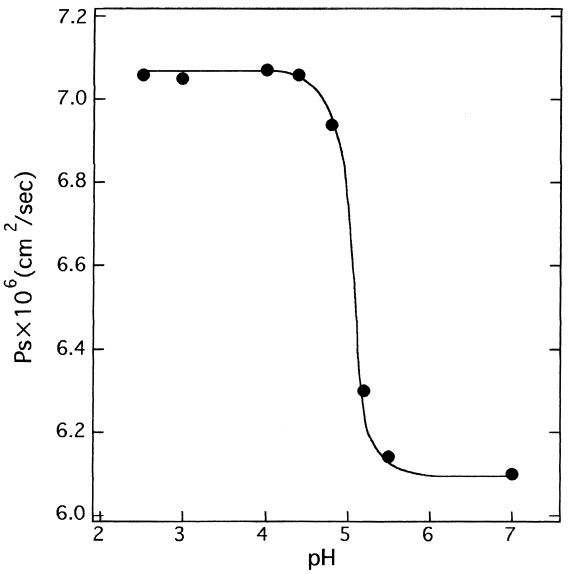
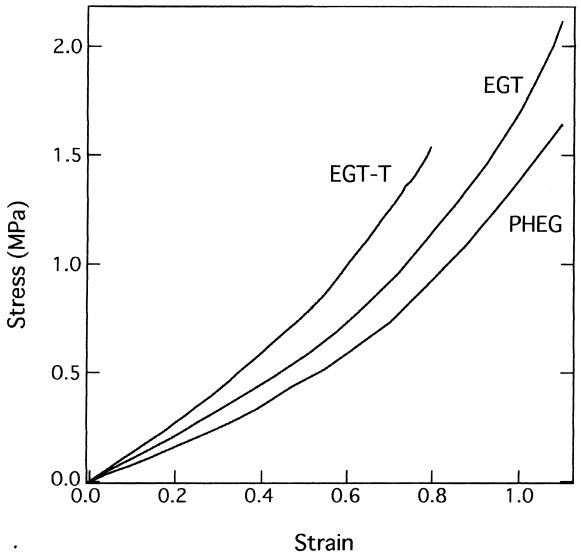


Figure 11 pH dependence of solute permeability coefficient (P_s) of EGT-T membrane



	E(MPa)	Qw(%)
PHEG	0.94	820
EGT	1.40	658
EGT-T	1.54	550

Figure 12 Stress–strain curves and Young's moduli of PHEG, EGT and EGT-T membranes in buffer solution at pH 7.4

membrane was observed. This decrement of E is due to the increment of Q_w accompanied by the dissociation of Trp residues. From these results, it is confirmed that the mechanical properties of the EGT-T membrane are not influenced by treatment with TFA, and that E of EGT-T membrane decreases with increasing Q_w accompanied by the dissociation of Trp residues.

Thus, a new pH response hydrogel with accompanying colour change can be prepared by treatment with strong acid, and it is expected to be applicable as a material that can monitor the molecular recognition of Trp by colour change.

CONCLUSIONS

The major conclusions of this investigation are as follows.

(1) *N*-acetyl Trp ethyl ester treated with TFA (ATE-T) shows reversible colour change from red (below pH 4.0) to yellow (above pH 5.5).

(2) ATE-T was purified by column chromatography, and the structure of coloured compound (ATE-T 62) was identified by various spectroscopic measurements. (i) ATE-T 62 changes in colour with the variation of pH, and forms a tricyclic structure because the nucleophilic nitrogen atom bonded to the acetyl group reacts at the 2-position of the indole. (ii) ATE-T 62 dissociates in acidic pH, and the variation of the electronic state of the aromatic ring accompanied with this dissociation caused a reversible colour change.

(3) The pH response material, EGT-T, can be prepared by the treatment of a copolypeptide hydrogel containing Trp residues, EGT, with TFA. (i) EGT-T shows reversible colour change against pH as well as in ATE-T, and its physical properties vary drastically in the pH region of 4.0–5.5. (ii) The degree of swelling of EGT-T membrane increases by the dissociation of indole ring. (iii) Young's modulus and solute permeability depend on the degree of swelling.

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